ROLE OF A NOVEL PATTERN RECOGNITION RECEPTOR IN ANTIBACTERIAL INNATE IMMUNITY

by

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(Under the Direction of Donald L. Evans)

ABSTRACT

Teleosts are an evolutionarily appropriate model for studying immunology. The innate immune system of fish is the principal defense mechanism. The acquired immune system is less developed in fish than mammals. Nonspecific cytotoxic cells (NCC) are a key cell in the teleost innate immune response and are the equivalent of mammalian NK cells. NCAMP-1, a histone-like protein on NCC, is an active participant in the antibacterial immunity of teleosts as both a pattern recognition receptor and an antimicrobial peptide. Recombinant NCAMP-1 and NCAMP-1 from NCC granule extracts had specific antimicrobial activity against *Escherichia coli* and *Edwardsiella ictaluri*. NCAMP-1 was expressed on the membrane of NCC and could be up-regulated by calcium ionophore activation. *In vivo* infection of channel catfish with *Edwardsiella ictaluri* resulted in increased membrane expression of NCAMP-1 on NCC and cell trafficking from the anterior kidney to the spleen. NCAMP-1 is involved in antibacterial innate immunity of teleosts.

INDEX WORDS: Innate Immunity, Antimicrobial Peptide, Histone, NCC, Channel Catfish, Pattern Recognition Receptor, *Edwardsiella ictaluri*

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B.S., University of Georgia, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

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May 2008

DEDICATION

This research is dedicated to my wonderful parents Lee and Kathy, my sister Lindsay, and my brother Sean. Their constant love and support have been instrumental throughout my life and in keeping me motivated while completing this degree. I also dedicate this research to my fiancé, Ryan, who has encouraged me every step of the way.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank and acknowledge the following people who have helped me complete this degree and for their contributions to me as a scientist and a person:

Dr. Donald L. Evans and Dr. Liliana Jaso-Friedmann for their scientific guidance, advice, support, hard work, encouragement and patience.

Dr. Thomas Krunkosky for his contributions and advice as a committee member.

John Leary III for his suggestions, supervision, and support. His guidance and assistance were essential for my success.

Lauren Moss for her friendship, collaboration and laboratory assistance. Her companionship in the lab and classes was valuable.

The faculty, staff, and graduate students of the Department of Infectious Diseases for their support and advice.

Most of all my family, Dad, Mom, Lindsay and Sean, and my fiancé, Ryan, for their constant support, understanding, and love, and for making me the person I am today.

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CHAPTER 1

INTRODUCTION

Teleosts are a relevant and evolutionarily appropriate model for studying innate immunology. The evolution of fish and tetrapods diverged hundreds of millions of years ago. Teleosts can be used as a model to investigate the evolution of lymphoid tissue and the development of the immune system. Teleosts have a less developed immune system than mammals. The adaptive immune system is more limited in fish than mammals. Teleosts have a limited antibody repertoire and slow lymphocyte maturation. Thus the innate immune response is the primary mechanism of defense. Fish also lack specialized lymphoid organs such as lymph nodes and bone marrow. Teleosts have adapted to the use of more primitive methods of immune defense. An important reason to study teleost immunology is to better understand the innate immune system in the absence of highly developed adaptive immunity. Fish, especially zebrafish, can be used as a model of vertebrate immunology.

Nonspecific cytotoxic cells (NCC) were first isolated from channel catfish. NCC are the teleost equivalent of natural killer (NK) cells in mammals and play an important role in innate immunity. The antimicrobial peptide NCAMP-1 (NCC cationic antimicrobial peptide-1) was initially isolated from catfish NCC but has also been found in other species of fish and mammals. NCAMP-1 is both a histone-like antimicrobial peptide and a pattern recognition receptor that binds bacterial DNA.

The objective of the present study is to further characterize the role of NCAMP-1 in the antibacterial innate immunity of channel catfish. The aims of the study are: 1) Determine the antimicrobial activity of recombinant NCAMP-1 against a wide range of Gram-negative clinical isolates; 2) Compare the antimicrobial activity of recombinant NCAMP-1 with natural NCAMP-1 isolated from catfish granules; and 3) Study the *in vivo* role of NCAMP-1 by following expression patterns during *Edwardsiella ictaluri* infection. We hypothesize that NCAMP-1 is an active participant on NCC in the antibacterial immunity of teleosts as both a pattern recognition receptor and an antimicrobial peptide.

CHAPTER 2

LITERATURE REVIEW

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are a diverse group of proteins that function as part of the innate immune system to kill a variety of microbes including bacteria, fungi and viruses. Cationic antimicrobial peptides are 12-50 amino acids in length, have a net positive charge and typically have a β-sheet structure [1, 2]. Most AMPs have amphipathically arranged hydrophilic, hydrophobic and cationic amino acids [3]. Over 800 different AMPs have been discovered [4]. Antimicrobial peptides have been identified in bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals [1]. Antimicrobial peptides are found in blood, granules [5], epithelial cells, biofilms, skin, GI tract [3] and cells and tissues involved in host defense against infection [2]. The synthesis of AMPs occurs quickly (only a few hours) which makes them an essential part of the innate immune response [6].

The mechanism of action of antimicrobial peptides is not entirely understood. Antimicrobial peptides target bacterial membranes that have highly negatively charged phospholipids on their outer membrane [3, 7]. However, plant and animal cell walls are not targeted because their outer leaflet is composed of lipids that have no net charge [3]. The general mechanism of action of cationic AMPs is attraction of the AMP to the bacterial membrane, followed by attachment and eventually peptide insertion into the bacteria causing membrane

permeability [7]. The Shai-Matsuzaki-Huang model [8-10] gives a more detailed description of the mechanism by which AMPs enter and kill bacteria. According to this model, the peptide interacts with the bacterial outer membrane, displacing the lipids and altering the membrane surface and enters the target cell [3]. The bacteria is then killed due to physical damage such as depolarization of the bacterial membrane, leaking of cellular contents through holes in the membrane or degradation of the cell wall by hydrolases [3]. Antimicrobial peptides can also cause damage to intracellular processes, such as inhibition of cell wall synthesis, nucleic acid synthesis, protein synthesis or enzymatic activity. These lead to the death of the bacteria [3, 7].

All targets of antimicrobial peptides must contain a target membrane. Enveloped viruses, fungi, parasites and cancer cells are good targets of AMPs [1]. Gram-negative bacteria are also highly susceptible to killing because their cell membrane is very negatively charged [3]. Cationic AMPs easily displace the metal ions that hold the LPS in the outer membrane [3]. The outer membrane is damaged and other peptides are allowed to enter the bacteria. This leads to bacterial cell death [3].

Antimicrobial peptides elicit effects on the host organism. Antimicrobial peptides alter the quality and effectiveness of the innate immune and inflammatory response in many ways [1]. The effects of AMPs may be the release of inflammatory stimuli, mast cell degranulation, neutrophil chemotaxis, non-opsonic phagocytosis, recruitment of T cells, enhanced chemokine production, increased immunoglobulin production and stimulation of apoptosis of macrophages and lymphocytes [1, 2]. Some AMPs are also capable of altering adaptive immune responses and acting as signaling molecules [11].

Antimicrobial peptides are an appealing option for the treatment of infection. Antimicrobial peptides are active against a broad spectrum of microbes including antibiotic

resistant bacteria [6, 12]. Antimicrobial peptides rapidly kill pathogens [12]. Microbial resistance to AMPs is less common than resistance to antibiotics because the outer membrane of the pathogen is targeted and it would be costly for the microorganism to change the composition or organization of its membrane to avoid lysis [3, 12]. Additionally, cationic AMPs can block endotoxin (LPS) activation of macrophages by binding to LPS binding protein (LBP). This prevents TNF-α production and septic shock [5, 12]. Problems with using AMP therapies are translating *in vitro* results to *in vivo* results, cost and ensuring that microbes will not develop antimicrobial resistance [11].

Many subfamilies of cationic antimicrobial peptides exist that are categorized broadly based on their secondary structure [3]. A single animal contains a variety of classes of antimicrobial peptides that may contribute to better protection against microbes [1]. The main classes of AMPs are defensins and cathelicidins [2]. Defensins are found at high concentrations in the granules of mammalian epithelial cells and phagocytes [2]. Cathelicidins are similar to defensins in both abundance and distribution [2]. Thionins are plant peptides that may be a new group of DNA binding proteins [13]. The class of AMP that is amino-acid enriched has a broad spectrum of activity against fungi, enveloped viruses and gram-positive and negative bacteria [13]. Histone derived AMPs are cationic helical peptides that have bactericidal activity against bacteria and fungi and were originally isolated from toad and fish epithelial cells [13]. The histone derived AMPs may be important in teleost innate immunity [13]. Cecropins and beta-hairpin AMPs are two other classes of potent antimicrobials [13].

In mammals, cationic antimicrobial peptides can be isolated from many cells and tissues. One example of a mammalian AMP is NK-lysin that was isolated from pig small intestine and cloned from porcine bone marrow cDNA [14]. Stimulation of NK and T cells with IL-2

promoted synthesis of NK-lysin [14]. NK-lysin has antibacterial and antiviral activity and also lyses the mouse tumor cell line (YAC-1) [14].

Antimicrobial peptides play an important role in teleost innate immunity and have been reported in many species of fish [12]. Antimicrobial peptides have been isolated in rainbow trout, Atlantic salmon, hybrid striped bass, winter flounder, channel catfish, zebrafish, carp, Atlantic halibut, hagfish, rockfish, soapfish, red sea bream, shark, red sea moses sole and mudfish [12, 15]. Teleost AMPs have been purified from different species of fish and have diverse killing activities.

Fish AMPs are primarily isolated from the mucus [6, 16]. Mucus acts as a first barrier of defense against pathogens [6, 16]. Pleurocidin is a fish AMP that was isolated from skin secretions of white flounder where it is localized in the granules of skin mucus and goblet cells [17]. An antimicrobial peptide, HLP-1, was isolated from acid extracts of channel catfish skin [16]. HLP-1 is closely related to histone H2B and has broad spectrum antimicrobial activity against *Aeromonas hydrophila* and *Vibrio alginolyticus*, but not *Edwardsiella ictaluri* [16]. Onchorhycin II was isolated from skin secretions of rainbow trout [18]. Onchorhycin II is a histone-like protein that can permeabilize bacterial membranes without pore formation. This AMP has antimicrobial activity against *M. luteus*, *E. coli*, and *L. anguillarum* [18]. Acid extracts of rainbow trout and sunshine bass skin, gills and spleen have histone-like antimicrobial peptides that are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum* [19].

If a fish has lost scales, is scratched or is stressed, AMPs located in the mucus layer may be unable to eliminate microorganisms [6]. Thus other AMPs must be present within the fish to eliminate pathogens that evade mucus AMPs. Liver expressed AMP-2 (LEAP-2) has been cloned and sequenced from channel and blue catfish [12]. LEAP-2 is primarily found in the liver

[12]. In response to infection with *Edwardsiella ictaluri*, LEAP-2 is modestly up-regulated in the spleen, but not altered in the head kidney or liver [12]. A defensin-like antimicrobial in rainbow trout has been shown to have antiviral activity [20].

Histones and Histone-like Proteins

Histones have traditionally been known to localize in the cell nucleus in association with chromatin fibers. A complex of four core histones (H2A, H2B, H3 and H4) form an octamer and create the nucleosome in the nucleus that stabilizes DNA [21]. Histone H1 is a compartment of nuclear chromatin that resides in the nucleus and contributes to packaging nucleosomes into higher order structures [21]. Histone H1 also stabilizes the nucleosome and is located at regions of entry and exit of DNA in the nucleosome [22]. Evidence suggests that intact or cleaved histone fragments are released during apoptosis and have antimicrobial activity that may be an important component of innate immunity [21].

Histones contribute to transcriptional regulation by altering the structure of chromatin [23, 24]. Histone H1 can regulate gene activity by repressing various chromatin activities [25]. Linker histone H1 also plays a role in cell proliferation and differentiation, apoptosis, and ageing [26]. Cytotoxicity mediated by histone H1 could be useful in carcinoma treatment [27]. Histone H1 that is released from epithelial cells during apoptosis may contribute to host protection following microbial invasion [28]. Histone H1 has been shown to bind to LPS [21] and prevent bacterial proliferation in villus epithelial cells [28].

Histone H1 is located in the cytoplasm and on the cell surface [21]. Histones are mainly found in chromatin regions that are protected from nuclease digestion [26]. Recent evidence

suggests that histones are not only found in the nucleus and on cell membranes, but are also secreted as antimicrobials from granules [29]. Histone H1 is a highly motile linker histone that is capable of moving around the cell [29]. A protease that cleaves histone H1 into smaller fragments may be the method of detachment of H1 from the nucleosome [21].

Human monocytes have histones H2A and H2B located on their cell membrane [30-32]. Histones H2A and H2B on the monocyte membrane are regenerating receptors that are a binding site for DNA and contribute to the endocytosis and degradation of exogenous DNA [30]. Monocyte activation results in increased expression of the histones on the cell membrane [31]. The human transformed B cell line, Raji, has 2 histone-like membrane proteins of 14-18 and 33-34 kDa [33]; and T-cells express membrane H2B (17 kDa) [33, 34] and H3 (29 kDa) [35, 36]. Other examples of the widespread expression of membrane histones include neurons [37] and macrophages [38] shown to have 30-33 kDa histone H1 membrane receptors that bind LPS and thyroglobulin, (respectively).

Histones with antimicrobial activity have been isolated from a number of teleost species. SAMPH1 is a histone-like antimicrobial peptide that was isolated from the skin mucus of Atlantic salmon and has antibacterial activity against both Gram-negative and Gram-positive bacteria [39]. Another histone H1 protein was identified in acid extracts from the liver, intestine and stomach of Atlantic salmon [40]. A protein (HDSF-1) that has an identical sequence to trout histone H1 was found in the mucus and blood of Coho salmon [41]. HDSF-1 is a poor membrane permeabilizer and may rely on synergy with another antimicrobial peptide (e.g. pleurocidin) to have bactericidal effects [41]. Parasin-1 is isolated from catfish and is an antimicrobial peptide that is cleaved from histone H2A by a specific protease and has strong antimicrobial activity [42]. Parasin-1 is an inducible antimicrobial peptide that is only found in

the skin mucus layer of injured catfish. This allows the AMP to provide protection against invading microbes at the site of injury [42].

Histones are not only found in the cytoplasm and on the membrane of cells, but also exist as binding components of neutrophil extracellular traps (NETs) [43]. Histones (especially histone H1), DNA and granular proteins are the main components of NETs [44]. Upon activation, neutrophils release granule proteins and chromatin that form extracellular fibers that bind bacteria [43]. NETs are an important form of innate immunity because they bind and kill bacteria, degrade virulence factors and prevent the spread of microbes [43]. The fibrous structure of NETs is necessary to trap the bacteria and ensure that a high local concentration of antimicrobial peptide is delivered to the bound microbes [43]. NETs are released from fish kidney neutrophils and as such may be important in teleost innate immunity [44].

Channel Catfish (*Ictalurus punctatus*)

The channel catfish (*Ictalurus punctatus*) is the most important commercially cultured aquatic species in the United States. They were first described by Samuel Rafinesque in 1818 [45, 46]. The channel catfish is native to St. Lawrence-Great Lakes, Hudson Bay, and the Missouri-Mississippi River basins from lower Quebec south through the United States to the Gulf of Mexico [45, 47]. They have been introduced throughout most of the United States, Cyprus, Romania, Slovakia, Spain and the Czech Republic [45, 47, 48].

Farm raising catfish is a multimillion dollar business in the United States. Commercial aquaculture of catfish first became economically practical in the 1950s allowing the catfish farming industry to grow rapidly [46]. At least 90% of farm-raised catfish are still produced in

the Mississippi River Valley region [46]. The main catfish producers are the United States, Russia, Guatemala, Cuba, Costa Rica and Paraguay [46].

Channel catfish are mostly found in freshwater lakes, reservoirs, ponds, streams, rivers and creeks, but can also survive in salt water and brackish water [45, 49]. During the day, catfish dwell in deep holes, overhangs, other shelter or at the bottom of the water [45]. Channel catfish are opportunistic omnivores (they eat any plant or animal material that can be found) [46]. They prefer to live in clear water with muddy surface bottoms [50]. They detect food through their sense of taste using taste buds that are present over the entire external surface of the catfish and in the mouth, gills and barbels [49]. Channel catfish grow best in temperate climates that have warm water because their metabolism increases and decreases with temperature fluctuations [49].

Channel catfish are ray-finned fish that have cylindrical bodies with no scales [46, 49]. They have spotted, darker colored backs and white bellies [45, 46, 49]. The color of the catfish changes depending on the color of the water they inhabit [49]. They have hard spines on their pectoral and dorsal fins that are used for protection and the remaining rays are soft [45, 46]. The characteristic feature of the channel catfish is a deeply forked caudal fin where the top of the fin is larger than the bottom portion [45, 49]. Catfish also have 2 barbels on their upper jaw and 4 on their lower jaw [45].

***Edwardsiella ictaluri*: Characteristics and Pathology**

Edwardsiella ictaluri is the causative bacteria of enteric septicemia of catfish (ESC). *E. ictaluri* was first isolated from catfish farms in Georgia and Alabama in 1976 [51]. *E. ictaluri* is

an important disease in farm-raised channel catfish due to annual economic losses. In 2002, 60.6% of food-size catfish farms reported ESC, making it the highest reported problem in catfish ponds [52]. In 2003, 300 million kilograms of food-size fish, valued at approximately $400 million, were produced [52]. Typically 45-50% of the fish will be lost to disease causing a loss of as much as $100 million annually to the catfish industry [52]. ESC outbreaks occur at a temperature range of 18-28°C making spring and autumn peak times for infection [53].

ESC occurs throughout the geographic range of the catfish industry, although it is primarily a pathogen of the Southeastern United States [54]. The channel catfish is most susceptible to infection by *E. ictaluri*. White catfish, brown bullhead, walking catfish, and some ornamental fish (danio, green knife fish, and rosy barb) are also susceptible [54].

Edwardsiella ictaluri is a Gram-negative rod shaped bacteria [55]. It is a member of the Enterobacteriacea family along with *Klebsiella pneumoniae* and *Proteus mirabilis* [55, 56]. Members of the Enterobacteriacea family are gram-negative, straight rod shaped bacteria that are found worldwide in soil, water, plants and animals [56]. *E. ictaluri* is a facultative anaerobe that is oxidase-negative, glucose-positive and nitrate-negative [55]. It is an obligate pathogen [53] and has peritrichous flagella that makes it weakly motile [55]. *E. ictaluri* lacks proteases, lipases, esterase, collagenase, chitinase and hyaluronidase [55]. The ability of *E. ictaluri* to degrade chondroitin sulfate (a component of cartilage) allows the bacteria to form a "hole-in-the-head" lesion on catfish [55]. Since *E. ictaluri* is very biochemically and serologically homogenous, it is a good vaccine candidate [55, 57].

The clinical presentation of *Edwardsiella ictaluri* is characterized by both behavioral and physical (both internal and external) signs. Infected fish begin to swim in tight circles and hang in the water column with their head up and tail down [54]. Fish with ESC also stop eating

shortly after infection [54]. External signs of *E. ictaluri* infection are red and white ulcers on the skin, petechial hemorrhaging (pinpoint red spots) under the head and in the ventral region, longitudinal "pimples" between the eyes at the cranial foramen that can turn into a "hole-in-the-head" appearance and a swollen abdomen [54]. The internal signs of ESC are clear, straw-colored, or bloody fluid in the ceolomic cavity, pale areas of tissue destruction in the liver, petechial hemorrhaging in the muscles, intestine and fat and bloody fluid in the intestine [54].

ESC can either be an acute or chronic infection. The acute infection is characterized by petechial hemorrhages in the mouth, throat, abdomen and at the base of the fins [53, 58]. The earliest signs of acute infection occur at 2 days post-infection with the appearance of cutaneous hemorrhage and ulceration [59]. Gross lesions, mostly hemorrhaging at the base of the fins, are more evident at 4 days post-infection [59]. The bacteria can quickly pass through the mucosa. Positive bacterial cultures have been identified from the trunk kidney within 15 minutes of exposure to the pathogen [60]. At 24 hours post-infection, bacteria can be found in phagocytes in the blood and intestine [60]. By 72 hours post-exposure, bacteria are found in the vacuoles of enterocytes and hepatocytes [60]. Chronic ESC results in granulomatous inflammation of the olfactory bulb and erosion of the skin and muscle of the skull that results in exposure of the brain [53, 58]. Signs of chronic ESC do not appear until 3-4 weeks post-infection [59].

Diagnosis of *Edwardsiella ictaluri* infection is typically performed by identifying cultured bacteria. Bacteria will be cultured and isolated from either organs (such as the liver or spleen) or brain tissue [54]. The bacteria is streaked onto blood agar plates and allowed to grow for 48 hours. The appearance of extremely small, white punctate colonies is characteristic of *E. ictaluri* [54].

Certain factors increase catfish susceptibility to *E. ictaluri* infection. Handling, close confinement, fluctuating water temperature, improper diet and poor water quality can stress the catfish increasing their susceptibility to ESC [54]. Fish on a regular diet that are fed daily or every other day are more resistant to ESC [61]. Improperly fed fish become anemic and have less macrophage chemotaxis during *E. ictaluri* infection, making them more susceptible to disease [61].

The most effective treatment of ESC consists of feeding antibiotic-containing feeds to infected fish [54]. *E. ictaluri* is susceptible to most antimicrobial agents that are active against Gram-negative bacteria. These agents include: aminoglycosides, cephalosporins, penicillins, quinolones, tetracyclines, chloramphenicol and nitrofuratonin [62]. *Edwardsiella ictaluri* is resistant to antimicrobial agents against Gram-positive bacteria [62]. Studies have shown that *E. ictaluri* has no evidence of developing resistance and has no unusual antimicrobial resistance [62].

Methods to prevent ESC outbreaks have the most potential to protect channel catfish. Current methods include minimizing stress, improving nutrition and genetically improving stocked fish [54]. Vaccination is another possible method of preventing ESC. The first vaccine, a killed-bacteria vaccine, had low effectiveness [53]. However vaccination with low doses of *E. ictaluri* resulted in strong acquired immunity [63]. Vaccination with a highly attenuated, auxotrophic strain of *E. ictaluri* led to a significant decrease in mortality following infection [64]. A live-attenuated *E. ictaluri* strain that can invade, persist, and stimulate cell mediated immunity would be the best vaccine target [54, 63, 64].

Differences in immunological function can be detected between control and infected catfish during infection with *Edwardsiella ictaluri*. Microarray analysis showed that ESC caused

the induction of several components of the MHC-class 1 pathway that were detectable in the liver 3 days post-infection [65]. Expression of TLR3 and TLR5 changed following infection with *E. ictaluri* [66]. TLR5 had increased expression in the kidney and liver on day 5 post-exposure [66]. TLR3 increased to a lesser extent in the kidney and the spleen over time [66].

Edwardsiella ictaluri infection can alter the expression of antimicrobial peptides in catfish. Hepicidin, an antimicrobial that is found in a variety of catfish tissues, is induced after infection with *E. ictaluri* [67]. Hepicidin is up-regulated in the spleen and head kidney at 1-3 days post-infection and returns to normal levels by day 7 [67]. This shows that antimicrobial peptides may play a role in ESC.

Teleost Immunology

The evolution of fish and tetrapods diverged 300 million years ago [68]. Fish can be studied to investigate the evolution of lymphoid tissue and the development of the immune system because fish have a less developed immune system [68, 69]. The innate immune system is the primary defense mechanism of fish [70]. Thus teleosts are an excellent model for studying innate immunity. Fish, especially zebrafish, can be used as a model of vertebrate immunology [68]. Fish are also a useful tool for monitoring the quality of the environment [68]. Another important reason to study fish immunology is the prevalence of disease outbreaks in the aquaculture industry that leads to substantial economic losses [68].

The innate immune system of fish is more active and diverse than the mammalian counterpart [70]. Innate immune defenses are non-specific, do not rely on specific recognition of invaders, have no or little time lag before action and are relatively temperature independent [71].

The innate immune system consists of physical barriers and cellular and humoral components [70]. Fish scales, mucus and the epidermis act as a first barrier of defense [70, 72]. Key cells of the teleost innate immune system are phagocytic cells (e.g. granulocytes/neutrophils and monocytes/macrophages), nonspecific cytotoxic cells (NCC), epithelial cells and dendritic cells [70].

Two molecular patterns are capable of inducing a teleost innate immune response: foreign or pathogen associated molecular patterns can induce innate immunity [70] and molecular patterns exposed through damage of host tissue due to infection, necrosis or a danger signal to the innate immune system can cause activation of defense mechanisms [70]. In response to bacterial pathogens, broad-spectrum antimicrobial substances are produced, the non-classical complement system is activated, cytokines are released and inflammation and phagocytosis occur [71]. The anti-viral immune response in fish consists of the cytotoxic activity of NCC against viral infected cells and interferon production [69, 71]. Another function of the teleost innate immune system is to initiate and direct the limited adaptive immune response [73].

Teleost acquired immunity is inefficient due to a limited antibody repertoire and slow lymphocyte maturation [70]. Teleosts have a more short-lived adaptive immune response than mammals [18]. Teleosts have the functional equivalents of B (IgG positive) and T cells (IgG negative) [68]. Teleosts only express one type of immunoglobulin (Ig), not the usual 5 types, in plasma, skin and gut mucosa, and bile [18]. Teleosts also lack specialized lymphoid organs [18]. The major lymphoid organs of fish are the thymus, kidney, spleen and gut-associated lymphoid tissue [68]. Lymphocytes reside primarily in the spleen and kidney and do not enter the thymus

while circulating [68]. Lymphocyte trafficking may be modulated by physiological (endocrine) mechanisms [68].

Fish lack both lymph nodes and bone marrow [68]. The head kidney is more structurally analogous to bone marrow than lymph nodes [74]. The kidney is divided into two distinct sections: the anterior (or head) kidney and the middle and posterior (or trunk) kidney [74]. The anterior kidney has no renal activity, phagocytic cells, proliferative hematopoietic activity, no lymphatic vessels or high venous endothelium, thin walled arteries (characteristic of bone marrow) and plasmacytes [74]. The trunk kidney has less hematopoeitic activity and has renal tubes [74].

The anterior kidney primarily contains macrophages, but granulocytes and NCC are also present [68, 75]. The anterior kidney is the major site of B cell development [76]. B cells migrate from the anterior kidney to peripheral blood to the immune tissue where they encounter antigen. The B cell then becomes a plasmablast, proliferates and migrates back to the anterior kidney [76]. The total number of lymphocytes in the anterior kidney is greater than the number in the peripheral blood and spleen (ratio of 10:4:1) [76].

Stress plays a major role in teleost innate immunity. Stress from handling and crowding can suppress the immune system of fish [70]. Fluctuations in water temperature, oxygen tension and water salinity can also increase the susceptibility of fish to disease [69, 70].

Nonspecific Cytotoxic Cells (NCC)

Nonspecific cytotoxic cells (NCC) are the teleost equivalent of mammalian natural killer (NK) cells [77, 78]. NCC are small nucleated cells found in the anterior kidney, spleen, liver,

and peripheral blood of several species of fish [75]. NCC have been described in catfish [77], tilapia [75], rainbow trout [79] and gilthead seabream [80]. The discovery of granzymes in tilapia cytotoxic cells suggests that NCC contain small granules [81].

NCC from fish were first shown to lyse human B-cells, erythroblastoid cells, and mouse cells [77]. The highest NCC activity was found in the anterior kidney, but NCC were also active in the spleen and peripheral blood [77]. NCC have cytotoxic activity against tumor cells, virus transformed cells and protozoans. NCC specifically lyse *Tetrahymena pyriformis*, an opportunistic parasite in fish, by binding to cell surface determinants on the protozoan [82]. Tumor cells (K562) and virus transformed cells (YAC-1) are also targets of NCC cytotoxicity [83]. NCC recognize a target cell ligand called NKTag. The specific determinant of NKTag is 7-9 amino acids long and is found on protozoan and tumor cells [83, 84]. A study using MAPs (multiple antigenic peptides) that are synthetic peptides of NKTag suggested that ligand activation of NCC was specific to the antigen and that NCC receptor crosslinkage was required to activate NCC cytotoxic activity [84].

Nonspecific cytotoxic cell receptor protein-1 (NCCRP-1) is a type III membrane receptor protein that was isolated from NCC of catfish and zebrafish [85-87]. NCCRP-1 is a proline rich protein that has 2 glycosylation sites [85]. Eighteen percent of the amino acids are serine, threonine or tyrosine and function as potential phosphorylation sites [85]. The N-terminus of the protein has proline rich-motifs (PRM) that are docking sites for JAK kinases [86]. After NCC activation, NCCRP-1 comes in contact with JAK kinases and STAT6 translocates to the nucleus [86]. NCCRP-1 has two roles in NCC: NCCRP-1 is an antigen recognition molecule needed to induce target cell lysis and cytokine release [86].

An anti-NCCRP-1 monoclonal antibody was produced [78]. The monoclonal antibody (5C6) binds to NCC and based on flow cytometric analysis, NCC from the anterior kidney are 25% positive, spleen are 42% positive and peripheral blood 2.5% positive [78]. Binding of 5C6 to NCCRP-1 on NCC initiates signaling that leads to increased cytotoxic activity [85].

NCC and NK cells are similar innate immune lymphocytes regarding many fundamental characteristics. Mammalian natural killer cells and NCC are non-T and non-B cells (NK cells however are large granular lymphocytes) [75, 81]; both cell types contain cytotoxic granules [75]; NK cells and NCC are found in the peripheral blood, spleen, and liver [75]; both cell types lyse transformed target cells and mediate non-lytic immunoregulatory processes; and NK cells respond to cytokine activation by producing altered growth characteristics and target cell specificity [75]. NK cells have a cytotoxicity response characterized by granule exocytosis, receptor-ligand binding and soluble lytic factor and cytokine release [75].

NCC and NK cells are also similar in granule content and in mechanisms of granule exocytosis [88]. Catfish store pre-formed, tryptase-like granzymes that are released toward the target cell upon conjugate formation. Teleost NCC have multiple effector molecules of the granule exocytosis pathway that are very similar to mammalian cytotoxic lymphocytes [88].

Stress and temperature change can lead to altered NCC cytotoxic activity in fish. Measurment of the acute innate cytotoxic activity showed that NCC isolated from the peripheral blood of stressed tilapia had 30 times greater cytotoxic activity compared to unstressed fish [89]. However, NCC isolated from the anterior kidney and spleen had decreased cytotoxic activity [89]. Low *in vivo* temperatures increased NCC activity whereas high temperature had no effect on cytotoxicity [90]. This indicated that temperature had a direct effect on immune function and that non-specific immunity offset specific immune suppression at low temperatures [90].

NCC participate in anti-bacterial immunity by releasing cytokines upon interaction with bacterial products thus enhancing the inflammatory response at the site of infection [82, 91]. The distribution of NCC in catfish shifted following infection with *Ichthyopthirius multifiliis* [91]. NCC activity decreased in the anterior kidney of infected fish and increased in the peripheral blood [91]. The peripheral blood of infected catfish had an increased percentage of NCC with increased killing activity suggesting that active NCC function in enhancing the immune response at the site of infection [91]. Following infection of tilapia with *Streptococcus iniae*, the cytotoxic activity of NCC isolated from the peripheral blood increased 100 percent compared to control [92]. NCC from the spleen and anterior kidney had no increased cytotoxicity [92].

Oligodeoxynucleotides, motifs that mimic bacterial DNA, specifically bind to NCC [93, 94]. Oligodeoxynucletides (dG20 and CpG) induce cellular DNA synthesis, proliferation and calcium flux (evidence of cellular signaling) [94]. ODN binding to NCC also up-regulates membrane expression of NCCRP-1 [93]. The ODN binding protein on NCC and YT-INDY cells has been identified as the 29 kDa protein. It is refered to as NCAMP-1 (NCC antimicrobial protein-1) [93, 94]. Oligodeoxyguanosine (dG20) activated NCC cytotoxicity, but dT20, dA20, and dC20 had no effect on cytotoxicity [95]. Bacterial DNA from isolates of *S. iniae* activated cytotoxicity. This data suggested that NCC recognize bacterial nonmethylated DNA (ODN) motifs [95].

NCC as well as NK cells involve soluble Fas ligand (sFasL) in the regulation of programmed cell death [96-98]. Apoptosis in teleost cells is characterized by chromatin condensation, nuclear peripheralization, formation of apoptotic bodies and the appearance of electron-dense micronuclei [96]. The Fas ligand must interact with the Fas receptor on the target

cell to initiate programmed cell death. NCC produce and secrete FasL following binding of NCCRP-1 and single-base oligodeoxynuclotides (dG20) to the cell membrane and tumor cell ligation [96, 97, 99]. Soluble FasL has been detected in the anterior kidney, peripheral blood, and liver of tilapia [98]. Catfish and tilapia NCC also constitutively express cytosolic FasL [92, 96]. Stress serum treated NCC from the peripheral blood produced increased levels of sFasL showing that cytokine factors in the serum of stressed teleosts can increase cytotoxicity by stimulating the expression of proteins involved in the activation of programmed cell death [92]. Activation of cytotoxic activity following stress may be a consequence of increased apoptotic control [96]. Bacteria (e.g. *S. iniae*) may also immunoregulate NCC by reducing normal levels of apoptosis while increasing necrosis which would lead to increased cellular recruitment and better primary and secondary antibody and cell mediated immune responses [100].

NCC also participate in tumor surveillance and can bind and lyse tumor cells [101]. NCC activation following binding of mAb 5C6 produced increased levels of expression of cytoplasmic *src* family proto-oncogenes (*lck*, *fyn*, and *src*) [102]. NCC receptor stimulation also increased the expression of protein kinase C [102].

The responses of NCC to pathogen associated molecular pattern (PAMP) ligands and associated expression of pattern recognition receptors was previously directly demonstrated by showing that NCC express a membrane Scavenger receptor orthologue that bound to polyguanosine PAMP [103]. NCC from the anterior kidney, spleen, and peripheral blood of catfish and tilapia expressed Scavenger receptor class A (SR-A) on their membrane [103]. NCC expression of a teleost orthologue of mammalian SR-A suggested that NCC participate in innate immune functions [103].

NCAMP-1

NCAMP-1 (Nonspecific cytotoxic cell cationic antimicrobial peptide-1) is a novel pattern recognition receptor that was first purified from catfish NCC [88]. It is expressed on catfish, zebrafish, mouse and tilapia cells [88]. Analysis of cDNA showed that NCAMP-1 is similar to histone H1X in xenopus, zebrafish, mouse and humans and is not identical to any other previously described protein [88]. NCAMP-1 is a strongly basic, 29 kDa protein that contains 58 strongly basic, 55 hydrophobic and 50 polar amino acids [88]. The N-terminus portion is lysine, alanine, and proline rich [88]. NCAMP-1 is a pattern recognition receptor that binds oligodeoxynucleotides (ODNs) that are bacterial DNA motifs [88]. Based on flow cytometric analysis using anti-NCAMP-1 polyclonal and monoclonal antibodies, NCAMP-1 is expressed on the membranes of catfish NCC [88]. NCAMP-1 is an antimicrobial peptide. Full-length and truncated forms of recombinant NCAMP-1 effectively kill *Escherichia coli* and *Streptococcus iniae* isolates *in vitro* [88].

Pattern Recognition Receptors/Toll-like Receptors

An important function of innate immunity is the recognition of non-self. The ability of the innate immune system to differentiate between self and non-self developed in early metazoan evolution [104]. Pattern recognition receptors (PRRs) are germ-line encoded receptors that recognize conserved molecular patterns (pathogen associated molecular patterns, or PAMPs) on microbial pathogens that are essential to microbial survival [104, 105]. PAMPs are recognized

by the innate immune system as a molecular signal of infection, only produced by microbes (not host cells), not subject to high mutation rates, and invariant between organisms of the same class [104, 106]. Some examples of PAMPs are lipopolysaccharide (LPS) of Gram-negative bacteria, techoic acid of Gram-positive bacteria, double-stranded RNA (dsRNA) of viruses and mannans of yeast cell walls [105]. Pattern recognition receptors recognize PAMPs directly and bind to the microbial cells marking them for destruction by complement or phagocytosis [104].

Toll-like receptors (TLRs) are an important class of PRR that were first identified in Drosophila [106]. TLRs are expressed on host cell surfaces and activate signaling pathways [104, 107]. TLRs recognize endogenous and exogenous ligands (PAMPs) [108]. TLRs are type 1 transmembrane receptors [109]. TLRs are identified as genes that have a code for both an extracellular, N-terminal leucine-rich repeat (LRR) domain and a C-terminal Toll-Il-resistance (TIR) domain (initiator of signal transduction) [108, 109].

TLRs activate signaling pathways that induce an immune response against microbes [104, 107]. All TLRs use the common adaptor protein, MYD88, that has a TIR domain (an evolutionarily conserved protein-protein interaction molecule) and a death domain [106, 107]. MYD88 associates with the TLR during activation and recruits IRAK [107]. All TLRs activate NF-κB transcription factors during the signaling cascade [107]. NF-κB binds to promoters on different genes and activates the transcription of TNF-α, IL-1, and IL-6 [106, 107].

TLRs are classified into 6 major families with each family recognizing a general class of PAMP [108]. The TLRs and their associated PAMP are: TLR3 (dsRNA), TLR4 (LPS), TLR1,2, and 6 (lipoprotein and peptidoglycan of gram-positive bacteria), TLR5 (bacterial flagellin) and TL9 (unmethylated CpG dinucleotide motifs in bacterial and viral genomes) [107]. Teleosts

express either protein or mRNA from each of the six major families of vertebrate TLRs (TLR1, TLR3, TLR4, TLR5, TLR7, TLR11) [108].

Zebrafish express TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 [110]. They also have adaptor genes MYD88, MAL, TRIF and SARM [110]. Expression levels of TLR1, TLR2, TLR5, and TLR9 increased following infection with *Mycobacterium marinum* [110]. This study also showed that levels of expression of MYD88, TRIF and SARM were unchanged [110].

TLR4 is a central part of the receptor complex that induces the immune response to LPS [111, 112]. This complex involves CD14 on the cell surface binding LPS directly with the aid of LPS binding protein (LBP) which accelerates binding to TLR4 leading to signal activation [111]. The immune response to LPS leads to endotoxic shock in mammals. Fish, however, are resistant to endotoxic shock [112]. Differences in the biological response to LPS between fish and mammals are due to differences in receptor mediated recognition of LPS [112]. Some key differences between LPS recognition and response in mammals and fish are: trout LBP transfers LPS to HDL cholesterol instead of CD14, high concentrations of LPS are needed to activate fish leukocytes and CD14 is only found in mammals and birds [112].

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CHAPTER 3

ROLE OF NONSPECIFIC CYTOTOXIC CELLS IN BACTERIAL RESISTANCE: EXPRESSION OF A NOVEL PATTERN RECOGNITION RECEPTOR WITH ANTIMICROBIAL ACTIVITY[1]

[1]Connor, M.A., L. Jaso-Friedmann, J. H. Leary III and D. L. Evans. Submitted to *Infection and Immunity*, 2/19/2008.

Abstract

Pattern recognition receptors (PRR) recognize invariant bacterial, viral, protozoan and certain synthetic ligands. PRR may be expressed as outer membrane (or endosomal) or cytosolic proteins and function to signal cell activation processes during inflammation responses. In the present study, a novel membrane receptor (NCAMP-1) is described that is expressed on nonspecific cytotoxic cell (NCC) membranes and is found in granule preparations from these cells. In recombinant form, full-length and truncated forms of NCAMP-1 had antibacterial activity against bovine, avian and lab strain *E. coli*. Recombinant NCAMP-1 also killed the gram negative fish pathogen *Edwardsiella ictaluri*. Maximal bacterial killing of a representative avian *E. coli*, APEC 3721, occurred at 60 minutes post-treatment with 2μg/ml of NCAMP-1. Polyclonal antibody anti-NCAMP-1 specifically neutralized the antimicrobial activity of recombinant NCAMP-1 against *E. coli* APEC 3751. Expression of NCAMP-1 as a NCC membrane protein was analyzed by flow cytometry using anti-NCAMP-1 monoclonal antibody 9C9. NCAMP-1 has been shown to have a two-fold up-regulation of expression on NCC membranes following *in vitro* activation (1 hour) with PMA/calcium ionophore. Merged images from immunofluorescence microscopy showed that NCAMP-1 and the NCC receptor protein (NCCRP-1) are co-expressed on NCC membranes. NCAMP-1 was identified as a constituent of acetic acid granule extract preparations from catfish NCC by Western blot analysis using polyclonal anti-NCAMP-1 antibodies. These studies were confirmed by determining the specific bactericidal activity of granule preparations from catfish NCC. The data suggested that NCAMP-1 is a membrane signaling protein and may participate in antibacterial innate immunity by granule exocytosis during inflammatory responses in teleosts.

Introduction

Nonspecific cytotoxic cells (NCC) are the teleost equivalent of mammalian NK cells [1-2]. The similarities that exist between NCC and the mammalian counterpart relate to specific target cells that are susceptible to lysis [2]; similarity in granule content and mechanisms of exocytosis [3]; and NCC as well as NK cells involve Fas ligand [4-6] in the initiation of apoptosis of target cells. The participation of NCC at other levels of innate immunity of teleosts has been shown. The responses of NCC to pathogen associated molecular pattern (PAMP) ligands and associated expression of pattern recognition receptors was previously directly demonstrated [7] by showing that NCC express a membrane Scavenger receptor orthologue that bound to polyguanosine PAMP. The direct expression of other PRR proteins by teleost cells has been more elusive.

The major class of PRR that has been described in teleosts belongs to the toll-like receptor (TLR) family. Teleosts express either protein or mRNA from each of the six major families of vertebrate TLRs [8]. These classes include TLR1, TLR3, TLR4, TLR5 and the TLR7 and TLR11 subfamilies [8]. In the present study, we introduce another class of proteins that demonstrate all of the characteristics of PRR: promiscuous binding to multiple ligands; constitutive expression on the cell membrane; up-regulated expression following PMA/calcium ionophore treatment; and expression by histologically different types of cells. This new class has been suggested by others [9-18] as consisting of cytosolic and membrane histones, especially linker histone-1. These studies have demonstrated the expression of H1 linker proteins on the membranes of many different types of cells. More recently [19] a membrane histone-like protein was identified from catfish NCC. This protein was referred to as NCC cationic antimicrobial

protein-1 (NCAMP-1). In recombinant form NCAMP-1 had relatively high levels of bacterial cell killing activity. Although previous studies [20-23] by others demonstrated the antimicrobial activity of soluble H1 and H2-like histones derived from various fish tissue, these antimicrobial histones were not also expressed as membrane receptors and they were not obtained from granules such as is the case for NCAMP-1 [19]. Additionally, as a membrane protein, NCAMP-1 demonstrated PRR-like properties such as binding to dinucleotides GpC and nonmethylated CpG [19]. In the present study, we propose that NCAMP-1 is a membrane protein that is also expressed in granules. As such it is expressed in many vertebrate species and may be an important effector of innate immune responses. We further characterize the kinetics and specificity of NCAMP-1 killing of enteropathogenic *E. coli* and the fish pathogen causing hemolytic septicemia (*Edwardsiella ictaluri*). Membrane and granule extracts from NCC are also shown to contain NCAMP-1 and granule extracts from NCC produced high specific activity killing.

Materials and Methods

Cell preparations. Fingerling channel catfish (*Icatalurus punctatus*) were maintained in tanks at 23-25°C in a flow-through water system. Single cell suspensions of anterior kidney cells (bone marrow equivalent) from channel catfish were obtained by physical disruption through a mesh screen. The cells were placed in RPMI-1640 (Mediatech, cat. no. 50-020-PB, Manassas, VA) supplemented with sodium bicarbonate, gentamycin (50 mg/ml), 2-mercaptoethanol, HEPES (25mM), L-glutamine (200mM) (Mediatech, cat. no. 25-005-CI, Manassas, VA), Sodium pyruvate (100mM) (Mediatech, cat. no. 25-000-CI, Manassas, VA),

MEM Vitamin Solution (Mediatech, cat. no. 25-020-CI, Manassas, VA), MEM Amino Acid Solution (Mediatech, cat. no. 25-030-CI, Manassas, VA), MEM Nonessential Amino Acid Solution (Mediatech, cat. no. 25-025-CI, Manassas, VA) and 10% fetal calf serum (Atlanta Biologicals, cat. no. S11150, Norcross, GA). Using a 45.5% Percoll cushion (Sigma, P1644, St. Louis, MO), leukocytes were separated from red blood cells by centrifugation for 1 hour at 700 x g. Cells were collected from the cushion and washed 2 times with RPMI-1640. The cells were suspended in PBS (phosphate buffered saline) and counted.

Anti-NCAMP antibodies. Monoclonal antibodies with specific activity against NCAMP-1 were developed in-house. Full-length recombinant NCAMP-1 was used as antigen and prepared by excising the protein from stained SDS-PAGE gels. Briefly, after electrophoresis, gels were stained with 0.3 M $CuCl_2$ and rinsed with dH_20. The gel band corresponding to NCAMP-1 was excised, cut into pieces, destained with 0.25 M Tris-Cl and 0.25 M EDTA (pH 9.0) and lyophilized. Dried gel pieces were reduced to a powder by grinding in a tissue homogenizer fitted with a Teflon coated pestle. The fine powder was transferred to a 2 ml glass syringe and re-swollen with 500µl of sterile saline. Incomplete Freund's adjuvant was added (500µl) and the mixture was emulsified using a dual hub emulsifying needle. The emulsion was injected intraperitonealy into female Balb/c mice. Mice were boosted with similar antigen preparations in incomplete Freund's adjuvant every 21 days. Serum was tested by ELISA for the presence of anti-NCAMP-1 antibodies. Mice were sacrificed 5 days after the last boost. Spleens were harvested and single cell suspensions were prepared for fusion with SP2/0 myeloma cells using the ClonaCell™-HY Hybridoma Cloning Kit (Stem Cell Technologies, Vancouver, BC, Canada) as per manufacturer's instructions. Isolated colonies were picked from the semi-solid selection media after 10-14 days and transferred to 96 well plates for expansion.

Supernatants were evaluated for anti-NCAMP-1 activity by ELISA. Positive clones were refed and retested by ELISA and for binding to NCC by flow cytometry. Several positive wells were selected for recloning according to manufacturer's instructions.

Tissue culture supernatants (TCS) from clone 9C9 (IgM isotype) or IgM isotype control were first concentrated approximately 20-fold by tangential flow ultrafiltration (MiniKros Module, 500,000 kDa molecular weight cutoff, Spectrum Laboratories, Rancho Domiguez, CA). Concentrated TCS was further purified using HiTrap IgM purification columns (GE Healthcare) according to manufacturer's instructions. Purified IgM was quantitated using an antibody capture ELISA developed in house. Purified mab was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to manufacturer's instructions.

Rabbit polyclonal anti-NCAMP-1 [19] and monoclonal antibody 5C6 (anti-NCCRP-1 were prepared as previously described [2].

ELISA Detection of Anti-NCAMP mabs. An indirect sandwich ELISA was used to detect NCAMP-1 specific antibodies in tissue culture supernatants. Recombinant NCAMP-1 was diluted in PBS with 0.05% sodium azide to 5μg/ml and 50μl/well was added to Immulon II (High Binding) 96 well flat bottomed plates. Plates were incubated overnight at room temperature followed by extensive washing with water. Blocking buffer (borate-buffered saline with 0.05% Tween 20, 1 mM EDTA, 0.25% BSA, 0.05% sodium azide) was added and incubated for 1 h at 37°C or overnight at 4°C. Blocked wells were washed 4 times with dH_2O. Test supernatants (or serum) were added and incubated for 1 h (room temperature) followed by 4 water washes. Peroxidase-conjugated goat anti-mouse IgM (Pierce #31440, Rockford, IL) or IgG (Pierce #31437, Rockford, IL) was diluted (1:2000) in blocking buffer and added to the appropriate wells for 1 hour at room temperature. Plates were washed 4 times with water. Turbo

TMB substrate (Pierce, 34022, Rockford, IL) was added and incubated in the dark for 15-20 min. Sulfuric acid (2 N, 25µl) was added to stop the reaction. Absorbances were read at 450 nm with a Biotrak II plate reader (GE Healthcare).

MACS separation. Flow cytometry experiments were performed using non-permeabilized NCC in PAB (phosphate buffered saline containing 0.1% sodium azide and 1.0% bovine serum albumin (Sigma, St. Louis, MO)). The $5C6^+$ NCC were enriched using the MACS separation technique with 5C6 monoclonal antibody and rat anti-mouse IgM microbeads (Militenyi Biotec, Auburn, CA, no.130-047-302) according to manufacturer's instructions. After incubation, the cells were stained with goat anti-mouse IgM-FITC conjugate (Sigma, F9259, St. Louis, MO). The $5C6^+$ enriched NCC were used for flow cytometry and microscopy.

Flow cytometry. Two-color flow cytometric analysis was performed using $5C6^+$ enriched NCC. The cells were stained with biotinylated 9C9 or IgM isotype control at 4°C for 30 minutes. Streptavidin-PE conjugate (eBioscience, 12-4317-87, San Diego, CA) was added for 20 minutes at 4°C. The cells were analyzed using an EPICS XL-MCL four color analyzer (Coulter Electronics Corp, Hileah, FL) equipped with a 15mW air cooled argon-ion laser. FITC was detected by photomultiplier tube 1 (PMT1) using the 525 nm bandpass filter. PE was detected by PMT2 using the 575 nm bandpass filter. Data was analyzed using Coulter's System II software, version 3.0.

Immunofluorescent microscopy. NCC were harvested from the anterior kidney of channel catfish. $5C6^+$ NCC were enriched using MACS separation with rat anti-mouse IgM microbeads (Militenyi Biotec, Auburn, CA, no.130-047-302). Viability counts were performed yielding 95-100% viability. The cells were stained with biotinylated 9C9 at 4°C for 30 minutes. Streptavidin-PE conjugate (eBioscience, 12-4317-87, San Diego, CA) was added for 20 minutes

at 4°C. Cells were settled onto Poly-L-Lysine coated slides for 30 minutes at room temperature, followed by fixation with 4% paraformaldehyde. Coverslips were mounted using Prolong Gold Anti-fade (Invitrogen, Carlsbad, CA). An Olympus IX70 fluorescent microscope with a 100W high pressure mercury burner was used. Pictures were taken with an Olympus DP 70 Digital Microscope Camera using DP Controller software and analyzed with DP Manager software.

Bacterial culture and identification of the isolates. Clinical isolates of bovine *Escherichia coli* SS1 and SS2 were kindly provided by Dr. Susan Sanchez (Athens, GA, University of Georgia, College of Veterinary Medicine). Isolates of avian *E. coli* APEC 3721 and APEC 96-3437 were donated by Drs. John Maurer and Margie Lee (Athens, GA, University of Georgia, Poultry Diagnostic Research Center Diagnostic Lab). *Edwardsiella ictaluri* isolates were obtained from Dr. Andy Goodwin (Pine Bluff, AR, UAPB, Aquaculture/Fisheries) (Table 1.1). *E. coli* was cultured in Muller-Hinton (MH) broth at 37°C and *E. ictaluri* was cultured in brain heart infusion (BHI) broth at 27°C. Cultures of both bacterial species were grown for 18 hours and then used to inoculate fresh media (1:4 dilution) for 2 hours until an optical density of A_{600}=1.0 was reached. The relationship between A_{600} and CFU per ml has been previously determined in our lab.

Antimicrobial killing assay using rNCAMP-1. Bacterial cultures were diluted to 10^5 CFU per ml in 10mM sodium phosphate buffer (pH 8.0). Recombinant NCAMP-1-NT (N-terminus) and NCAMP-1-CT (C-terminus), purified as previously described [19], were diluted in 10 mM sodium phosphate buffer (pH 8.0) to the indicated protein concentrations. Bacteria (5000 CFU) were added to each tube in an equal volume and incubated at either 37°C (*E. coli*) or 27°C (*E. ictaluri*) for 2 hours. Controls included Polymyxin b (1μg/ml) and Kanamycin (1μg/ml) (positive controls) and the His-tag column buffer used for recombinant protein purification

Table 1.1. Clinical isolates used for antimicrobial killing assays with NCAMP-1.

Bacterial Isolate	Source	Clinical Presentation
Escherichia coli DH5α	Reference strain	none
Escherichia coli APEC 3721	Chicken (air sac)	
Escherichia coli APEC 96-3437	Chicken (yolk)	
Escherichia coli SS1	Cow	Bovine mastitis
Escherichia coli SS2	Cow	Bovine mastitis
Edwardsiella ictaluri PB 04-93	Channel Catfish (blood)	Enteric septicemia
Edwardsiella ictaluri 97-773	Channel Catfish	Enteric septicemia
Edwardsiella ictaluri 97-779	Channel Catfish	Enteric septicemia
Edwardsiella ictaluri LN 01-379B	Channel Catfish (lymph node)	Enteric septicemia
Edwardsiella ictaluri PB 97-40	Channel Catfish (blood)	Enteric septicemia
Edwardsiella ictaluri PB 97-30	Channel Catfish (blood)	Enteric septicemia
Edwardsiella ictaluri PB 99-129	Channel Catfish (blood)	Enteric septicemia
Edwardsiella ictaluri PB 06-395	Channel Catfish (blood)	Enteric septicemia
Edwardsiella ictaluri AL 98-75	Channel Catfish (vaccine strain)	Enteric septicemia

(elution buffer control; 50mM NaH_2PO_4, 0.3M NaCl, and 100mM imidazole, pH 8.0). Colony counts of surviving bacteria were determined by plating serial dilutions of bacteria on MH agar and incubating at either 37°C (*E. coli*) or 27°C (*E. ictaluri*) for 20 hours. Results are expressed as CFUs/mL.

Kinetics of NCAMP-1 Killing. The antimicrobial assay was used as described above. Briefly, recombinant NCAMP-1-NT (2µg/ml), Polymyxin b, and elution buffer were diluted in 10mM sodium phosphate buffer (pH 8.0) to a total volume of 50µl. *E.coli* APEC 3721 (5000 CFU) were added to each tube and incubated at 37°C for 2 hours. Colony counts of residual bacteria were performed after incubation for 0, 15, 30, 45, 60 or 120 as described above. Plates were incubated at 37°C and CFUs were determined after 20 hours.

Specificity of NCAMP-1 Killing. Recombinant NCAMP-1-NT was incubated with either anti-NCAMP-1-NT rabbit polyclonal antibody or pre-immune rabbit serum in 50µl of 10mM sodium phosphate buffer (pH 8.0) for 30 minutes at 37°C. *E. coli* APEC 3721 (5000 CFU) was added in a volume (50µl) and incubated for 2 hours at 37°C. Colony counts were determined by plating serial dilutions of surviving bacteria on MH agar plates and incubating at 37°C for 20 hours. CFUs were determined at 20 hours.

Granule extract preparation. Granule extracts were prepared as previously described [13, 24]. Briefly, cells were harvested from the anterior kidney of channel catfish, passed through a mesh screen to create a single cell suspension, and placed on a 45.5% Percoll cushion to remove red blood cells. Cells were collected from the cushion interface and washed 2 times with RPMI-1640. Washed cells were suspended in hypotonic swelling buffer (10mM TrisCl, pH 7.6, 0.5mM $MgCl_2$) with 1µg/ml each of four protease inhibitors (Aprotinin (Sigma, A1153, St. Louis, MO), Pepstatin (Sigma, P4265, St. Louis, MO), Leupeptin (Sigma, L2023, St. Louis,

MO), and Phenylmethylsulfonyl Fluoride (Sigma, P7626, St. Louis, MO))) and incubated for 10 minutes (4°C) followed by homogenization with a Dounce homogenizer (60 strokes). Tonicity restoration buffer (10 mM TrisCl, pH 7.6, 0.5mM $MgCl_2$, 0.6M NaCl) with protease inhibitors was added to the homogenized cell lysate. The lysate was spun at 400 x g for 5 minutes at 4°C to remove the nuclei, cellular debris, and intact cells. The supernatant was spun at 27,000 x g for 20 minutes at 4°C. The resulting pellet was resuspended in 5% acetic acid, sonicated on ice, and extracted for 24 hours with rocking at 4°C. The extract was spun at 27,000 x g for 20 minutes at 4°C to remove the acid-insoluble residue. The extracts were vacuum centrifuged (Speed-Vac) to remove acetic acid. Dried extracts were resuspended in dH_2O and the drying process was repeated 2 additional times. The final dried product was resuspended in dH_2O and stored at -70°C. The protein concentration of the acetic acid granule extract was determined using the Bio-Rad Protein (Bradford) Assay (Bio-Rad, cat. no. 500-0006, Hercules, CA).

Antimicrobial assay using acetic acid granule extract. Acetic acid granule extracts in dH_2O were mixed with an equal volume of 20mM sodium phosphate buffer (final concentration of 10mM sodium phosphate buffer) and the pH was adjusted to 8.0. Acetic acid granule extract was incubated with either anti-NCAMP-1 rabbit polyclonal antibody or pre-immune rabbit serum in 50µl of 10mM sodium phosphate buffer (pH 8.0) for 30 minutes at 37°C. Alternatively, immobilized rProteinA beads (RepliGen, Cambridge, MA, IPA-300) were coated with anti-NCAMP-1 polyclonal antibody or normal rabbit IgG and incubated with 10µg/ml of granule extract protein for 30 minutes at 37°C. The beads were removed by centrifugation before testing the remaining antimicrobial activity as previously described. Acetic acid granule extract was also diluted in 50µl of 10mM sodium phosphate buffer (pH 8.0) and used alone in antimicrobial assays. *E. coli* APEC 3721 (5000 CFU) was added to each treatment in an equal

volume (50µl) and incubated for 2 hours at 37°C. Serial dilutions of residual bacteria were plated on MH agar plates and incubated at 37°C for 20 hours.

Western blots. Acetic acid granule extracts from NCC were prepared as described above. Extracts were resolved on a 12.5% SDS gels and transferred to nitrocellulose. Filters were blocked with Super Block® Blocking Buffer (Pierce, 37545, Rockford, IL) (15 minutes at room temperature), incubated with rabbit anti-NCAMP-1 polyclonal antibody (or control rabbit pre-immune serum) for one hour followed by washing with TBS with 0.05% Tween-20 (TBST), and incubated for 30 minutes with goat anti-rabbit IgG- horseradish peroxidase (HRP) conjugate diluted 1:50,000 in blocking buffer. Blots were developed with Super Signal® West Pico Chemiluminescent Substrate (Pierce, 34079, Rockford, IL).

Results

Amino acid sequence of NCAMP-1. Table 1.2 compares the percentage sequence homology between NCAMP-1, H1X and H1 proteins from other species. NCAMP-1 full-length, N-terminus, and C-terminus sequences were also determined as previously described [19] (Figure 1.1).

Antimicrobial Killing of *E. coli* Isolates. The antimicrobial activity of both the recombinant N-terminus (rNCAMP-1-NT) and recombinant C-terminus (rNCAMP-1-CT) of the NCAMP-1 protein was determined using both avian and bovine *E. coli* clinical isolates. rNCAMP-1-NT killed 99% of lab strain *E. coli* using 1µg/ml of protein ($1.7x10^5$ CFU/ml reduced to $2.0x10^1$ CFU/ml), compared to the same concentration of rNCAMP-1-CT ($1.7x10^5$ CFU/ml reduced to $4.0x10^3$ CFU/ml) (Figure 1.2A). rNCAMP-1-NT killed the avian *E. coli*

Table 1.2. Amino acid identity of NCAMP-1 with other histone-like proteins. H1XHUM: H1 histone family member X from human (Accession # BAA11018); H1XMUS: H1 histone family member X from mouse (Accession #XP_144949); H1X-Xenopus: H1 histone family member X from Xenopus levis (Accession #AAH41758); H1TRT: histone H1 from trout (Accession #CAB37646), H1HUM: histone H1 from human (Accession #P10412).

	H1X				H1	
	Danio	Human	Mouse	Xenopus	Trout	Human
NCAMP-1	51.2	42.4	43.9	42.9	30.3	33.1

A **NCAMP-1 N-Terminus Residues:**

10 PEAAAPVQPSQPAA**KKK**GPAS**K**A**K**PASAE**KK**N**KK**A**K**G**K**GPG 50

B **NCAMP-1 C-Terminus Residues:**

139 –TA**K**PT**KK**PA**KK**AA**KKKK**RVSGV**KK**ATPPPE**K**TS**K**P**KK**AD**K**SPAVSA**KK**AS**K**P**K**
KA**K**QT 196

C **Full-length NCAMP-1**:

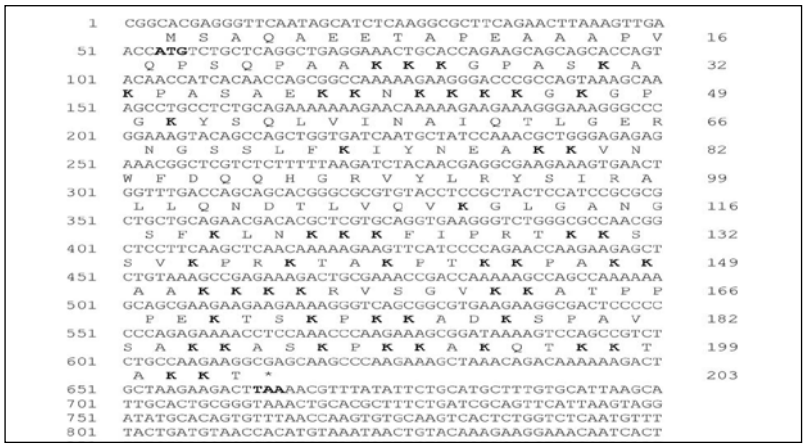


Figure 1.1. Sequence of NCAMP-1. Sequence of N-terminus (A), C-terminus (B), and full-length (C) NCAMP-1 from catfish NCC. Lysine residues are in bold.

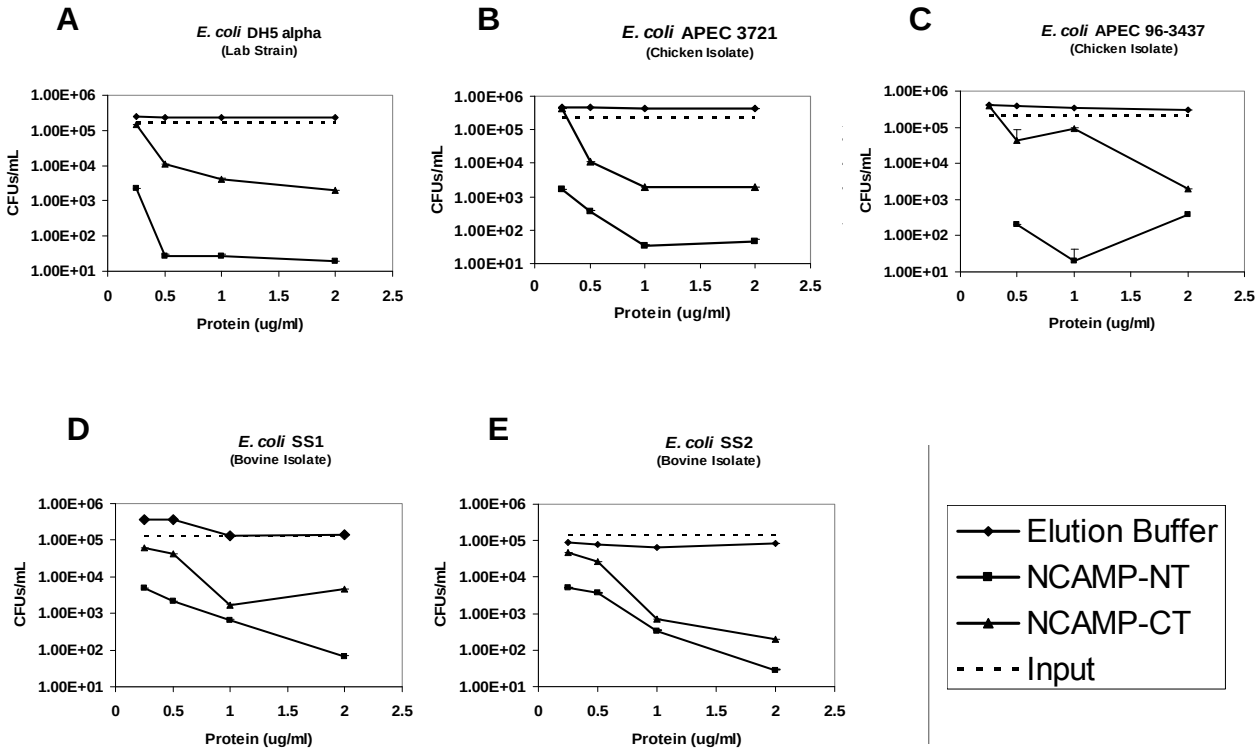


Figure 1.2. Avian and bovine *E. coli* antimicrobial killing assays. Antimicrobial assays were performed using 0, 0.5, 1.0, or 2.0 μg/ml of rNCAMP-NT or rNCAMP-CT. *E. coli* isolates from different species were incubated for 2 hours with the antimicrobial peptide. Elution buffer was a negative control. Polymyxin b was used as a positive control yielding 0 CFU/ml after plating (data not shown). (A) Lab strain *E. coli*, DH5α, was used as a control for bactericidal activity. (B and C) Avian *E. coli* isolates, APEC 3721 and APEC 96-3437, were tested for killing by recombinant NCAMP. (D and E) Bovine *E. coli* isolates, SS1 and SS2, were killed. Error bars indicate ± SEM from 3 experiments.

isolates, yielding a 99.9% reduction in the number of bacteria from $2.3x10^5$ CFU/ml to $3.3x10^1$ CFU/ml using 1µg/ml of recombinant protein. rNCAMP-1-CT killed 99.2% ($2.3x10^5$ CFU/ml reduced to $2.0x10^3$ CFU/ml) of bacteria using 1µg/ml of recombinant protein (Figure 1.2B and C). Bovine *E. coli* isolates were killed by 2µg/ml of both rNCAMP-1-NT and –CT. The percentage reduction in viability was 99.98% and 99.85% ($1.4x10^5$ CFU/ml reduced to $2.7x10^1$ CFU/ml and $2.0x10^2$ CFU/ml), respectively (Figure 1.2D and E).

Kinetics and Specificity of NCAMP-1-NT. The ability of rNCAMP-1-NT to kill gram-negative *E. coli* was time-dependent. The kinetics of rNCAMP-1-NT killing was evaluated by counting CFU/ml at 0, 15, 30, 45, 60 or 120 minutes. Maximum killing of *E. coli* APEC 3721 with 2µg/ml of rNCAMP-1-NT occurred at 60 minutes post-treatment (Figure 1.3A). The elution buffer (negative control) showed no killing.

Both anti-NCAMP-1-NT and full-length anti-NCAMP-1 polyclonal antibodies neutralized the bactericidal activity of rNCAMP-1-NT. The anti-NCAMP-1-NT antibody specifically inhibited killing of *E. coli* by binding to rNCAMP-1-NT ($3.02x10^2$ CFU/ml compared to $1.42x10^5$ CFU/ml) (Figure 1.3B). These results indicated that the rNCAMP-1-NT has specific bactericidal activity *in vitro*.

Bactericidal Activity of NCAMP-1-NT against *Edwardsiella ictaluri*. Antimicrobial activity of rNCAMP-1-NT against *E. ictaluri* was dose dependent. Protein concentrations of rNCAMP-1-NT used in the assay and CFU/ml were inversely related. Incubation with 2µg/ml of rNCAMP-1-NT produced $5.8x10^4$ CFU/ml (60% increase); 4µg/ml of rNCAMP-1-NT yielded $1.3x10^4$ CFU/ml (64% reduction); and 8µg/ml of rNCAMP-1-NT yielded $4.4x10^3$ CFU/ml (88% reduction). Kanamycin and Polymyxin b killed 100% of the bacteria. The elution buffer had no bactericidal effect (Figure 1.4A). Nine *E. ictaluri* clinical isolates were tested for sensitivity to

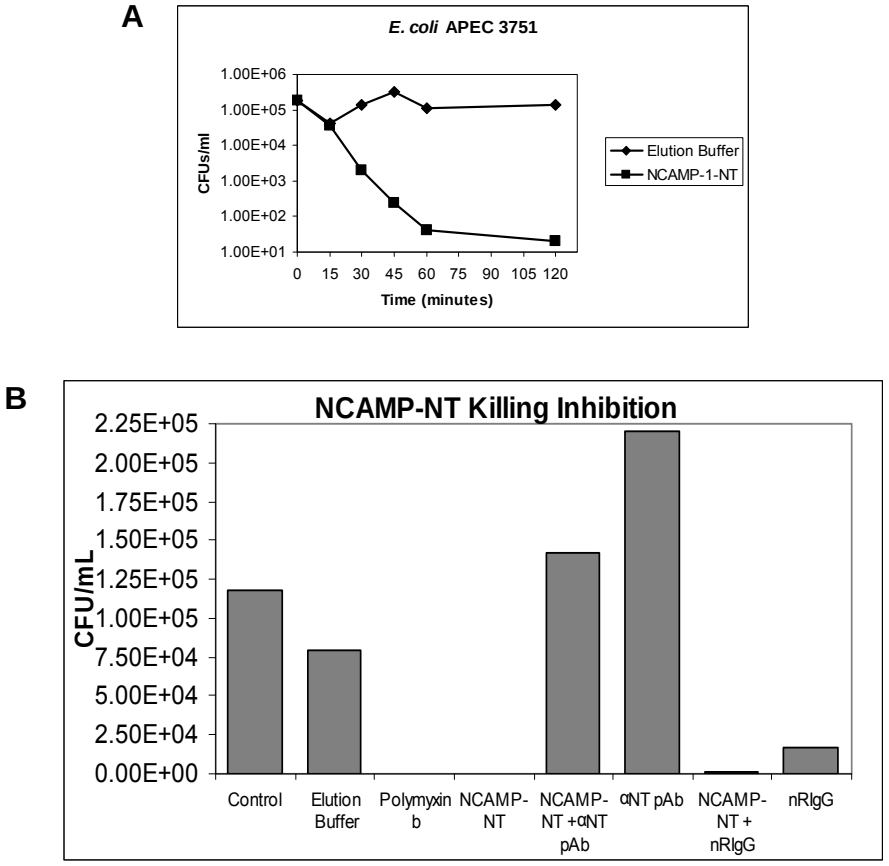
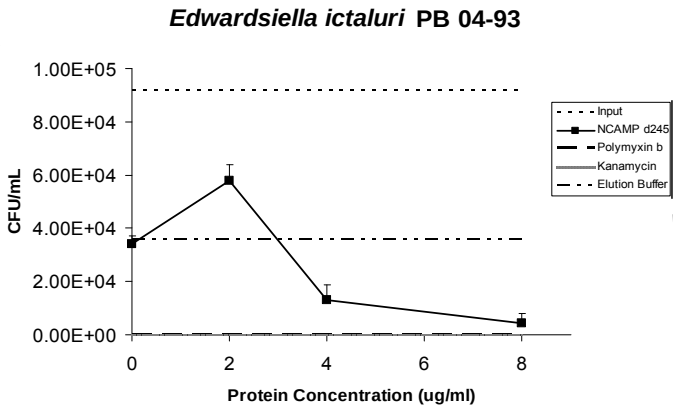


Figure 1.3. Kinetics and specificity of NCAMP-1-NT killing. (A) An antimicrobial assay was performed using 2 μg/ml of rNCAMP-1-NT or BSA in elution buffer. Avian *E. coli* APEC 3721 was incubated for 0, 15, 30, 45, 60, and 120 minutes. Serial dilutions were made to determine CFU/ml at each time point. (B) Specificity of recombinant NCAMP-1-NT killing was determined by using an anti-NCAMP-1 polyclonal antibody to inhibit specific bactericidal activity. *E. coli* APEC 3721 was incubated with 2 μg/ml of rNCAMP-1-NT alone or with 70 μg/ml of anti-NCAMP-1 polyclonal antibody or normal rabbit IgG. Data is representative of 3 separate experiments.

A



B

Edwardsiella ictaluri isolate	%Killing (4ug/ml NCAMP NT)
PB 04-93	75
97-773	70
97-779	82
LN 01-379B	77
PB 97-40	72
PB 97-30	81
PB 99-129	61
PB 06-395	72
AL 98-75 (vaccine strain)	50

Figure 1.4. *Edwardsiella ictaluri* killing assays. (A) 2, 4, or 8 μg/ml of NCAMP-1-NT was incubated with *E. ictaluri* PB 04-93. Kanamycin and Polymyxin b (1 μg/ml each) were used as positive controls. Elution buffer was used as the negative control. Data is representative of three separate experiments. (B) Antimicrobial killing assays were performed on nine strains of *E. ictaluri*. Data is representative of at least 3 separate experiments.

NCAMP-1-NT. Seven out of nine isolates had a 70-82% reduction in bacterial count after incubation with 4μg/ml rNCAMP-1-NT for 2 hours (Figure 1.4B).

Membrane Expression of NCAMP-1 on Catfish NCC. Acetic acid granule extracts of purified NCC were examined by Western blot analysis for the presence of NCAMP-1 [13, 24]. NCAMP-1 was present in catfish NCC acetic acid granule extracts (Figure 1.5A) and was also expressed on the membranes of NCC. The MACS sorted cells from the catfish anterior kidney were 90% $5C6^{+}$ and 95% $9C9^{+}$ by flow analysis (data not shown). NCCRP-1 and NCAMP-1 were co-expressed on the membrane of NCC as indicated by 2-color flow cytometric analysis (Figure 1.5B and C). Approximately 76.6% of CF NCC constitutively co-expressed membrane NCAMP-1 and NCCRP-1.

Fluorescent Microscopy of NCAMP-1 on NCC. Immunofluorescence microscopy showed NCAMP-1 and NCCRP-1 on the membrane of enriched NCC (Figure 1.6). Merged images of NCC stained with monoclonal antibodies 9C9 and 5C6 demonstrated that NCAMP-1 and NCCRP-1 are both located on the same cell membrane.

Antimicrobial Killing with Catfish Granule Extracts. The antimicrobial activity of acetic acid granule extracts from catfish NCC was investigated. *E. coli* APEC 3721 was incubated with 0, 5, 10, or 20μg/ml of acetic acid granule extract. The bactericidal activity of the granule extract was concentration dependent. Pre-incubation of *E. coli* with 5μg/ml, 10μg/ml, and 20μg/ml of extract produced $2.34x10^{3}$ CFU/ml, $2.0x10^{1}$ CFU/ml, and $1x10^{1}$ CFU/ml, respectively (Figure 1.7A). Pre-incubation of the catfish granule extract with anti-NCAMP-1 polyclonal antibody reduced the killing of *E. coli* by 99.85% ($4.0x10^{1}$ CFU/ml remained compared to $2.8x10^{4}$ CFU/ml) (Figure 1.7B). Pre-incubation of the acetic acid extract with anti-

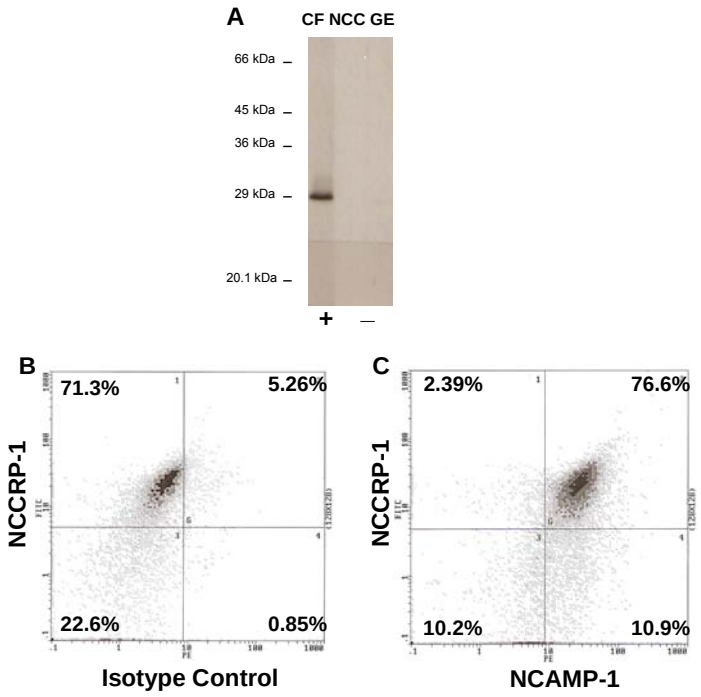


Figure 1.5. Western blot of granule extract and flow cytometric analysis of NCAMP-1 membrane expression. (A) Granule extracts from catfish NCC were probed with anti-NCAMP-1 polyclonal antibody (+) or control normal rabbit IgG (-). (B) 5C6+ (FITC) NCC were stained with isotype control IgM antibody (PE) or (C) with monoclonal antibody 9C9 (PE).

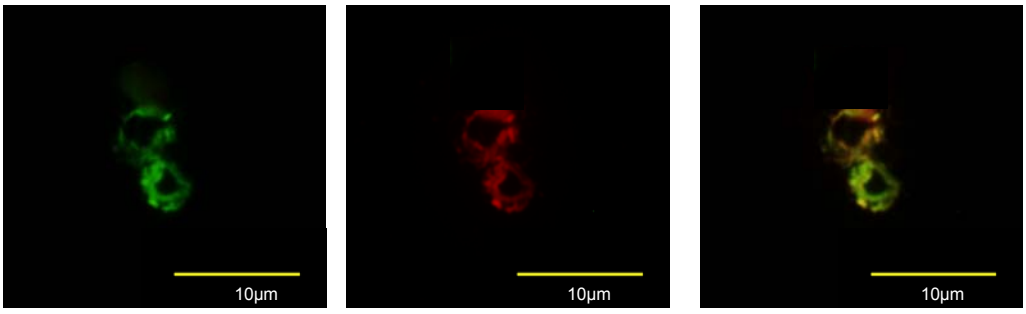


Figure 1.6. Fluorescent microscopy of CF NCC. NCCRP-1(green) indicated by staining with monoclonal 5C6. NCAMP-1 (red) indicated by staining with monoclonal 9C9. Overlapping images show that NCCRP-1 and NCAMP-1 are both located on CF NCC membranes (yellow).

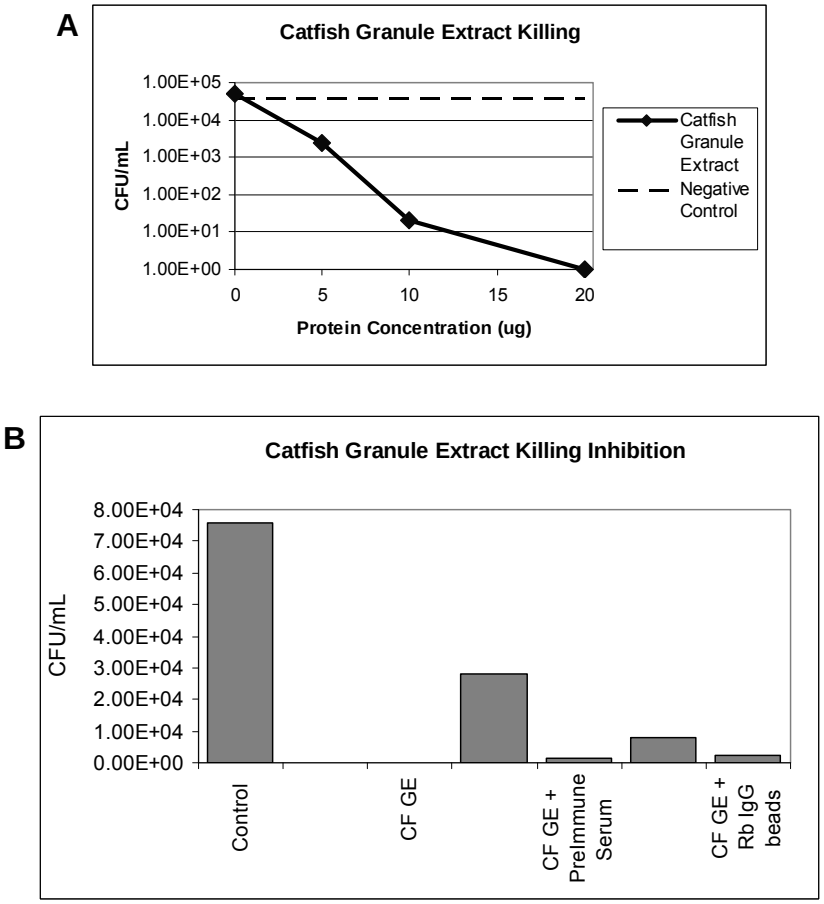


Figure 1.7. Antimicrobial assays using catfish NCC granule extract. (A) *E. coli* APEC 3721 was incubated with 0, 5, 10, or 20 µg/ml of CF NCC granule extract (CF NCC GE) for 2 hours. Polymyxin b was used as a positive control. Data is representative of 5 individual experiments. (B) *E. coli* APEC 3721 was incubated with 10 µg/ml of CF NCC GE and/or 35 µg/ml anti-NCAMP-1 polyclonal antibody or rabbit preimmune serum or with 7.5 µl of anti-NCAMP polyclonal coated Protein A beads (pAb beads) or normal rabbit IgG coated beads (nRbIgG beads). Polymyxin b (1 µg/ml) was used as a positive control. Data is representative of 4 separate experiments.

NCAMP-1 polyclonal antibody coated Protein A beads also reduced killing of bacteria ($4.0x10^1$ CFU/ml compared to $8.0x10^3$ CFU/ml).

MIC_{50} of NCAMP-1. rNCAMP-1-NT has a lower 50% minimal inhibitory concentration (MIC_{50}) than CF NCC GE against *E. coli* (Table 1.3). Lower concentrations of recombinant NCAMP-1 (0.38μg/ml) are needed to kill gram-negative bacteria than are needed of the natural NCAMP found in CF NCC granules (1.50μg/ml). *E. ictaluri* (MIC_{50}=3.75μg/ml) is less susceptible to killing by NCAMP-1 than *E. coli* (MIC_{50}=0.38μg/ml). rNCAMP-1-CT has the lowest MIC_{50} (0.32μg/ml).

Discussion

We previously demonstrated [25] that NCC from teleosts could be directly activated by CpG ODNs to induce cytolytic activity against tumor target cells and release effector molecules such as Fas ligand [6]. An ODN hierarchy for activation of cytotoxicity was directly dependent on dinucleotide sequences where 5' purines are preferably GpT and 3' pyrimidines are TpT. The DNA binding protein on NCC recognized both cytosine and guanosine as well as single base-guanosine. This protein was subsequently purified from NCC membranes and identified. The message for the ODN binding protein was partially sequenced and the RT-PCR generated cDNA product was translated. The complete amino acid sequence was submitted to NCBI (accession numbers AAQ99138 and AY324398). The cDNA product was referred to as nonspecific cytotoxic cell cationic membrane protein-1 (NCAMP-1). It was similar in amino acid sequence to mammalian histone 1X but was otherwise determined not to be identical to any previously described protein (Table 1.2). The gene encoding ncamp-1 has a typical polyadenylation signal

Table 1.3. MIC_{50} for recombinant NCAMP-1 vs. CF NCC Granule Extracts.

Protein	Bacteria	MIC_{50} (µg/ml)
NCAMP-1-NT	*E. coli*	0.38
NCAMP-1-CT	*E. coli*	0.32
NCAMP-1-NT	*E. ictaluri*	3.75
CF NCC GE	*E. coli*	1.50

and poly-A tail [19]. Recent sequencing projects have identified EST's identical (e values of 0) to NCAMP-1 from a catfish NK-like cell line (CB937576) and from head kidney cDNA (BE469379) preparations.

Although histones have traditionally been known to localize in the cell nucleus in association with chromatin fibers, there is considerable new evidence that histones (e.g. linker histones) are expressed on cell membranes as well as existing as secreted antimicrobials in cytosolic granules [26] and as binding components of neutrophil extracellular traps (NETs) [27]. Studies performed in higher vertebrates have shown that many cells also express membrane forms of histones. Human monocytes have membrane H2a and b [9, 12, 14]; a human transformed B cell line, Raji, has 2 histone-like membrane proteins of 14-18 and 33-34 kDa [15]; and T-cells express membrane H2b (17 kDa) [15-16] and H3 (29 kDa) [17-18]. The mechanism of transport of histones to the cell membrane is not understood, as these proteins do not contain traditional trans-membrane domains. Other examples of the widespread expression of membrane histones include neurons [10] and macrophages [11] shown to have 30-33 kDa histone H1 membrane receptors that bind LPS and thyroglobulin, (respectively). These studies clearly demonstrated that histone-like proteins are expressed on cell membranes. However, little progress has been made in defining a role for these proteins in anti-bacterial *in vivo* immunity.

In teleosts, histone-like proteins and peptides with antimicrobial activity have been isolated from salmon blood, liver, intestine and mucus [21-22]. Catfish skin, epithelial cells and mucus contain H2a-like (Parasin-I) and H2b-like molecules [20, 24]. In these studies though, membrane expression of histone-like proteins was not determined, thus release from cells required either tissue injury or exocytosis.

Several mechanisms of the biology of membrane insertion and/or cellular release have been proposed for cationic proteins with antibacterial activity that lack signal sequences. Some are expressed in granules (granulysin and some histone-derived peptides) and are thus released by degranulation. Alternatively, their repeated alpha-helix structure allows them to cross the cellular membrane in a screw-like motion. This issue must also be addressed with NCAMP-1 as the gene does not have an apparent signal sequence nor does the protein have a sequence typical of the transmembrane portion of a receptor protein [19].

The biochemical and genetic characteristics of the H1X histone subtype as well as the partial aa comparisons of this gene family with NCAMP-1 remain an enigma. For example, as a member of the tenth H1 subtype, H1X transcription is not replication dependent and the H1X mRNA contains multiple tandem linked polyadenylation signals [26, 28]. This might explain the ability of the H1X-like NCAMP-1 to be expressed at the levels of the membrane as well as in cytosolic granules, or NCAMP-1 differential expression may be the result of alternative poly(A) site choice or of RNA processing (e.g. alternative splicing). Because of the multiple (potential) polyadenylation sites, it appears to be less likely that the membrane and secreted forms of NCAMP-1 are generated in the golgi by post-translational modifications. Although there are a relatively small number of genes with alternative tandem poly(A) sites (e. g. CD40, Histone $H1^0$ subtype, Ig heavy chains, IL-1R type I, TNF-R, etc.) [29-30], it remains very possible that NCAMP-1 represents yet an additional member of this group.

In summary, a new class of PRR exists on nonspecific cytotoxic cells (NCC) that we refer to as NCAMP-1. NCAMP-1 recognizes bacterial DNA, oligodeoxynucleotides/ODNs and polyguanosine motifs. Similar to scavenger receptors, mannose receptors and Toll-like receptors in other vertebrates, we hypothesize that NCAMP-1 participates in innate immune responses of

teleosts. Certain of the ligand specificities of scavenger receptor A (SR-A) are polyguanosine/G-tetrads, polyvinyl sulfate and dextran sulfate. These ligand specificities are based on charge densities (e.g.) as well as patterns and structures. NCC also recognize ligands based on charge and structural conformation of the antigen. These are by definition, characteristics of a PRR. Unlike SR-A however, NCAMP-1 in soluble form is a potent antimicrobial protein. Thus the similarities of NCAMP-1 to other PRR are compelling.

In soluble form, NCAMP-1 has bactericidal activity against a wide-range of microorganisms, including gram-negative *E. coli* and *E. ictaluri*. Both the N-terminus and C-terminus of recombinant NCAMP-1 kill bovine and avian clinical isolates of *E. coli*. NCAMP-1-NT has rapid bactericidal activity that is detectable within 15 minutes of exposure to microbes. Polyclonal antibodies against NCAMP-1 specifically neutralize bacterial killing of rNCAMP-1-NT.

Using Western blot analysis, we show that NCAMP-1 is present in the acetic acid granule extracts of catfish NCC. The extracts have bactericidal activity against gram-negative pathogens. Pre-incubation of the acetic acid granule extract with anti-NCAMP-1 polyclonal antibody reduces the killing of *E. coli*. Therefore, NCAMP-1 is a potent antimicrobial peptide in the catfish NCC granule.

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CHAPTER 4

IMMUNOREGULATION OF A NOVEL PATTERN RECOGNITION RECEPTOR, NCAMP-1, ON CATFISH NONSPECIFIC CYTOTOXIC CELLS DURING INFECTION WITH *EDWARDSIELLA ICTALURI* [1]

[1]Connor, M.A., L. Jaso-Friedmann, J.H. Leary III, A.C. Camus and D.L. Evans. To be submitted to *Developmental and Comparative Immunology*.

Abstract

Teleosts are a useful model for studying innate immunity. The innate immune system of fish is principal method of defense. The acquired immune system is less developed in fish than mammals. Nonspecific cytotoxic cells (NCC) are a key cell in the teleost innate immune response and are the equivalent of mammalian NK cells. In the present study, the role of a novel pattern recognition receptor, NCAMP-1, in innate immunity during infection with the catfish pathogen *Edwardsiella ictaluri* is described. NCAMP-1 is expressed on NCC and can be up-regulated on the membrane by phorbol ester/calcium ionophore activation. An *in vivo* model of *Edwardsiella ictaluri* infection was developed to study the innate immune response in infected catfish. Cell count determination showed that cells left the anterior kidney and traveled to other immune organs, such as the spleen, during infection. The membrane expression of NCAMP-1 was analyzed by flow cytometry using an anti-NCAMP-1 monoclonal antibody. NCAMP-1 membrane expression was up-regulated on NCC in the spleen of infected fish. The NCAMP-1 membrane expression remained unchanged on the NCC from the anterior kidney. The gene expression of NCAMP-1, NCCRP-1, and TNF-α was analyzed using QPCR. NCAMP-1 message is down-regulated in the anterior kidney and spleen during ESC. TNF-α and NCCRP-1 were up-regulated during the course of infection in both the anterior kidney and spleen. In the present study, we have shown that NCAMP-1 on the NCC primarily of the spleen and moderately of the anterior kidney plays a role in the innate immune response of catfish during *Edwardsiella ictaluri* infection.

Introduction

The innate immune system is the fundamental defense mechanism of fish [1]. Therefore, teleosts are an excellent model for studying innate immunity [2]. Fish scales, mucus, and the epidermis act as a first barrier of defense [1, 3]. Key cells of the teleost innate immune system are phagocytic cells (e.g. granulocytes/neutrophils and monocytes/macrophages), nonspecific cytotoxic cells (NCC), epithelial cells, and dendritic cells [1]. Nonspecific cytotoxic cells (NCC) are the teleost equivalent of mammalian natural killer (NK) cells [4, 5]. NCC are small nucleated cells found in the anterior kidney, spleen, liver, and peripheral blood of several species of fish [6]. NCC have been described in catfish [4], tilapia [6], rainbow trout [7], and gilthead seabream [8]. Fish lack both lymph nodes and bone marrow [2]. The head kidney is more structurally analogous to bone marrow than lymph nodes [9]. The total number of lymphocytes in the anterior kidney is greater than the number in the peripheral blood and spleen (ratio of 10:4:1) [10].

Edwardsiella ictaluri is the causative gram-negative bacteria of enteric septicemia of catfish (ESC) [11-14]. *Edwardsiella ictaluri* is an important disease in farm-raised channel catfish. In 2002, 60.6% of food-size catfish farms reported ESC, making it the highest reported problem in catfish ponds [15]. Typically, 45-50% of food-size fish will be lost to disease causing a loss of as much as $100 million annually to the catfish industry [15].

During infection with *Edwardsiella ictaluri*, differences in immunological function can be detected between control and infected catfish. Microarray analysis showed that ESC caused the induction of several components of the MHC-class 1 pathway that were detectable in the liver 3 days post-infection [16]. The gene expression of TLR 3 and 5 changed following

infection with *E. ictaluri* [17]. TLR5 had increased expression in the kidney and liver on day 5 post-exposure [17]. TLR3 increased to a lesser extent in the kidney and the spleen over time [17].

Edwardsiella ictaluri infection can alter the expression of antimicrobial peptides in catfish. Histones with antimicrobial activity have been isolated from a number of teleost species including Atlantic salmon [18, 19], Coho salmon [20], channel catfish [21-23] and flounder [24]. A histone-like antimicrobial peptide, NCAMP-1 (Nonspecific cytotoxic cell cationic antimicrobial peptide-1), has been previously identified [25]. NCAMP-1 is a novel pattern recognition receptor that was first purified from catfish NCC (nonspecific cytotoxic cells) and is also found on tilapia, zebrafish and mouse cells [25]. The cDNA sequence of NCAMP-1 is similar to histone H1X in xenopus, zebrafish, mouse and humans, but is not identical to any other previously described protein [25]. NCAMP-1 is a basic 29 kDa protein [25]. Previous data suggests that NCAMP-1 may be a pattern recognition receptor. NCAMP-1 binds dinucleotides and polyguanosine oligodeoxynucleotides (ODNs) and bacterial DNA [25]. NCAMP-1 is expressed on the membranes of catfish and zebrafish NCC and on mouse macrophages (unpublished data). NCAMP-1 is also an antimicrobial peptide. Full-length and truncated forms of recombinant NCAMP-1 and NCAMP-1 isolated from the granules of catfish effectively kill *Escherichia coli, Edwardsiella ictaluri*, and *Streptococcus iniae* isolates *in vitro* [25].

In the present study, the effect of infection with *Edwardsiella ictaluri* on the innate immune system of channel catfish was investigated. NCAMP-1 expression on the cell membrane of NCC was determined over a time course of infection. Spleen and anterior kidney cell counts were done to determine cell trafficking patterns during infection. Gene expression of NCAMP-1, NCCRP-1 and TNF-α was examined by QPCR analysis to investigate transcriptional

activation in response to ESC. We hypothesize that NCAMP-1 found in the spleen and anterior kidney plays a role in innate immunity during infection with *Edwardsiella ictaluri*.

Materials and Methods

Cell preparations. Fingerling channel catfish (*Icatalurus punctatus*) were maintained in tanks at 23-25°C in a flow-through water system. The anterior kidney (bone marrow equivalent) and spleen were excised from channel catfish. Single cell suspensions were obtained by physical disruption through a mesh screen. The cells were placed in RPMI-1640 (Mediatech, cat. no. 50-020-PB, Manassas, VA) as previously reported [25]. Leukocytes were separated from red blood cells by centrifugation on a 45.5% Percoll (Sigma, P1644) cushion for 1 hour at 700 x g. Cells were collected from the cushion and washed 2 times with RPMI-1640. The cells were suspended in PBS (phosphate buffered saline) and counted.

NCC activation with PMA/calcium ionophore. Cells were harvested from the anterior kidney of channel catfish, passed through a mesh screen to create a single cell suspension, and placed on a 45.5% Percoll cushion to remove red blood cells. Cells were collected from the cushion interface and washed 2 times with RPMI-1640. The cells were suspended in RPMI-1640 supplemented with 10% fetal calf serum and counted. Cells were suspended in a volume of 10^6 cells/ml in RPMI-1640 with 10% FCS (control) or RPMI-1640 with 10% FCS containing 30ng/ml of phorbol ester/PMA (Sigma, Cat. No. P1585, St. Louis, MO) and 150ng/ml of calcium ionophore/A23187 (Sigma, Cat. No. C7522, St. Louis, MO) (treatment). The cells were incubated at 27°C and analyzed by flow cytometry at 0, 1, 1.5, 2, and 3 hours post-activation. Briefly, the cells were washed and suspended in cold PAB (phosphate buffered saline containing

0.1% sodium azide and 1.0% bovine serum albumin (Sigma, St. Louis, MO)) to stop activation. Monoclonal antibody 9C9 or isotype control was added for 1 hour at 4°C. Anti-mouse IgM-FITC conjugate (Sigma, F9259, St. Louis, MO) was added for 30 minutes at 4°C. The cells were washed, suspended in PBS azide and analyzed by flow cytometric analysis.

Monoclonal antibodies. Monoclonal antibody 9C9 (IgM isotype) with specific activity against NCAMP-1 was prepared in-house using the ClonaCell™-HY Hybridoma Cloning Kit (Stem Cell Technologies, Vancouver, BC, Canada) as previously described (manuscript in progress). Monoclonal antibody 5C6 (anti-NCCRP-1) was also prepared as previously described [5].

Bacterial isolates and culture. *Edwardsiella ictaluri* isolate S94-887 was obtained from Dr. Andy Goodwin (Pine Bluff, AR, UAPB, Aquaculture/Fisheries). *E. ictaluri* was cultured in brain heart infusion (BHI) broth at 27°C. Bacterial cultures were grown for 16 hours until an optical density of A_{600}=1.5 was reached. The relationship between A_{600} and CFU per ml has been previously determined in our lab to be A_{600}=1.0 is 1.5 x10^8 CFU/ml. Dilutions of bacteria were made (10^5, 10^6, 10^7 CFU/ml) in BHI broth.

LD50 Determination. Fish were acclimated in tanks for one week prior to infection. Tanks (15L) were stocked at a density of 8 fish per tank at 25°C. On the day of infection, the tank water level was lowered to 1 inch. Fish were infected by immersion (1 hour) with 10^5, 10^6, 10^7 CFU/ml of *Edwardsiella ictaluri* or 4 ml of BHI broth (control). After 1 hour, the tanks were slowly refilled. Morbidity and mortalities were monitored every 12 hours for 10 days post-infection.

***In Vivo* Infection Model**. Fish were infected as described above except on the day of infection fish were infected with 10^7 CFU/ml of *E. ictaluri* S94-887 (infected) or treated with

BHI broth (control). Fish were monitored daily for 10 days. Daily, 3 infected and 3 control fish were sampled, their anterior kidney and spleen were removed and single cell suspensions were made as previously described. Red blood cells were removed, the cells were counted daily and cells were stained with anti-NCAMP-1 antibody (9C9) for flow analysis. The remaining cells were placed in RNA later (QIAGEN, Cat. No. 76106, Valencia, CA) and saved for PCR analysis.

***Edwardsiella ictaluri* Identification and Histopatholgy**. Bacteria from the brain tissue of control and infected catfish were cultured on blood agar plates and incubated for 48 hours at 27°C. Bacteria were identified using the API 20E system for Enterobacteriaceae (bioMerieux, Inc., Durham, NC) as per manufacturer's instructions.

Following incision of the abdomen for tissue culture, whole fish were fixed in 10% neutral buffered formalin (100 ml Formalin (40%), 4g monobasic sodium phosphate, 6.5g dibasic sodium phosphate in 1L dH_2O), decalcified in a commercial decalcifying solution (Cal-Ex, Fisher Scientific, Fair Lawn, NJ), and cut into approximately 3 mm cross sections along their body lengths. Tissues samples were processed for embedding in paraffin, and sectioned at 5 µm for staining with hematoxylin and eosin [26]. Images were captured using an Olympus Q Color 3 microscope camera.

Flow cytometry. Flow cytometry experiments were performed using cells from the anterior kidney and spleen that were suspended in PAB (phosphate buffered saline containing 0.1% sodium azide and 1.0% bovine serum albumin (Sigma, St. Louis, MO)). The cells were incubated with 9C9 or isotype control for 1 hour at 4°C, washed with PBS, and stained with goat anti-mouse IgM-FITC conjugate for 30 minutes (Sigma, F9259, St. Louis, MO). The cells were analyzed using an EPICS XL-MCL four color analyzer (Coulter Electronics Corp, Hileah, FL)

equipped with a 15mW air cooled argon-ion laser. FITC was detected by photomultiplier tube 1 (PMT1) using the 525 nm bandpass filter. PE was detected by PMT2 using the 575 nm bandpass filter. Data was analyzed using Coulter's System II software, version 3.0.

Isolation of RNA and cDNA from Catfish Cells. Cells that had been collected from control and infected catfish and placed in RNA later were used. The cells were pelleted at 14,000xg. RNA later was removed and RNA was isolated from the cells using either the QIAGEN RNeasy Mini Kit (anterior kidney cells) (QIAGEN, Cat. No. 74104, Valencia, CA) or the QIAGEN RNeasy Micro Kit (spleen) (QIAGEN, Cat. No. 74004, Valencia, CA) according to manufacturer's instructions. The RNA from each sample was quantified using the Quant-It™ RiboGreen® RNA Reagent (Molecular Probes, Cat. No. R11491, Carlsbad, CA) as per manufacturer's instructions. cDNA was synthesized, normalizing for RNA quantity (40ng/ml of total RNA per reaction), using the First-Strand cDNA Synthesis with SuperScript III for RT-PCR protocol from Invitrogen (Invitrogen, Cat. No. 18080-093, Carlsbad, CA).

QPCR. Quantitative PCR analysis was performed using Brilliant® SYBR® Green QPCR Master Mix according to manufacturer's instructions (Stratagene, La Jolla, CA) on the MX3000P Instrument (Stratagene, La Jolla, CA). A volume of 0.5 µl of cDNA from each sample was added to the reaction. Catfish actin was used as the internal control for normalization. The non-degenerate primer sets (catfish actin, NCAMP-1, NCCRP-1, and TNF-α) used in the QPCR analysis are listed in Table 2.1. Uninfected fish were used as a calibrator to compare control versus infected fish. cDNA from the anterior kidney and spleen of 3 fish per treatment per day were analyzed. The fold change to calibrator (log base 2) was quantified by averaging fluorescence (dRn) values for the 3 fish in each treatment at each time point using the MXPro QPCR Software.

Table 2.1. Primers for QPCR Analysis.

Gene	Primer Sequence	Product Length	Optimum Concentration (nM)	Efficiency
CF Actin	Forward: 5'-CAGACATCAGGGTGTGATGG-3'	153	300	112%
	Reverse: 5'-GGTGTGGTGCCAGATCTTCT-3'		150	
NCAMP-1	Forward: 5'-AACGAGGCGAAGAAAGTGAA-3'	169	300	200%
	Reverse: 5'-TCTTCTTGGTTCTGGGGATG-3'		300	
NCCRP-1	Forward: 5'-CGAAGACTGTTATGAGGAGAG-3'	284	150	117%
	Reverse: 5'-GACTTTGTTGCCTTGACGACA-3'		150	
TNF-α	Forward: 5'-GTACGAGAGAAGGCCTCGTG-3'	167	150	83%
	Reverse: 5'-CTGGGAAATCTGCCTGAGAG-3'		300	

Statistical Analysis. An unpaired two-tailed t-test was used for statistical analyses. P-values are indicated in the figure legends as $^{*}P < 0.05$ and $^{**}P < 0.005$.

Results

Activation of NCC with Calcium Ionophore. Flow cytometric analysis using anti-NCAMP-1 monoclonal antibody 9C9 demonstrated that PMA/calcium ionophore treatment of NCC increased NCAMP-1 membrane expression on NCC (Figure 2.1). NCC were treated with phorbol ester (PMA) and calcium ionophore (A23187) for 1, 1.5, 2, or 3 hours. The activated cells were then stained with monoclonal antibody 9C9 and analyzed by flow cytometry. A two-fold increase in NCAMP-1 expression (25% compared to 11%) was found after 1.5 hours of PMA/calcium ionophore activation. Maximum NCAMP-1 expression occurred at 1.5 hours. Binding by the isotype control mab remained at 11% positive during activation of the NCC.

Histopathology of Catfish with ESC. Fish exposed to *E. ictaluri* exhibited signs typical of acute and chronic enteric septicemia [27, 28]. External changes included ventral petechiation, abdominal swelling, multifocal shallow white 1-2 mm dermal ulcers and hemorrhagic ulceration above the dorsal cranial fontanel, often referred to as the "hole in the head" lesion. Gross internal changes included clear yellow ascites, multiple pale foci of hepatic necrosis, and splenomegaly. Microscopic lesions in the sections examined were predominated by widespread small foci of hepatocellular and pancreatic coagulative necrosis, containing clusters of free and phagocytized bacterial rods (Figure 2.2).

LD50 for Catfish Infected with *E. ictaluri*. Channel catfish were infected with 3 concentrations (10^7, 10^6, 10^5 CFU/ml) of *Edwardsiella ictaluri* and the number of mortalities

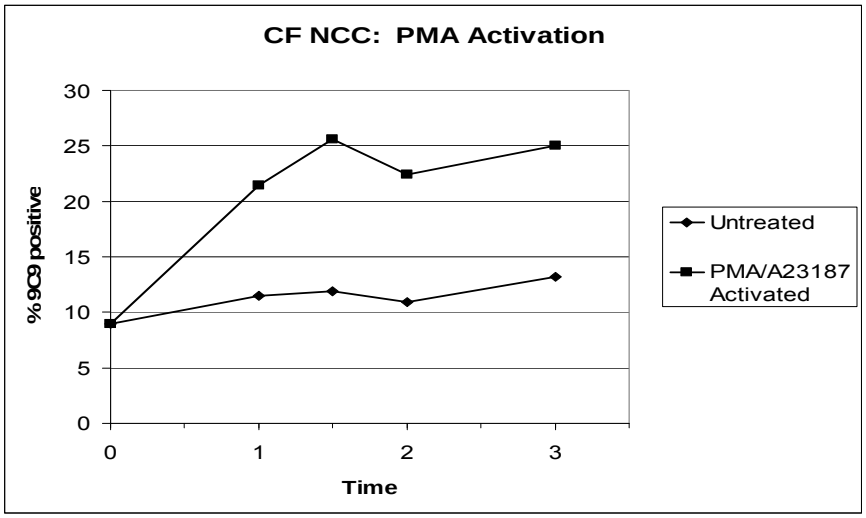

Figure 2.1. Activation of NCC with Phorbol Ester/Calcium Ionophore. Cells harvested from the anterior kidney of channel catfish were suspended in a volume of 10^6 cells/ml in RPMI-1640 with 10% FCS (control) or RPMI-1640 with 10% FCS containing 30ng/ml of phorbol ester/PMA and 150ng/ml of calcium ionophore/A23187 (treatment). The cells were incubated at 27°C for 0, 1, 1.5, 2, and 3 hours. After treatment, the cells were stained with anti-NCAMP-1 mAb 9C9 or isotype control and analyzed by flow cytometry. Data is representative of 5 separate experiments.

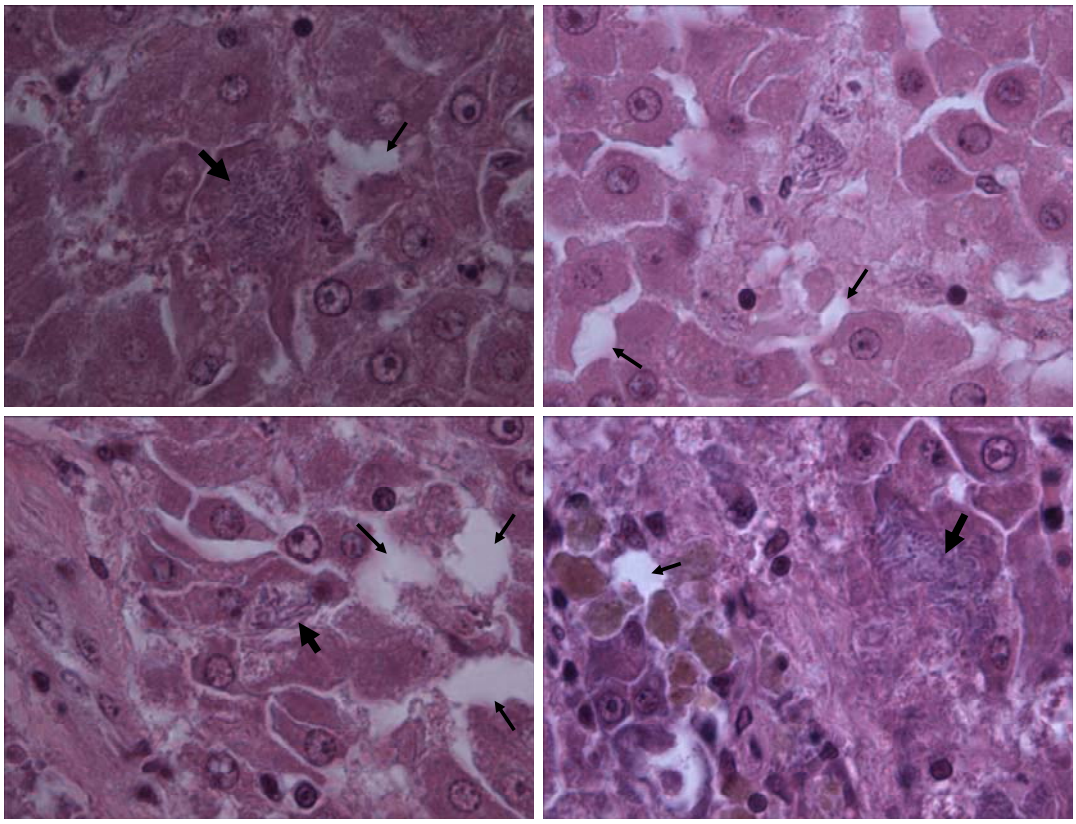

Figure 2.2. Histology of liver of catfish infected with ESC. Tissues samples from the liver of infected catfish were processed, embedded in paraffin, and sectioned at 5 µm. Prepared slides were stained with hematoxylin and eosin. Microscopic lesions in the liver sections are characterized by widespread small foci of hepatocellular and pancreatic coagulative necrosis (small arrows), containing clusters of free and phagocytized bacterial rods (large arrows).

were recorded daily (Figure 2.3). The first signs of morbidity, which were characterized by petechial hemorrhaging on the fins, appeared on day 3. The first deaths occurred on day 5 yielding a 62.5% survival rate in the 10^7 CFU/ml infected tank and 87.5% survival rate for fish infected with 10^5 CFU/ml (Figure 2.3). The LD50 in 10 days occurred on day 8 in the 10^7 CFU/ml tank. Recovered bacterial isolates produced identical API 20E numeric codes (4004000) consistent with published profiles for E. i*ctaluri* [29].

Cell Count Determination and NCAMP-1 Membrane Expression during *Edwardsiella ictaluri* Infection. Cell counts of the anterior kidney and spleen of infected and control fish were performed daily. There were 1- to 3-fold fewer cells in the anterior kidney of infected fish compared to control fish on days 1 through 7 (Figure 2.4A). Significantly fewer cells were present in the anterior kidney of the infected catfish compared to control on day 3. On day 8, there was little difference in cell number between the infected and control fish. A 1.5-fold increase ($4.18x10^7$ CFU/ml in control compared to $5.89x10^7$ CFU/ml in infected) of cells in the anterior kidney of infected fish occurred on day 9, followed by a significant 3-fold increase ($3.56x10^7$ CFU/ml in control compared to $7.00x10^7$ CFU/ml in infected) on day 10.

The membrane expression of NCAMP-1 was analyzed by flow cytometry using monoclonal antibody, 9C9, over a period of 10 days. No difference between NCAMP-1 expression on the surface of NCC from the anterior kidney of control and infected catfish was detected (Figure 2.4B).

The daily number of cells in the spleen differed from the anterior kidney. The number of cells in the spleen of infected and control catfish were similar on days 1 through 3 (Figure 2.5A). A significant 4-fold increase ($1.52x10^6$ CFU/ml in control fish versus $6.45x10^6$ CFU/ml in infected fish) in the number of cells in the infected spleen occurred on day 6. By day 7, the

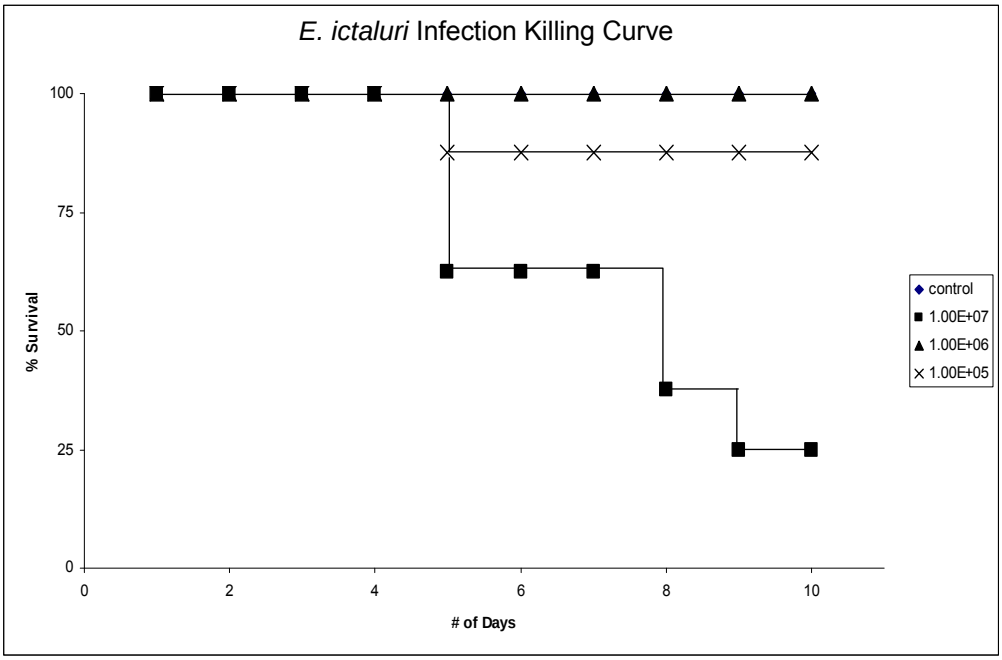

Figure 2.3. LD50 determination following *E. ictaluri* infection. Tanks were stocked with 8 channel catfish fish/ tank. On the day of infection, the tank water level was lowered to 1 inch. The dilutions of *Edwardsiella ictaluri* (10^5, 10^6, 10^7 CFU/ml) or 4 ml of BHI broth (control) were added to the respective tank. After 1 hour, the tanks were slowly refilled. The fish were checked for mortalities every 12 hours for 10 days. The LD50 in 10 days occurred on day 8 in the 10^7 CFU/ml tank. Data is representative of 3 independent experiments.

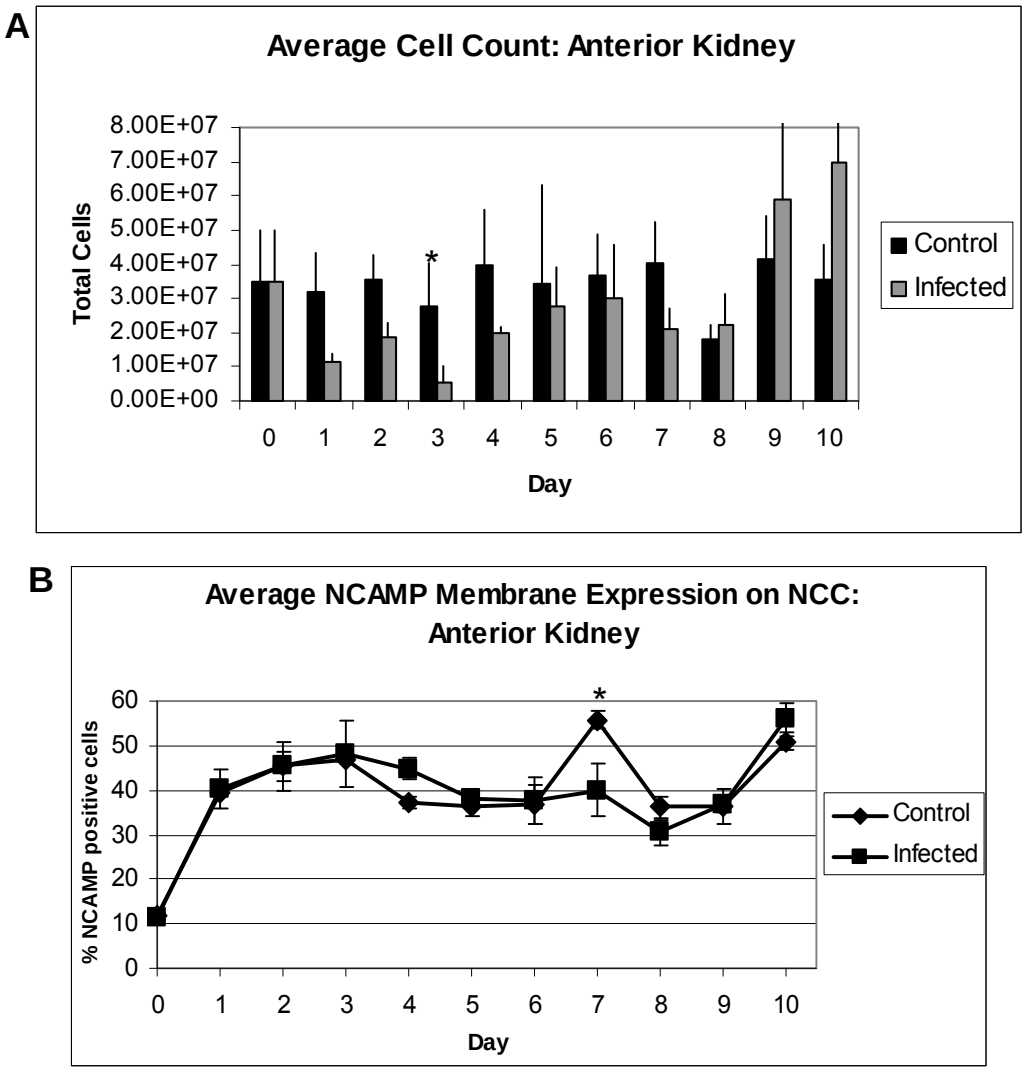

Figure 2.4. Cell Count and NCAMP membrane expression in the Anterior Kidney.

Channel catfish were infected with 10^7 CFU/ml of *Edwardsiella ictaluri* (infected) or were not exposed to bacteria (control). Every day for 10 days, 3 control and 3 infected catfish were sacrificed. The anterior kidney was excised from the fish and a single cell suspension was made. (A) Cell counts were performed from the individual fish anterior kidneys. (B) Flow cytometric analysis was performed to determine NCAMP-1 membrane expression following staining with mAb 9C9. Data is represented as an average of 3 fish and is representative of 2 independent experiments. Errors bars represent ± SEM. ($^*P < 0.05$)

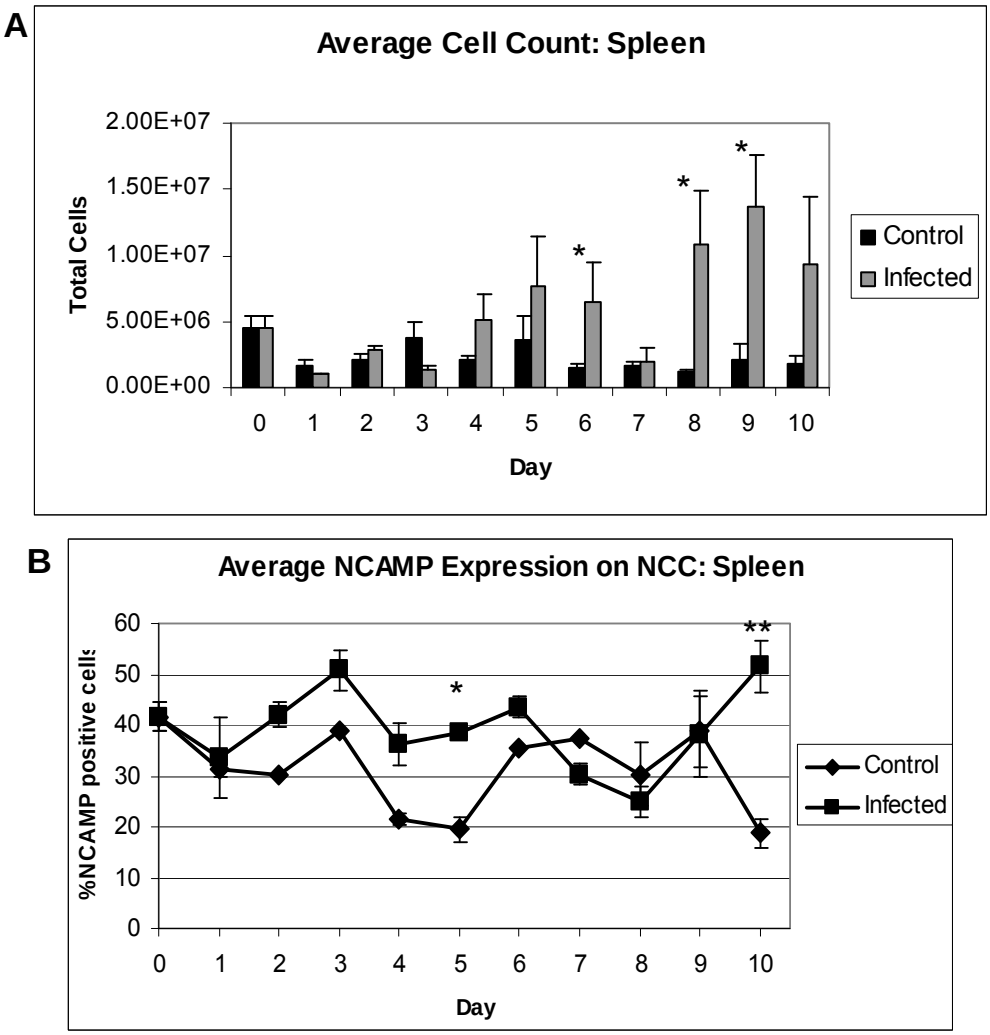

Figure 2.5. Cell Count and NCAMP membrane expression in Catfish Spleen. Channel catfish were either infected with 10^7 CFU/ml of *Edwardsiella ictaluri* (infected) or were not exposed to bacteria (control). Three control and 3 infected catfish were sacrificed once daily for 10 days. The spleen was removed from the catfish and a single cell suspension was made. (A) Cell counts were performed from the individual fish spleens. (B) Flow cytometric analysis was performed to determine NCAMP-1 membrane expression following staining with mAb 9C9. Data is represented as an average of 3 fish and is representative of 2 independent experiments. Errors bars represent ± SEM. ($^*P < 0.05$ and $^{**}P < 0.005$)

number of cells in the spleen of infected and control catfish was the same. Another significant peak that was characterized by a 6.5-fold increase ($2.08x10^6$ CFU/ml in control fish versus $1.37x10^7$ CFU/ml in infected fish) occurred on day 9 of infection.

Differences between control and infected fish NCAMP-1 membrane expression were identified in the spleen. A 12-15% increase in binding of NCAMP-1 was detected on days 3 through 5 (Figure 2.5B). An 18.95% increase occurred on day 5, followed by an 8.2% increase in NCAMP-1 surface expression on NCC on day 6. NCAMP-1 membrane expression decreased by 7% on day 7. Levels of NCAMP-1 on the membranes of NCC from control and infected fish were equal on day 9. On day 10, NCC from infected fish significantly had 32.7% more NCAMP-1 expressed on their membrane (2.7-fold increase).

Regulation of NCAMP-1, NCCRP-1, and TNF-α Gene Expression during Infection. Gene expression of NCAMP-1 in the anterior kidney was unaltered on day 1, was up-regulated (4-fold) on day 2 and was down-regulated (0.5 to 2-fold) on days 3 through 9 in infected fish compared to control fish (Figure 2.6A). Gene expression of NCCRP-1 in the anterior kidney of catfish infected with *E. ictaluri* was characterized by slight up-regulation (1-fold on day 2), down-regulation (0.5-1 on days 1, 3, 4, 6, and 7) or no change (days 5, 8, and 9) (Figure 2.6B). TNF-α gene expression was up-regulated in the anterior kidney of catfish with ESC on day 3 (2-fold increase) and days 6 through 10 (1.5 to 4.5-fold up-regulation) (Figure 2.6C).

NCAMP-1 gene expression in the spleen was increasingly down-regulated on days 1, 2, 3, and 4 (0.5 to 2 to 3-fold, respectively) in infected catfish (Figure 2.7A). NCAMP-1 expression was up-regulated in the spleen on day 6 (1-fold) and down-regulated on days 7 through 10 (0.7, 0.2, 1.1, 1.3-fold respectively). In the spleen of catfish infected with *E. ictaluri*, NCCRP-1 gene expression was up-regulated on days 1 through 6 with the peak up-regulation

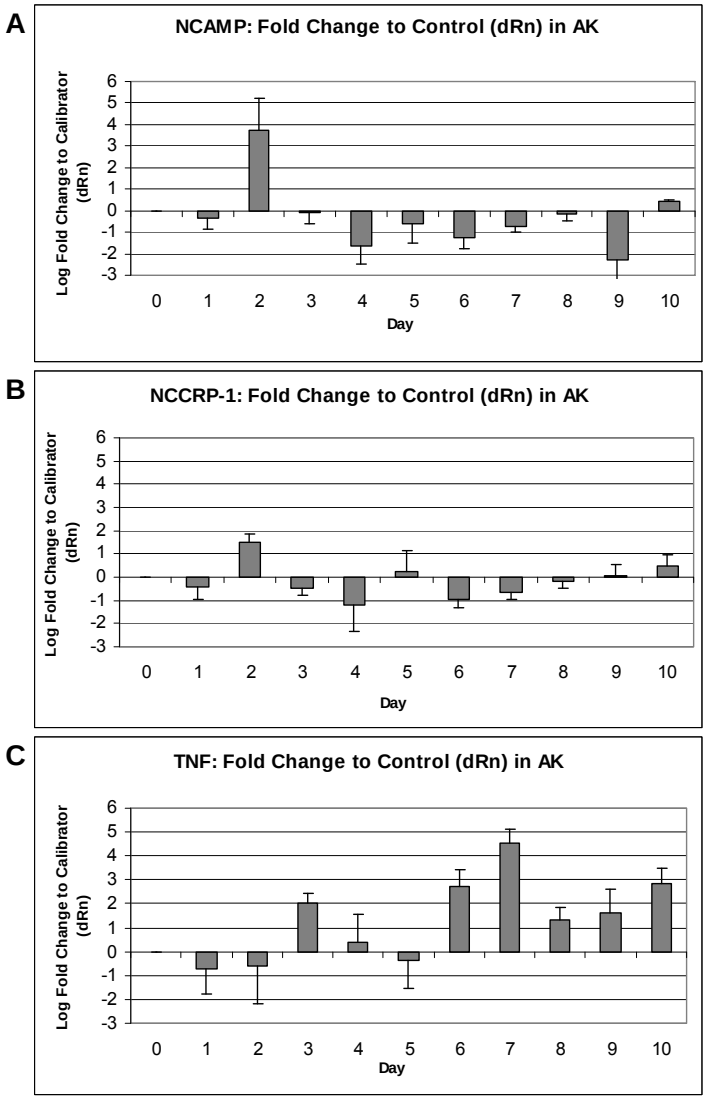

Figure 2.6. Fold Change in Gene Expression of NCAMP-1, NCCRP-1, and TNF-α in the Anterior Kidney. Quantitative PCR analysis was performed on cDNA isolated from the anterior kidney of 3 infected and 3 control fish on each day (0-10). Catfish actin was used as the internal control for normalization. Non-degenerate primer sets of NCAMP-1 (A), NCCRP-1 (B), and TNF-α (C) were used in the QPCR analysis. Uninfected fish were used as a calibrator to compare control versus infected fish. The fold change to calibrator (log base 2) was quantified by averaging fluorescence (dRn) values for the 3 fish in each treatment at each time point. Error bars are representative of ± SEM.

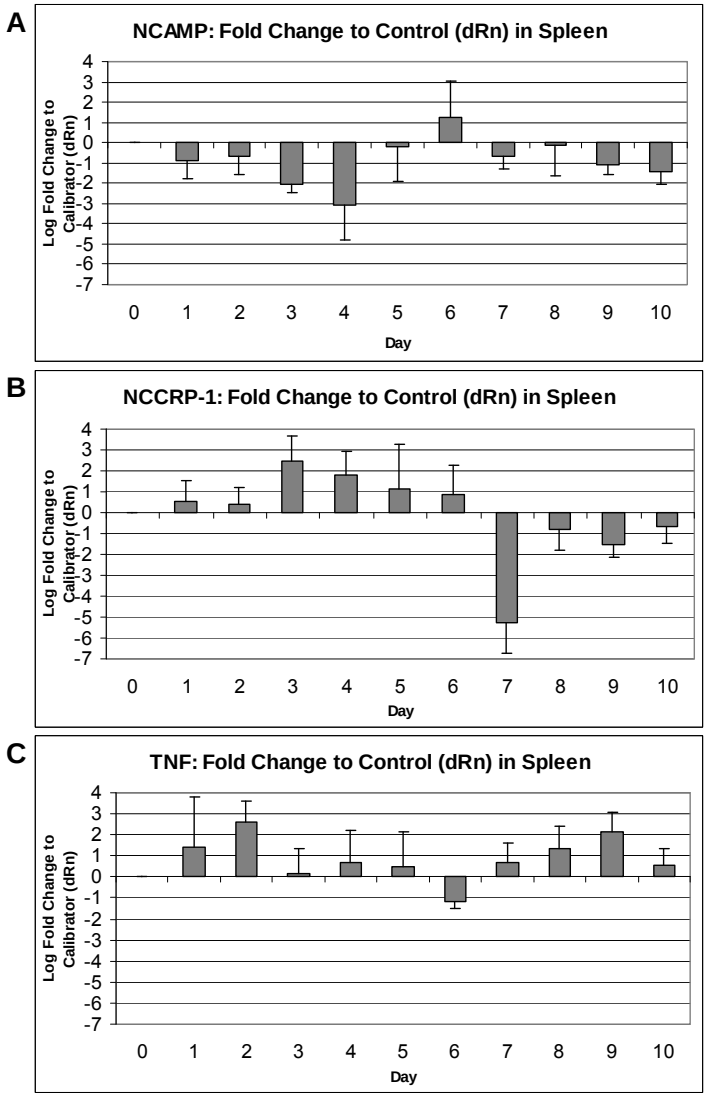

Figure 2.7. Fold Change in Gene Expression of NCAMP-1, NCCRP-1, and TNF-α in the Spleen. Quantitative PCR analysis was performed on cDNA isolated from the spleenocytes of 3 infected and 3 control fish on each day post-infection (0-10). The primer sets, NCAMP-1 (A), NCCRP-1 (B), and TNF-α (C), were used in the QPCR analysis. Catfish actin was used as the internal control for normalization. Uninfected fish were used as a calibrator to compare control versus infected fish. The fold change to calibrator (log base 2) was quantified by averaging fluorescence (dRn) values for the 3 fish in each treatment at each time point. Error bars are representative of ± SEM.

occurring on day 2 (2.4-fold) and was down-regulated on days 7 through 10 (5.2-fold down-regulation on day 7 and approximately 1-fold down-regulation on days 8 through 10) (Figure 2.7B). TNF-α gene expression was up-regulated on every day post-infection except day 6 (1-fold down-regulation) (Figure 2.7C). The peak days of TNF-α up-regulation were days 2 and 9 (2.6 and 2.2-fold up-regulation, respectively).

Discussion

Previous studies have investigated the role of stress on the immune response of channel catfish to *Edwardsiella ictaluri* infection. Handling, close confinement, fluctuating water temperature, improper diet, and poor water quality can stress the catfish increasing their susceptibility to *E. ictaluri* infection [12]. Few have looked exclusively at the innate immune response in catfish following infection with *E. ictaluri*. In the present study, we examined the change in cell count in the spleen and anterior kidney during the course of infection. Cell counts decreased in the anterior kidney beginning on day 1 (Figure 2.4A) suggesting that cells leave the anterior kidney and are recruited to other organs as part of the innate immune response. In contrast, more cells are present in the spleen starting on day 4 compared to control (Figure 2.5A). This indicates that NCC may leave the anterior kidney and travel to the spleen, where they encounter bacteria, during infection. Two peaks of increased cell numbers in the spleen occurred suggesting that there may be two significant influxes of immune cells to the spleen over time. Large variances occur in cell counts due to the variability between outbred fish.

Expression of TLR 3 and 5 during infection with *E. ictaluri* has been studied [17]. Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR). As a pattern recognition

receptor, NCAMP-1 binds oligodeoxynucleotides (ODNs) [25], or bacterial DNA motifs, and can be up-regulated on the membranes of NCC following activation with phorbol ester/calcium ionophore (Figure 2.1). NCAMP-1 membrane expression also changes in response to ESC in the spleen (Figure 2.5B) but not the anterior kidney (Figure 2.4B). In previous studies TLR5, which binds bacterial flagellin [30], had increased expression in the kidney and liver on day 5 post-exposure [17]. TLR3, which recognizes double-stranded RNA [30], increased in the spleen and, to a lesser extent, in the kidney over time [17]. Our results and the results of the TLR study show a similarity that more immunological function occurs in the spleen than the anterior kidney during *E. ictaluri* infection. *Edwardsiella ictaluri* is a blood-borne pathogen that enters through the gills. Thus it follows that the spleen would be a major site of infection. The NCC in the spleen are activated and express more NCAMP-1 on their membrane which makes them more efficient killers of bacteria at the site of infection.

The gene expression of different antimicrobial peptides in catfish has been studied. Hepicidin, an antimicrobial that is found in a variety of catfish tissues, is induced after infection with *E. ictaluri* [31]. Hepicidin is up-regulated in the spleen and head kidney at 1-3 days post-infection and returns to normal levels by day 7 [31]. Liver expressed AMP-2 (LEAP-2) has been cloned and sequenced from the livers of channel and blue catfish [21]. In response to infection with *Edwardsiella ictaluri*, LEAP-2 is modestly up-regulated in the spleen, but not in the head kidney or liver [21]. Changes in NCAMP-1 gene expression can be observed during ESC. NCAMP-1 is up-regulated in the anterior kidney on day 2 suggesting that more NCAMP-1 is being produced in response the initial infection (Figure 2.6A). NCAMP-1 is modestly down-regulated or unchanged in the anterior kidney following the initial production of the

antimicrobial peptide on day 2. In the spleen, NCAMP-1 is down-regulated over time (Figure 2.7A).

Nonspecific cytotoxic cell receptor protein-1 (NCCRP-1) is a type III membrane receptor protein that was isolated from the NCC of catfish and zebrafish [32-34]. NCCRP-1 is up-regulated in the spleen on days 3 through 6 suggesting that NCC are being recruited into the spleen during this time period of infection with *E. ictaluri* (Figure 2.7B). The gene expression of NCCRP-1 in the anterior kidney is moderately down-regulated during infection (Figure 2.6B). These results correlate to the trafficking seen in the cell count data. The expression of TNF-α in the spleen and anterior kidney is up-regulated mainly at the end of the course of infection (days 6 through 10) (Figures 2.6C and 2.7C).

In the present study, we have shown that NCAMP-1 in the NCC primarily of the spleen and moderately of the anterior kidney plays a role in the innate immune response of catfish during *E. ictaluri* infection. NCAMP-1 is a pattern recognition receptor that can be up-regulated on the membrane by calcium ionophore activation. Cells traffic from the anterior kidney to other immune organs (e.g. the spleen) during the infection. The membrane expression of NCAMP-1 is increased in the spleen during *E. ictaluri* infection. NCAMP-1 message is down-regulated in the spleen during ESC while NCCRP-1 gene expression in up-regulated as more NCC enter the spleen.

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CHAPTER 5

CONCLUSIONS

Nonspecific cytotoxic cells (NCC) are active participants in the antibacterial immunity of teleosts as both a pattern recognition receptor and an antimicrobial peptide. The purpose of the study was to determine the role of NCAMP-1 in the innate immune response of channel catfish against bacterial pathogens.

Recombinant NCAMP-1 had antimicrobial activity against a wide-range of microorganisms, including gram-negative *E. coli* and *E. ictaluri*. The N-terminus and C-terminus of recombinant NCAMP-1 killed bovine and avian clinical isolates of *E. coli*. Recombinant NCAMP-1-NT had rapid bactericidal activity that is detectable within 15 minutes of exposure to microbes. Polyclonal antibodies against NCAMP-1 specifically neutralize bacterial killing of rNCAMP-1-NT.

Using Western blot analysis, we have shown that NCAMP-1 is present in the acetic acid granule extracts of catfish NCC. The extracts had bactericidal activity against gram-negative pathogens. Pre-incubation of the acetic acid granule extract with anti-NCAMP-1 polyclonal antibody reduced the killing of *E. coli*. Therefore, NCAMP-1 is a potent antimicrobial peptide in the catfish NCC granule.

NCAMP-1 is a pattern recognition receptor that is present in the granules and on the membrane of NCC. Immunofluorescent microscopy and two-color flow cytometric analysis

demonstrated that NCAMP-1 is located on NCCRP-1 positive NCC. NCAMP-1 is a novel pattern recognition receptor that can be up-regulated on the cell membrane of NCC by phorbol ester/calcium ionophore activation.

NCAMP-1 in the NCC primarily of the spleen and moderately of the anterior kidney plays a role in the innate immune response of catfish during *Edwardsiella ictaluri* infection. Lymphocytes travel from the anterior kidney to other immune organs (e.g. the spleen) during the course of infection. The membrane expression of NCAMP-1 is increased on NCC in the spleen during *E. ictaluri* infection. NCAMP-1 message is down-regulated in the spleen during ESC while NCCRP-1 gene expression in up-regulated as more NCC enter the spleen.