NCAMP-1: A NOVEL CLASS OF DANGER MOLECULES IN TELEOSTS

by

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ABSTRACT

The nature and mechanism of action of host-derived molecules termed alarmins or damage-associated molecular patterns (DAMPs) in the inflammatory response remains elusive. The hypothesis in this work states that a soluble histone H1x-like protein, NCAMP-1 functions as an endogenous danger molecule in zebrafish. NCAMP-1 was present in the cytoplasm of different cell types and tissues and was released from immune cells. To identify pathways utilized by NCAMP-1 in cellular binding and activation, its effects were compared to those of ATP, known to act through the ligand-gated ion channel, $P2X_7$ receptor. Binding of either agonist to zebrafish leukocytes initiated intracellular calcium mobilization, pore formation and increased cytotoxic killing. While some of the effects between the two agonists are similar, significant differences in their mechanisms of action were found. Therefore, NCAMP-1 may utilize a unique mechanism of cellular binding and activation in its role as a multi-functional effector molecule and inflammatory mediator.

INDEX WORDS: alarmins, zebrafish, ATP, $P2X_7$ receptor, calcium mobilization, cytotoxic killing, inflammatory mediator

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## DEDICATION

This work is dedicated to my parents Robert and Addie Ann Monette. With out their foundation of love and support I would not have been provided with the many opportunities in life that I have.

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# CHAPTER 1

## INTRODUCTION

The zebrafish (*Danio rerio*), typically a model used for studies of developmental biology, is increasingly being used as an animal model for studies of immunity and infectious disease. The zebrafish has been useful specifically in studies of innate immunity such as the acute-phase response to infection, the interaction of host and pathogen and chemotactic responses to injury. There are many economical advantages to the using zebrafish as a model to study immunological responses, especially since the immune system of zebrafish has been shown to be very similar to humans. Also, the entire zebrafish genome has been sequenced and has been found to share a high degree of synteny to the human genome. A lavage technique has been developed using the coelomic cavity of zebrafish that obtains a heterogeneous population of leukocytes with immune mediated functions. Granulocytes make up a majority of this population (60-80%), while lymphocytes and NCC make up 10-20% and 4-10%, respectively.

In this study, we propose the use of this tissue to investigate possible alarmin characteristics of NCAMP-1 (NCC cationic antimicrobial peptide-1), a histone-like antimicrobial peptide and pattern recognition receptor that binds bacterial DNA. Alarmins are host-derived molecules with a previously identified intracellular role, which upon release into the extracellular milieu stimulate inflammatory processes in a manner similar to microbial products. Unregulated inflammation due to alarmins has been implicated in many autoimmune and inflammatory diseases. Examples of such mediators are cytosolic proteins such as heat shock

proteins, high-mobility group box 1 (HMGB1), and antimicrobial peptides such as the cathelicidin-derived LL37 peptide, and defensins. Other, non-protein alarmins include ATP, lysophophatidylcholine (LPC), uric acid, and DNA. The effects of soluble NCAMP-1 on zebrafish coelomic cavity cells (CC) were compared to those observed of ATP. Immunohistochemical analysis of whole zebrafish was performed to demonstrate the presence of NCAMP-1 in tissues of immunological significance. Confocal analysis of CC was performed to observe the nature of intracellular expression of NCAMP-1. Functional assays were performed to investigate alarmin activities on zebrafish CC such as ligand binding, pore-formation, and intracelullar calcium mobilization. This work provides a new avenue in which properties of alarmin molecules can be investigated on zebrafish leukocytes harvested from the coelomic cavity, as well as further characterization of NCAMP-1's role in innate immunity. It also reaffirms the use of the zebrafish coelomic cavity as an immunological model.

# CHAPTER 2

## LITERATURE REVIEW

### Inflammation

Inflammation is the immediate coordinated response in animals to microbial invasion or tissue damage designed to resolve infections and repair tissues with the goal to return to a state of homeostasis. In order for this inflammatory process to be efficacious and advantageous, there needs to be a balance between the ability to mount a rapid response appropriate to the particular trigger and the ability of the immune system to limit the damage associated with the mediators of inflammation. The significance of maintaining this balance is demonstrated by the observations that in certain chronic infections or inflammatory disorders, the inflammatory response causes more damage to the host than to the microbe. Thus, understanding the molecules that trigger inflammation and the pathways that ensue, are essential to animal health.

**Inflammatory mediators.** The initial inflammatory response that follows infection or injury represents widespread activation of the innate immune system [1]. The best characterized inflammatory triggers are microbial products, termed pattern-associated molecular patterns (PAMPs). PAMPs bind pattern recognition receptors (PRRs), germ-line encoded receptors such as the toll-like receptors (TLRs) and the Nod-like receptors (NLR). Extracellular and intracellular PRRs are expressed in effector cells such as macrophages, neutrophils, and dendritic cells. PAMP recognition stimulates signaling networks that result in the activation of transcriptional elements including nuclear factor-κB (NF-κB), interferon regulatory factor 3

(IRF-3), and activating protein 1 (AP-1) [1]. Activation of these transcription factors induces the expression of pro-inflammatory cytokines, chemokines, and other mediators of inflammation [2], ultimately leading to the activation of the adaptive immune system. Interferon-gamma (IFNγ) activates both neutrophils and macrophages for intracellular killing of microbes [3]. Patients with defects in the IL12/IFNγ activation pathways are at increased risk of severe mycobacterial and *Salmonella* infections [4], and recombinant IFNγ is an established therapy for patients with chronic granulomatous disease [5]. In addition to TNF and IFNγ, the proinflammatory cytokines of the IL-1 family, such as IL-1β and IL-18, also play a crucial role in host defense. Different subsets of T helper responses may be activated by the polarizing pro-inflammatory stimuli. For example, IL-1α and IL-1β, which bind and activate the same receptor [6], activate the release of additional proinflammatory cytokines such as TNF and IL-6, and induce a Th17 bias in the cellular adaptive responses [7]. IL-18 induces IFNγ expression and secretion to promote differentiation of T cells, inducing a Th1 response [8-9]. IL-33 has recently been identified as the ligand of the IL-1 receptor family protein ST2 and promotes responses mediated by Th2 T cells [10].

**Role of IL-1β in inflammation.** L-1β is largely responsible for inducing the acute phase inflammatory response such as fever and acute protein synthesis [6]. Although inflammation is a critical response to clear infections and repair tissue damage, it can lead to disorders when it is not tightly controlled. The important role of IL-1β in inflammation is exemplified by the autoinflammatory syndromes associated with the disruption of IL-1β activity and are characterized by attacks of sterile inflammation in joints, serositis, fever, and skin lesions [3]. Some of the diseases include familial Mediterranean fever (FMF) [11], cryopyrin-associated syndromes, such as familial cold auto-inflammatory syndrome (FCAS) [12], Muckle-Wells

syndrome (MWS) [13], TNF receptor-associated periodic syndrome (TRAPS), and adult-onset Still's disease [14]. An abnormal production of this cytokine has been proposed to be the underlying cause of these diseases as blood monocytes from patients with some of these disorders readily release more IL-1β than monocytes from unaffected controls.

Pro-IL-1β is transcribed and translated as a biologically inactive 31-kDa precursor and must be cleaved into its mature 17-kDa form before release [15]. Cleavage of proIL-1β requires the enzyme caspase-1, previously termed interleukin-1β converting enzyme (ICE). ICE is a member of a large family of intracellular aspartate specific cysteine proteases and it is also synthesized as zymogen that needs to be activated by cleavage from its precursor. The organizational structure that brings all these enzymes together is a multimeric protein complex called the inflammaosme that is responsible for the recruitment and activation of pro-caspase-1 [16]. In addition to IL-1β, the inflammasome formation is necessary for additional caspase-1 substrates such as pro-IL-18, interferon-gamma-inducing factor (IGIF) [17], IL-33 [10] and the related IL-1 family member IL-1F7b [18]. Thus, the regulation of inflammasome formation is a crucial component in the control of inflammatory mediators.

**Inflammasome formation.** Several inflammasome complexes have been described and the components have profound effects on innate immunity and infectious and non-infectious diseases by acting as key regulators of inflammation, energy metabolism and cell death [19]. Activation of the inflammasome occurs by oligomerization of its components, eventually leading to the recruitment and activation of caspases. The inflammasome was first described as consisting of the intracellular NALP1 (NLR family, domain containing 1) and its adaptor protein ASC (apoptosis-associated speck-like protein) containing a CARD (caspase recruitment domain). NALPs belong to the NOD-like receptor (NLR) family of pattern recognition receptors

which sense danger signals and microbial motifs to initiate inflammatory and anti-microbial responses [20-21]. Similar to intracellular TLRs, cytosolic NLRs use LRR regions to detect intracellular pathogens. The NLR family is divided into several subfamilies including the CIITA, nucleotide binding and oligomerization domain (NOD), IPAF and NALP subfamilies [19]. NLRs are characterized by 3 functional domains, including a central oligomerization domain (NACHT), a C-terminal ligand sensing leucine-rich repeats (LRRs), and an N-terminal caspase binding region, or caspase recruitment domain (CARD), in IPAF and NOD proteins, three BIRs (Baculovirus IAP Repeats) in NAIP, or a PYD (pyrin domain) in NALP proteins [22]. The NACHT domain, also called nucleotide binding site (NBS) or NOD, facilitates the formation of the oligomers. In the NALP1 inflammasome, caspase-1 is recruited by NALP1 through the adaptor molecule ASC via its CARD domain. ASC is also known as PYCAES and is a bimodular adaptor composed of a N-terminal PYD and a C-terminal CARD that mediates interaction with NALPs and caspase-1, respectively [23].

The NLR family member, NALP3, previously called cryopyrin or CIAS, is similar to NALP1 in that it requires ASC for caspase-1 activation, but like NALP2, it also requires a second adaptor known as CARDINAL [19]. Containing both ASC and CARDINAL, two molecules of caspase-1 are recruited to the NLRP3 inflammasome [24]. Although the exact mechanism leading to the activation of NLRP3 inflammasome is unclear several bacterial molecules are known to stimulate the NLRP3 inflammasome, such as muramyl dipeptide (MDP) [25], bacterial RNA [26], and double-stranded RNA. Several endogenous danger molecules also stimulate the inflammasome, such as uric acid crystals [27], ATP and amyloid-beta, along with exogenous compounds such as asbestos, silica [28], or alum adjuvant [29-30]. The activation of

inflammatory responses via the inflammasome by molecules produced endogenously may explain one mechanism by which inflammation occurs in the absence of infection.

**Host Danger Signals**: Host molecules are released to signal danger as a result of tissue injury or inflammation. These mediators, termed alarmins, though host-derived, initiate an inflammatory response that is similar to that initiated by microbial products [31-32]. Recent research indicates that alarmins and microbial products are recognized by many of the same PRR, adding complexity to the recognition abilities of the innate immune system. For this reason alarmins have been suggested to pertain to host endogenous signals while the term damage-associated molecular patterns (DAMPs) encompasses both alarmins and PAMPs. The general characteristics of alarmins are: (1) they are rapidly released following non-apoptotic cell death, while they are not released upon apoptosis: (2) viable immune cells can be induced to produce and release alarmins through secretion; (3) they activate cells of the immune system, especially APCs; and (4) alarmins should restore homeostasis by promoting the reconstruction of tissue [33].

Alarmins vary greatly depending on the cell or damaged tissue that releases them and in their molecular nature. The majority of alarmins are intracellular proteins that have a previously identified role and do not initiate inflammatory pathways until they are released to the extracellular milieu. They range from nuclear and cytosolic proteins such as heat shock proteins, high-mobility group box 1 (HMGB1) and hyaluronan fragments to antimicrobial peptides such as the cathelicidin-derived LL37 peptide, protegrin, and defensins. Other, non-protein alarmins include ATP, lysophophatidylcholine (LPC), uric acid, heparin sulfate and DNA.

## Host proteins with Dual Intracellular and Extracellular Roles

**HMGB1.** High-mobility group box 1 protein (HMGB1) belongs to a superfamily of nuclesome-binding proteins that have functions in controlling chromatin architecture, gene transcription modification, regulating DNA repair, cell differentiation and ontogenic development [34]. Once released by injured necrotic cells or activated immune cells, HMGB1 has been shown to behave as a chemoattractant and activator for antigen presenting cells (APCs), ultimately resulting in inflammatory processes.

Although HMGB1 is released passively from necrotic cells [35], mononuclear leukocytes can be induced to actively secrete the protein in response to PAMPs or proinflammatory cytokines [36-37]. The secretion pathway of HMGB1 is not entirely defined but it was found to be distinct from the classical Golgi- and endoplasmic reticulum-dependent pathway [37]. Many of the lysine residues of HMGB1 are acetylated in the nucleus followed by transport to the cytoplasm and eventually exocytosis [38-39]. The role of HMGB1 as a chemoattractant occurs via receptor of advanced glycation end products (RAGE) and can be partially inhibited by anti-RAGE antibody [40]. However, data suggest that RAGE is not the only receptor involved because the use of pertussis toxin, a selective inhibitor of Gαi protein-coupled receptors (GiPCR), blocks HMGB1-induced migration [41]. HMGB1 binds DNA and LPS and forms DNA-complexes in serum of patients with systemic autoimmune diseases [42-43]. By this mechanism, HMGB1 can act through TLRs 4 and 9 to further enhance its own secretion and potentiate the inflammatory response.

Monocytes stimulated with HMGB1 release numerous cytokines and inflammatory mediators. Addition of purified recombinant HMGB-1 to human monocytes significantly stimulated the release of TNF, IL-1α, IL-1β, IL-1RA, IL-6, IL-18, macrophage inflammatory

protein (MIP)-1α, and MIP-1β, but not IL-10, or IL-12 [44]. When HMGB1 is administered to Balb/c mice significantly increased serum TNF levels were detected [44]. A more recent study demonstrated that both highly purified eukaryotic and bacterial recombinant HMGB1 treatment of mononuclear cells induced TNFα secretion and nitric oxide release [45]. HMGB1 also stimulates the production of reactive oxygen species in neutrophils through a TLR4 dependent activation of NAD(P)H oxidase [46], as well as NK-κB activation resulting in increased secretion cytokines [47-48]. Maturation of dendritic cells (DCs) is induced by HMGB1 as measured by the increased expression of many cell surface markers, as well as secretion of inflammatory cytokines [49-51]. Specifically, recombinant HMGB1 (full-length and the B box domain) induced increased phenotypic expression of CD83, CD54, CD80, CD40, CD58, and MHC class II and decreased expression of CD206, while stimulating secretion of IL-12, IL-6, IL-1α, IL-8, TNFα and RANTES in human dendritic cells [49].

Early work on HMGB1 demonstrated its role as a late mediator of sepsis [52]. Considering the vast implicationS of HMGB1 in inflammatory processes, it is not surprising that it has been linked in the pathogenesis of a variety of non-infectious inflammatory conditions including autoimmunity, cancer, trauma and hemorrhagic shock, and ischemia-reperfusion injury [53]. Anti-HMGB1 antibodies are present in the serum of patients with rheumatoid arthritis and drug-induced systemic lupus erythematosus (SLE) [54-55]. Overexpression of extracellular HMGB1 is detected in synovial biopsy specimens of patients with rheumatoid arthritis [56] and skin lesions in patients with cutaneous lupus erythematosus [57]. In its association with cancer, HMGB1 plays a role in the transcription of several genes that have been implicated in cancer development including E-selectin, TNFα, insulin receptor, and BRCA [58-60]. Extracellular HMGB1 can lead to chronic inflammatory/reparative responses that, in the setting of cancer,

may lead to tumor cell survival, expansion, and metastases [61]. After initiation of hemorrhagic shock in mice, pulmonary HMGB1 levels are increased as soon as four hours [62]. This was demonstrated to occur in a TLR4-dependent manner [63]. These findings correlate with clinical observation of elevated circulating HMGB1 levels in trauma patients with hemorrhagic shock as compared with normal volunteers [64]. Significant evidence also implicates HMGB1 in ischemia/reperfusion injury (IRI) in multiple organ systems including kidney, brain, heart, and liver [53]. Using a hepatic IRI model in mice, elevated levels of HMGB1 are detected in tissue as early as one hour following reperfusion and continue to increase for up to twenty-four hours following insult [65]. In the same study neutralizing antibodies to HMGB1 ameliorates damage in a TLR4-dependent manner [65].

**Heat shock proteins.** Heat shock proteins (HSPs) are a very diverse group of proteins found in the cytosol, nucleus, mitochondria and the endoplasmic reticulum (ER) of cells. Apart from their roles in promoting correct folding, HSPs serve an important role as initiator of innate immune responses due to cellular stress [66]. For example, via TLR4, HSP60 induces the production of NO and TNF-alpha [67], while HSP70 also uses TLR4 to induce IL-12 production in macrohages [68]. TLRs (4 and 2) are also involved in the activation of DCs by HSPs (gp96 and gp60), resulting in the production of pro-inflammatory cytokines and other co-stimulatory factors [68-69]. Their involvement in inflammatory response stimulation has lead investigators to use HSPs as experimental adjuvants for poorly immunogenic substrates [70]. The abundant expression of HSPB8 in synovial tissue of arthritis is consistent with its role in promoting inflammatory processes and contributing to autoimmune diseases [66].

**Hyaluronan fragments and fibronectin.** Extracellular matrix (ECM) components hyaluronan and fibronectin have also been implicated as endogenous danger signals. During

injury or inflammation, degradation of these components results in ligation to macrophages and potentiation of inflammatory processes [66]. Hyaluronan binds CD44, but interestingly also signals through TLR2 and 4 [71] to mediate inflammatory processes. Fibronectin has been shown to bind to macrophages via the $\alpha_5\beta_1$-integrin receptor and transduce signals that either activate cells directly or prime them for activation by other ligands [72]. Another interaction of fibronectin fragments with macrophages involves the enhancement of phagocytosis [73].

## Antimicrobial Peptides (AMPs) with a Dual Role as Alarmins

**Cathelicidin-derived proteins (LL37**). Antimicrobial peptides (AMPs) are crucial to host defense against pathogens and play an essential role in the innate immune system. These peptides are typically cationic and amphipathic in nature and kill bacteria by disrupting microbial cell membranes [74]. Recent evidence indicates a major role for AMPs in inflammation initiation induced by infection or injury, even though they were originally characterized as direct effector molecules [75]. One well studied family of AMPs is the cathelicidins, which are produced with a signal sequence at the N-terminus, a conserved cathelin-like domain and a variable C-terminal antimicrobial domain [76]. Once the signal sequence is cleaved off, the pro-protein is further processed by elastase or other proteases. The released AMP can vary in size and sequence between species but all peptides have an overall cationic and often amphipathic character [76]. The only human cathelicidin family member (hCAP18) is predominantly found in neutrophils and in cells of the bone marrow. It is produced in its immature form as an 18-kDa peptide that is cleaved into its biologically active form, LL37, by serine proteases [77]. The human cathelicidin is stored in neutrophils in its inactive proform with in peroxidase-negative granules. LL37 has anti-microbial activity toward gram (+) and gram (-) bacteria, and also neutralizes extracellular LPS [78]. Other sources of LL37 include epithelial cells and

keratinocytes. LL37 expression increases in keratinocytes by skin injury associated with disruption of the epidermal permeability barrier [79-80].

Another important role in LL37's ability to activate innate immune responses is as a chemoattractant and inducer of IL-1β secretion. By binding to N-formyl peptide receptor-like 1, LL37 attracts neutrophils, monocytes, and T cells [81]. The C-terminal portion is thought to be responsible for this activity. LL37 induces maturation and IL-1β secretion from mouse monocytes via the $P2X_7$ nucleotide receptor [78]. LL37 treatment induced ATP release, membrane permeability, caspase-1 activation, all with out cytotoxicity [78]. IL-1β secretion and cell permeability were both blocked by $P2X_7$ receptor specific inhibitors [78]. Evidence also suggests that LL37 is able to initiate an inflammatory response by binding self-DNA and activating the TLR9 pathway in plasmacytoid dendritic cells (pDCs) [82].

In skin wounds, as might be expected for an antimicrobial peptide, a transient upregulation of LL37 expression occurs [75]. LL37 persists at high levels, however, in psoriatic skin throughout the development of the lesion [83]. In psoriatic skin, LL37 may couple continuously with extracellular self-DNA and trigger excessive and sustained IFN responses in pDCs, leading to the initiation and maintenance of autoimmune skin inflammation [75]. Apart from excessive levels of LL37 found in psoriatic skin, recent studies associate LL37 to autoimmune skin inflammation through the activity of Th17 cells. Th17 cytokines, IL-17 and IL-22 induce keratinocyte expression of cationic antimicrobial peptides [84-85]. It is proposed that this leads to a self-sustaining feedback loop that maintains autoimmune skin inflammation in psoriasis [75].

**Defensins.** Traditionally functioning as antimicrobial peptides, there are three subfamilies of defensins, α, β, and θ. Human α-defensin-1-4 are conventionally called human

neutrophil peptides (HNP1-4) due to their presence in the primary granules of neutrophils [86]. They vary in structure and cell source. α-defensins reside in Paneth cells, while β-defensins predominate in epithelial cells of various tissues, including keratinocytes [87]. Apart from their antimicrobial activities, α-and β-defensins have been shown to act as chemoattracting and activating agents for APCs. HNP1-3 and several β-defensins are chemotactic for various subsets of leukocytes including dendritic cells (DC), monocytes, and macrophages [88-91]. This chemoattractant function mediated through CCR6 and Gαi protein-coupled receptors (GαiPCRs), is inhibited by the Gαi protein-specific inhibitor pertussis toxin and CCR6 specific antibodies [92]. In addition to their chemoattractant activities, several defensins have the ability to activate a variety of leukocytes [87]. Human β-defensin-2 (HMBD2) and several α-defensins can activate mast cells and epithelial cells [93]. This resulted in the release of prostaglandins and histamine along with the production of many cytokines and chemokines. Mouse beta-defensin-2 (MBD2) induces dendritic cell activation involving the upregulation of DC costimulatory and major histocomapatibility complex (MHC) molecules, along with cytokine expression including IL-12, all in a TLR4-dependent manner [94].

Alarmins play critical roles in host antimicrobial immunity by initiating and augmenting both innate (direct microbicidal activity) and adaptive immune responses (cell recruitment and activation) [87]. Other AMPs with alarmin functions are lactoferrin and eosinophil-derived neurotoxin (EDN). While these are examples of AMPs with dual functions, there are some that do not function as alarmins such as neutrophil-derived azurocidin, and transferrin, lysozyme, myeloperoxidase, elastase, and cathespin G [37].

**Antimicrobial peptides in teleosts.** Antimicrobial peptides (AMPs) have been isolated from many species of teleost fish such as rainbow trout, Atlantic salmon, channel catfish, zebrafish, carp, and Atlantic halibut, and play an important role in innate immunity [95-96]. AMPs purified from teleosts demonstrate diverse killing activities. Fish AMPs can be isolated from mucus, a first barrier of defense against pathogens, where these killing activities would be appropriate [97]. A fish AMP isolated from skin secretions of white flounder called pleurocidin is localized in the granules of skin mucus and goblet cells [98]. An antimicrobial peptide, HLP-1, was isolated from acid extracts of channel catfish skin [97]. HLP-1 is closely related to histone H2B and has broad spectrum antimicrobial activity against *Aeromonas hydrophila* and *Vibrio alginolyticus*, but not *Edwardsiella ictaluri* [97]. Onchorhycin II is an AMP isolated from skin secretions of rainbow trout [99]. 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* [99]. 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* [100].

The highly conserved nature of the cathelin domain has been used to search for cathelicidins in other species. Several cathelicidins have been identified in fish [101-104], which display the feature seen in mammalian cathelicidins, a conserved cathelin region containing four cysteine residues and a variable portion [76]. Both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) have two cathelicidin genes each (*rtCath 1/2* and *asCath 1/2* respectively). Rainbow trout cathelicidin 1 (*rtCath1*) was found to be expressed only after infection, while *rtCath2* was expressed constitutively in many tissues and upregulated further after bacterial challenge in fish [101]. Three cathelicidin genes were identified in Atlantic cod

(*Gadus morhua*) (*codCath1-3*), which display a conserved four-exon organization as seen in all mammalian cathelicidins [105]. The peptides were found to be very cationic in nature [105]. Since it has been reported in mammals that there is a direct correlation between positive charge and binding strength to bacterial membranes [106], this would suggest that the cod cathelicidin would also have strong antimicrobial function. In the same study the cathelicidin gene from Arctic charr (*Salvelinus alpinus*), termed *acCath*, was also identified and found to lack exon 3 in the conserved region of the cathelin domain [105]. These teleost cathelicidins are constitutively expressed in tissues of infected fish at low levels and upregulated after challenge with *A. salmonicida* spp. *achromogenes* (Asa) [105]. It has not been reported to date, whether any of these AMPs have a dual function as alarmins.

## Non-Protein Molecules with Roles as Alarmins

**Uric acid.** Uric acid is naturally produced as an end product during the catabolism of purines. It was recently reported that uric acid is released from injured cells by DNA and RNA degradation followed by conversion of purines to uric acid, leading to its extracellular accumulation [107]. While uric acid is soluble within cells, in the extracellular milieu it readily precipitates into monosodium urate (MSU) microcrystals, which are thought to be its biologically active form [108]. It was reported that MSU stimulates dendritic cells, induces increased expression of CD86 and CD80, and enhances CD8+ T cell mediated lysis of target cells [107]. MSU decrease by uricase and allopurinol treatment of mice significantly reduced CTL activity in the same study. MSU engages the inflammasome resulting in production of IL-1β and IL-18. Cytokine secretion and inflammation due to MSU treatment is reduced in macrophages from mice deficient in IL-1R or in various components of the inflammasome, caspase-1, ASC, and NALP3 [27]. Uric acid deposition is associated with gout, the development

of acute and chronic inflammatory responses in joints and periarticular tissues [27]. One study implicates the role of uric acid in tumor rejection. It is demonstrated that uric acid levels are elevated in tumors undergoing immune rejection and that inhibition of its production, by uricase or allopurinol, delayed tumor rejection, while local administration of uric acid increased the rate of tumor rejection in C57BL/6 mice [109].

**DNA and others.** Under some conditions, DNA-chromatin complexes thought to act through TLR9 have been shown to stimulate proinflammatory cytokine production in splenocytes and endothelial cells in vitro [110]. An intracellular sensor called DNA-dependent activator of IFN-regulatory factors (DAI) was shown to be involved in NFκB activation upon binding of DNA [111]. In addition to DNA, lysophosphotidyl choline (LPC), calcium pyrophosphate □eparin□e (CPPD) crystals [107], and □eparin sulfate are also non-protein cellular components that demonstrate alarmin characteristics and functions.

**ATP.** The intracellular functions of ATP functions in energy and metabolism, is present in the extracellular millieu of normal tissue at low concentrations. However, it is rapidly released from a variety of cells under conditions of cell damage, hypoxia, ischemia, inflammation or even mechanical stress [112]. This sudden increase in extracellular ATP is tightly controlled by ubiquitous ecto-ATPases and endonucleotidases that hydrolyze ATP to less reactive ADP and AMP [113-114]. When the balance between ATP release and its degradation is not regulated, uncontrolled inflammation ensues. This sudden ATP increase is believed to play a role in asthma. Epithelial cells of lung airways can release ATP which increases the contractile ability of the airway smooth muscle (ASM), leading to airway hyper-responsiveness in patients with asthma [115]. In vivo, it has also been shown that ATP levels increase in the bronchoalveolar lavage (BAL) of patients challenged with allergen. Furthermore, features of asthma, such as

eosinophilic airway inflammation and bronchial hyper-reactivity were abolished in mice treated with the apyrase, an ATP neutralizing enzyme [116]. In addition to the pro-inflammatory activity, ATP by itself or in conjunction with TNFα , has been reported to activate DCs [117-118]. Recent data has shown that long exposure to low ATP concentrations has a profound effect on dendritic cell differentiation towards a Th2- polarized phenotype [119].

**ATP and $P2X_7R$.** The best characterized role of ATP in inflammation is its involvement in the processing and secretion of leaderless cytokines such as IL-1β and IL-6 via the purinergic receptor, $P2X_7$ ($P2X_7R$), and subsequent signaling through the inflammasome. There are two families of purinergic receptors, termed P2Y and P2X, which are G-protein coupled and ligand-gated ion channel, respectively. The P2X receptor family consists of seven transmembrane ion-channels (1-7) that differ with respect to nucleotide agonist and antagonist sensitivity. $P2X_7R$ is specific for ATP and is predominantly expressed in cells of hematopoietic lineage, including monocytes, macrophages and lymphocytes [120]. P2X receptors consist of three subunits that form intrinsic pores that switch conformation from closed to open upon ATP binding, allowing the flow of ions. The flow of ions is a key step in signaling, because it changes the transmembrane potential as well as local ion concentrations [121]. Functional expression studies provide evidence that the P2X family subunits are able to form heteromultimeric receptors, however, the number of actual subunits that exist normally is inconclusive [122]. It has not been shown yet that $P2X_7$ subunits will co-assemble with any others and they are also most distinct in sequence [122].

**$P2X_7$ receptor characterization.** The $P2X_7$ receptor was initially called the P2Z receptor [123] and its distinct properties were: low sensitivity to ATP relative to other P2X receptors, high sensitivity to analogue 2',3'-O-(4-benzoylbenzoyl) ATP (BzATP) relative to ATP, and

lastly, marked potentiation of the response by reducing the concentration of extracellular divalent ions [121]. $P2X_7R$ is 595 amino acids in length. Each subunit has two hydrophobic, putative membrane-spanning segments (TM1 and TM2) separated by an ectodomain that contains ten conserved cysteine residues that form disulphide bonds [122]. Thus, the amino and carboxy termini are intracellular, and most of the molecule (about 280 amino acids) forms an extracellular loop [122].

The ability of ATP to induce pore formation was first observed in peritoneal mast cells [124] and is now more commonly studied in macrophages and lymphocytes by ATP-induced uptake of fluorescent dyes (such as ethidium and YO-PRO-1), which become fluorescent when they bind intracellular nucleic acids [121]. These dyes have the advantage that they can be added in relatively low concentrations (typically ~1uM) to an otherwise physiological solution [122]. In most respects, the properties of ATP-evoked YO-PRO-1 uptake closely resemble those of ATP-evoked ionic current [122]. Brief application of agonist renders dye and ion currents fully reversible, with effects similar between ATP and BzATP [125]. The uptake of calcium into cells following a stimulus is also measured with fluorescent dyes. This function of $P2X_7R$ activation has been measured in HEK293 cells transfected with rat $P2X_7R$ [108] and human $P2X_7R$ [108, 126-127].

It has been shown that three molecules of ATP bind to the extracellular portions of P2X receptors and mutagenesis experiments indicate that conserved lysines near the extracellular ends of TM1 and TM2 may contribute to its binding [128]. The same investigators found that aromatic residues within the extracellular loop are involved in coordinating the adenine group of ATP [128]. The C termini sequences of P2X receptors vary greatly among the seven subunit types but there is a conserved YXXXK sequence (where "X" is any amino acid) in the

juxtamembrane region [129]. The C-terminus of the $P2X_7R$ is much longer than the other receptors, at least 200 amino acids, and is thought to contribute to downstream signaling. Potassium efflux and calcium influx induced by ligand binding lead to the activation of various phospholipases involved in the activation of caspase-1 and IL-1β release [130-131]. This process has been shown to be mediated by the formation of a large pore formed by the protein pannexin-1, after P2X7 receptor activation [132-133]. This protein is proposed to serve as a non-selective hemichannel pore [133] and displays structural similarity to a family of non-mammalian proteins, innexins, that form gap junctions in invertebrate tissue [134-135]. ADP and AMP are very weak agonists for the $P2X_7R$ but it has been found that after a brief exposure to ATP, the effectiveness of ADP and AMP binding is augmented [136]. Additional mutagenesis studies have attempted to determine areas of function within the $P2X_7R$. For example, truncation of the protein (deletion of residues from 419 to 595) results in a receptor with much reduced uptake of YO-PRO-1 [125].

Individual natural polymorphisms have given rise to further characterization of $P2X_7R$ function. In about twenty percent of individuals, a Glu496 to Ala polymorphism (1513A->C), which is located in the carboxyl terminus of the $P2X_7R$ leads to a loss of function in homozygous individuals, while only about fifty percent reduction in heterozygous individuals [137-138]. A second polymorphism that involves the exchange of Asn for Ile568 (1729->A), prevents normal trafficking and surface expression of the receptor on the membrane as amino acid lies in the trafficking motif of the carboxyl terminus [139-140]. It is thought that the large amount of positively charged amino acids present in the extracellular loop, for example Lys193 and Lys311, are responsible for formation of an ATP binding site. It has been shown that a naturally

occurring polymorphism Arg307 to Gln results in a complete loss of receptor function, interpreted as a disruption in the ATP binding site [141].

There are five main types of $P2X_7R$ inhibitors. The first class consists of ions such as calcium, magnesium, zinc, copper, and protons and they all inhibit ATP-evoked currents of the rat $P2X_7$ receptor [122]. The second class consists of generic P2X receptor antagonists. They are suramin, PPADS and Brilliant Blue G [142] and oxidized ATP (oATP). Brilliant Blue G is a strong inhibitor as it blocks rat $P2X_7$ receptors at 10nM and human $P2X_7$ receptors at 200nM [122] . oATP (ATP with the 2'- and 3'-hydroxyl moieties oxidized to aldehydes by periodate treatment) irreversibly blocks currents at a concentration of 100uM. It is a selective inhibitor of $P2X_7R$ and completely blocks secretion of IL-1β from LPS primed human monocytes, after treatment of ATP [143]. The third group of blockers contains calmidazolium and KN-62, two large organic cations. Calmidazolium, {1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorophenylmethoxy)-ethyl]-1H-imidazolium}, was originally introduced as a calmodulin antagonist while KN-62 {4-[2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl ester}, a piperazine, was originally intended as an inhibitor of calcium/calmodulin-dependent portein kinase type II (CaM kinase II). KN-62 blocks currents in cells expressing the human $P2X_7R$ but has little effect on the rat P2X7R [144]. The last two classes of $P2X_7R$ blockers consist of 17β-estradiol [145] and a monoclonal antibody selective for human $P2X_7R$ [146].

**$P2X_7R$ mediated release of IL-β.** It has been shown that upon inflammatory insult, immune cells generate large quantities of pro-IL-1β but release very little of the mature, biologically active cytokine. It was first discovered that ATP is necessary for the post-translational processing and release of IL-1β in mouse peritoneal macrophages primed with

lipopolysaccharide (LPS) [147]. Since then, many studies have demonstrated the requirement of exogenous ATP, as a second signal to activate the inflammasome, for the production of active IL-1β and the subsequent impact of this on the inflammatory response [148].

Macrophages from $P2X_7R$ gene-deficient mice do not release IL-1β and are deficient in IL-6 in response to ATP, post-priming with LPS [148]. The results suggest that blocking IL-1β release can impair cytokine signaling cascades in vivo [149]. The $P2X_7R$-deficient mice develop significantly reduced arthritis on induction of collagen injection into their joints [150]. In addition, the mice also show reduced or absent behavioral responses to inflammation of the paw, as well as tactile allodynia [151]. These results appear to result from a reduced production of IL-1β in the paw itself [121].

The role of the inflammasome in infection has also been well characterized. Caspase-1 recruitment and activation by the inflammasome is important for the host immune response to pathogens as it has been demonstrated that caspase-1-deficient mice are susceptible to several bacterial infections such as *Escherichia coli* [152], *Shigella flexneri* [153], *Salmonella typhimurium* [154] and *Francisella tularensis*. Partial defects in clearance are seen in response to *Listeria monocytogenes* [155] or the fungal pathogen *Candida albicans* [156].

The examples explained highlight the significance of inflammasome activation as a critical component of innate responses to bacterial challenge as well as a its activation to release IL-1β in response to host-derived factors. Alarmins, such as uric acid crystals, amyloid-beta and calcium pyrophosphate, induce inflammation via the $P2X_7$ receptor [27]. In addition other factors such as the potassium ionophore, nigericin, and a potent marine toxin, maitotoxin, are also capable of stimulating the release of IL-1β via the $P2X_7R$ [29]. These molecules are thought to directly interact with the central component of the inflammasome, NALP3. ATP is a potent

stimulus for activation of the inflammasome via the $P2X_7R$ in a NALP3-dependent manner [157]. Since NLRs appear to act as direct receptors of PAMPS or alarmins it is hypothesized that rather than being classified as 'receptors', NLRs might appropriately be termed 'guard' proteins [19].

In addition to its role in inflammation, caspase-1 also has other implicated functions such as induction of macrophage cell death by bacteria [19] and induction of apoptosis after over-expression in fibroblasts [158]. Recently, caspase-1 has been implicated in unconventional secretion of proteins [159], including leaderless proteins that lack a leader sequence, such as IL-1α and fibroblast growth factor 2 (FGF2). Altogether, these functions suggest that caspase-1 activation leads to the processing and release of various proteins with functions in inflammation, tissue repair and maintaining homeostasis after tissue damage or stress in response to infection [19].

Although IL-1β is a necessary proinflammatory cytokine, it is responsible for pathological conditions at high concentrations [160]. Briefly mentioned previously, dysregulated production of IL-1β is associated with rheumatoid arthritis, inflammatory bowel disease, sleep sickness, acute and chronic myelogenous leukemia, insulin-dependent mellitus, atherosclerosis, asthma, and septic shock [161]. Muckle-Wells syndrome (MWS), an inherited autoinflammatory disorder in the category of familial periodic fever syndromes, is linked specifically to mutations in the *Nalp3* (*Cias1*) gene [162]. Functional studies have revealed that the *Cias1* mutation results in a gain of function phenotype, as the ensuing mutant proteins are constitutively active and able to induce NF-κB activation and IL-1β release [24, 163]. Another inflammatory disease associated with a mutation in a component of the inflammasome is Crohn's Disease (CD). It

involves frameshift mutations in the *Nod2* gene, affecting the LRR domain, leading to a defect in the ability of NOD2 to sense bacteria [164-165].

The $P2X_7R$ ortholog was identified in zebrafish, along with eight other identified P2X subunits [166]. Six of them are homologous to the mammalian counterpart, while 2 were identified as paralogs [166]. It remains unknown whether the last gene is a homolog, paralog or new subunit [166]. Further, multiple sequence alignment have shown that all the fish $P2X_7$ receptors sequenced maintain intact the five positively charged amino acids responsible for nucleotide binding (K64, K66, R294, R307, and K311) [141, 167-168] However, only three out of five mammalian amino acid residues of the intracellular domain (E496, I568, and R578) that have been implicated in receptor trafficking and function are also conserved in the non-mammalian receptors [137-138, 140, 169].

Although extensive work has been done to characterize functions of the $P2X_7R$ in humans, mice, and rats, little has been done in teleost species. In one study, endotoxin challenge led to a significant increase in IL-1β intracellular expression in giltehead seabream (*Sparus aurata*) macrophages [170]. The same investigators found that extracellular ATP promoted the release of IL-1β from a seabream fibroblast cell line (SAF-1 cells) after endotoxin priming consistent with stimulation of the $P2X_7R$ [171]. Use of the potent agonist BzATP led to permeabilization in a subset of acidophilic granulocytes and was completely reversible, but release of IL-1β did not result from these cells primed with different TLRs and Nod proteins as has been shown for ATP [171]. The zebrafish and seabream $P2X_7R$ genes were individually expressed in HEK293 cells to compare agonist and antagonist profiles to cells transfected with the rat $P2X_7$ gene [172]. It was found that responses to ATP and BzATP were greatly reduced in the cells expressing zebrafish and seabream $P2X_7R$ than the rat expressing cells [172]. These

studies suggest that although there are significant conserved similarities between the generation of pro-inflammatory mediators in mammals and teleost, there appear to be sufficient discrepancies that will merit a more in depth analysis.

**Inflammasome components in teleosts.** The zebrafish genome encodes the great majority of the components that mediate apoptosis and inflammation in mammals, including humans [173-174]. Two zebrafish caspases containing N-terminal pyrin domains, Caspy and Caspy2 have been identified [175]. Although, the processing of IL-1β was not assayed, expression of Caspy and Caspy2 induced apoptosis in mammalian cells, exhibited different substrate specificity and were inhibited by general caspase inhibitors [175]. Furthermore, Caspy, but not Caspy2 interacted with the zebrafish orthologue of the mammalian ASC (zAsc) via the signature homophilic pyrin-pyrin domain interaction [175]. Three distinct NLR subfamilies were identified by genomic mining of the annotated zebrafish sequences [176]. The first subfamily (NLR-A) resembles mammalian NODs and is highly conserved relative to human components, the second (NLR-B) resembles mammalian NALPs, while the third (NLR-C) appears to be unique to teleost fish [176]. The last subfamily contained several hundred genes, while NLR-A and NLR-B contained five and six genes, respectively. Using RT-PCR, the spatial expression of NLR genes were evaluated in zebrafish tissues. NLR-A1 through -A5 were all expressed in the intestine of the zebrafish [176]. All five genes were also identified in the liver as well as the spleen, with the exception of the absence of NLR-A3 in the spleen. NLR-B2 and NLR-C mRNA were detected in all three tissues [176]. Genomic and functional analysis of zebrafish components should provide insight into the mechanisms that mediate apoptosis and inflammation in mammalian systems.

## Zebrafish (*Danio rerio*) as an Immunological Model

The zebrafish (*Danio rerio*), typically used in studies for of developmental biology, is increasingly being used as an animal model for studies of immunity and infectious disease [177]. The zebrafish has been useful specifically in studies of innate immunity such as the acute-phase response to infection, the interaction of host and pathogen and chemotactic responses to injury [178]. The zebrafish serves as a useful animal model for many reasons, such as low cost and required laboratory space, high fecundity, and rapid development [177]. The majority of tissues form within 24 hours post-fertilization (hpf) and sexual maturity is reached with in 3 months [179]. Embryonic development can be observed freely due to its transparency and occurs externally to the mother. Also, the entire zebrafish genome has been sequenced and it has been found to share a high degree of synteny to the human genome [180-181].

The immune system of the zebrafish has been shown to be very similar to humans and other vertebrates while having cells and tissues not present in invertebrate models such as *C. elegans* and *Drosophila* [177, 180]. In contrast to these invertebrate models that lack adaptive immune systems, the zebrafish as a teleost species has both innate and adaptive capabilities [177-178, 180]. Studies in teleosts have demonstrated the inclusion of all major blood lineages [180], however the lack of standard reagents such as specific phenotyping antibodies has made the investigation of zebrafish immune cell function difficult.

The site of hematopoiesis changes through out the developmental process in teleost species. This occurs in zebrafish as primitive and definitive hematopoiesis [182]. Primitive hematopoiesis occurs in the intermediate cell mass (ICM), the equivalent to the mammalian yolk sac [182-185]. It then shifts to an area homologous to the mammalian AGM region (aorta, gonad and mesonephros), then to the caudal hematopoietic tissue [186] and finally to the pronephros

[178]. The major lymphoid organs in teleosts are the thymus, kidney, spleen and gut-associated lymphoid tissue [178]. The kidney is considered to be the fish equivalent of the mammalian bone marrow [184]. It is made up of two segments, the pronephros or head kidney, which predominantly consists of hematopoietic tissue, and the mesonephros or trunk kidney, which is mainly responsible for renal activity [178].

The ability of host derived molecules to initiate inflammatory responses and signal danger in these vertebrates is important to investigate due to their place in the evolution of the immune system. They are immersed in the environment and therefore must have a robust innate immune response to pathogens since activation of adaptive immunity would not provide immediate protection. There are several examples of innate immune parameters in fish that are more active and show more diversity than comparable components of mammalian species [187]. Examples of this are complement components like C3 and Bf and high spontaneous activity of the alternative pathway [188-189].

Fish orthologs of a fundamental pattern recognition receptor, toll-like receptors (TLRs), have been described [190-193]. Currently, a total of 24 putative variants of TLRs have been identified in zebrafish. Orthologues of ten human TLR genes have also been found [194]. Even with all of this machinery, one major difference between the immune response of mammals and fish is the response to LPS. Fish are more resistant to the toxic effects of LPS than mammals and as a result high concentrations of LPS are used in studies to investigate the immune responses to this type of activation [195-197]. Zymosan, MDP, particulate β-glucan, and poly(I:C) were able to stimulate the upregulation of TNF2 in trout mononuclear phagocytes (rtMOCs) with the same sensitivity as mammalian cells, but the concentration of LPS required was 1000-fold higher [198]. The difference in resistance is presently not understood.

Models of infection using the zebrafish play an important role in understanding the way pathogens cause disease. Mice have been the host model of choice for many years, but in some bacterial models, zebrafish are better at mimicking human disease characteristics [177]. For example, infection of zebrafish with *Mycobacterium marinum*, mimics the naturally occurring progression of tuberculosis in humans with the formation of granuloma-like lesions and the ability to establish acute or chronic infections based on inoculum [199-200]. Other models of infection using the zebrafish to evaluate the acute-phase response in zebrafish involve *Aeromonas salmonicida*, *Staphylococcus aureus* [201], *Francisella spp*. [202], *Edwardsiella spp*. [203-204], and *Streptococcus spp*. [205-206]. Infection resulted in a rapid upregulation of proinflammatory cytokines like IL-1β and TNFα. This early phase response is followed by increased expression of proteins such as fibrinogen, haptoglobin, complement components, and hepcidin [178]. The results from these studies demonstrate the temporal expression of acute-phase proteins in the zebrafish acute-phase response that closely mimics that seen in humans [178].

In addition to infection models, the zebrafish can serve as model of noninfectious diseases. Using transgenic procedures, a model of T cell acute lymphoblastic leukemia (T-ALL) was developed in which the onset of leukemia can be monitored in real time [207]. The zebrafish carry the mouse *c-myc* oncogene under the control of the *rag2* promoter [178] and molecular characterization of harvested tissue confirms that the contained cells are clonal and arrested at an early stage of T cell development [207]. A second T-ALL model was created by introducing the human oncogene, *Notch1*, into zebrafish under the *rag2* promoter [178]. Characterization of the leukemic cells shows they are oligoclonal and transplantable, however, in contrast to the *myc* model, they do not show increased expression of tal1/scl and lmo2 [208].

Although this is a new area of research for the zebrafish as a model, the ability to carry out forward genetic screens will speed the identification of genes, novel or with novel functions, affecting virtually all biological processes [209]. The use of novel approaches that probe genes involved with autoimmunity and leukemogenesis, show that zebrafish will be instrumental in elucidating physiology and pathology of the immune system [209].

## Extracellular Histones

Traditionally, histones associate with chromatin fibers in the cell nucleus. A complex of four core histones (H2A, H2B, H3 and H4) form an octamer to create the nucleosome that functions to stabilize DNA [210]. Histone H1 is a compartment of nuclear chromatin that contributes to packaging of nucleosomes into higher order structures [210]. It also stabilizes the nucleosome and is located at regions of entry and exit of DNA in the nucleosome [211]. Histones are also involved in transcriptional regulation by alteration of chromatin structure [212-213], while histone H1 also plays a role in cell proliferation and differentiation, apoptosis, and ageing [214].

Although, histone H1 is considered a nucleus-associated protein, it also localizes to the cytoplasm and cell surface [210]. Evidence suggests that intact or cleaved histone fragments are released during apoptosis and have antimicrobial activity, an important component in innate immunity [210]. Histone H1 that is released from epithelial cells during apoptosis may contribute to host protection following microbial invasion [215]. Evidence indicates that histones can also be secreted as antimicrobials from granules [216]. Histones H2A and H2B are located on the cell membranes of human monocytes [217-219] and serve as receptors for exogenous DNA, leading to endocytosis and degradation [217]. Monocyte activation results in increased expression of the histones on the cell membrane [218]. Other examples of the

widespread expression of membrane histones include neurons [220] and macrophages [221]. These two cell types have been shown to have a 30-33 kDa membrane histone H1, which appear to act as receptors for LPS and thyroglobulin, respectively.

Histones with antimicrobial activity have been isolated from a number of teleost species. For example, SAMPH1 is a histone-like antimicrobial peptide isolated from the skin mucus of Atlantic salmon, with activity against gram-negative and gram-positive bacteria [222]. Another protein, HDSF-1, has an identical sequence to trout histone H1 and was found in the mucus and blood of Coho salmon [223]. Parasin-1 is isolated from catfish and is an AMP that is cleaved from histone H2A by a specific protease and has strong antimicrobial activity against gram-negative and gram-positive bacteria, as well as fungi without significant hemolytic activity [224-225]. It is inducible and is only found in skin mucus of injured catfish, providing protection against microbe invasion at the site of injury [224].

Histones also exist as binding components of neutrophil extracellular traps (NETs) [226], an important form of innate immunity as they kill and prevent the spread of microbes. Histones, DNA, and granular proteins are the main components of NETs [227]. The fibrous structure of NETs is crucial to the ability to trap bacteria and ensure that a potent, local concentrations of AMP is delivered to bound microbes [227]. NETs are released from fish kidney neutrophils and as such may be important in teleost innate immunity [227]. They are released from neutrophils isolated from the zebrafish along with myeloperoxidase (MPO) upon stimulation with calcium ionophore, phorbol myristate acetate, and beta-glucan [228].

**NCAMP-1**

A 203 amino acid long (22 kDa molecular mass) cationic anti-microbial peptide, NCAMP-1 (Nonspecific cytotoxic cell cationic antimicrobial peptide-1), was purified from catfish NCC (nonspecific cytotoxic cells) [229]. NCC are the teleost equivalent of mammalian natural killer (NK) cells [230-231]. NCC are small nucleated cells found in the anterior kidney, spleen, liver, and peripheral blood of several species of fish [232-234]. The identification of granzymes in tilapia and catfish cytotoxic cells suggests that NCC contain small granules [235-236].The amino acid sequence of NCAMP-1 (Accession Numbers AAQ99138 and AY324398) shows that it is lysine-rich and shares 42.4% and 51.2% identity with human and zebrafish histone family member H1X, respectively [229]. NCAMP-1 is expressed on the membranes of catfish NCC as determined by flow cytometric analysis using anti-NCAMP-1 polyclonal and monoclonal antibodies [229]. Recombinant NCAMP-1 as well as the membrane form of NCAMP-1 on catfish NCC has been shown to bind GpC and CpG oligodeoxynucleotides (ODNs) [229]. As a direct antimicrobial protein, the recombinant expression of the protein has shown that full-length and truncated forms were effective in killing of *Escherichia coli* and *Streptococcus iniae* isolates in vitro [229]. A recent study shows that the N-terminal portion was able to effectively kill the pathogen responsible for hemolytic septicemia in catfish, *Edwardsiella ictaluri*. In the same study, acetic granule extracts from catfish NCC were found to have antimicrobial activity against *E. coli* APEC 3721 in a concentrations dependent manner and was inhibited by NCAMP-1 specific polyclonal antibody [237].

NCAMP-1 activity has been shown to be relevant in higher vertebrates as well. NCAMP-1 is expressed on the membrane of a human NK cell line, YT-INDY, and binds dGT20 and CpG ODNs [238]. Upon binding of YT-INDY cells, ODNs induced activation and calcium

influx within seconds of treatment [238]. NCAMP-1 is also expressed on a variety of mouse leukocytes derived from blood, mesenteric lymph nodes, and spleen [239]. NCAMP-1 expression was found to predominate in granulocytes, NK cells and monocytes [239]. Granule extracts from RAW 264.7 also had antimicrobial activity, which was abrogated by anti-NCAMP-1 polyclonal antibody, corresponding to the dual role of the protein seen in catfish NCC [239]. Confocal analysis of RAW cells demonstrated that NCAMP-1 may be associated with a secretory exocytosis pathway rather than a constitutive component of a secondary granule/phagolysosome endocytic pathway.

Recent unpublished data from our laboratory has demonstrated that serum levels of NCAMP-1 increase in catfish challenged with *E. ictaluri* and *Ichthyophthirius multifilis*. These results appear to implicate NCAMP-1 as an alarmin involved in the generation of inflammatory mediators in teleosts.

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# CHAPTER 3

## NCAMP-1: A NOVEL CLASS OF DANGER MOLECULES IN TELEOSTS[1]

[1]**Monette, M. M, J.H. Leary, D.L. Evans, T.M. Krunkosky, A. Camus and L. Jaso-Friedmann. To Be Submitted to Developmental & Comparative Immunology.**

## Abstract

Inflammation results from the binding of self molecules or microbial molecules to pattern recognition receptors (PRR). Most work has previously focused on microbial ligands and their receptors. The identification and the mechanism of action of self molecules, termed alarmins or damage-associated molecular patterns (DAMPs), in the inflammatory response have been difficult to elucidate. The present study proposes a novel role for an ancient class of molecules consisting of a soluble histone H1x-like protein (e.g., nonspecific cytotoxic cationic antimicrobial peptide-1/ NCAMP-1). NCAMP-1 may function as DAMP/alarmin. In the present study cells of the zebrafish coelomic cavity (CC) were developed as model targets for NCAMP-1 activities. The results demonstrated that NCAMP-1 is present in the cytoplasm of various cell types and tissues and is released upon cell damage. Once released by CC cells, soluble NCAMP-1 binds to receptors on CC cells and induces cellular activation. In an effort to identify the pathways utilized by NCAMP-1 in cellular binding and activation, its effects were compared to those of another alarmin ATP. This DAMP is thought to act through the ligand-gated ion channel, $P2X_7$ receptor ($P2X_7R$). The results show that similar to ATP, binding of NCAMP-1 to cells initiates intracellular calcium mobilization, pore formation and increased cytotoxicity. While the activating functions of the two agonists are similar, significant differences in their mechanisms of action were found. $P2X_7R$ inhibitors blocked ATP action but had no measurable effects on NCAMP-1 binding or activity. These data suggested that NCAMP-1 may utilize a different receptor mechanism of cellular binding and activation in its role as a multi-functional effector molecule and inflammatory mediator.

## Introduction

Alarmins are host-derived molecules that mediate inflammatory responses that are similar to those originated by microbial products (pathogen-associated molecular patterns, PAMPs) following engagement of their innate immune receptors [1-2]. Recent research indicated that alarmins and PAMPs are recognized by many of the same PRR, adding complexity to the recognition abilities of the innate immune system. For this reason, alarmins have been suggested to pertain to host endogenous signals while the term damage-associated molecular patterns (DAMPs) encompasses both alarmins and PAMPs [3]. Alarmins are rapidly released following cell death; they are not released upon apoptosis; viable immune cells can be induced to produce and release alarmins through secretion; they activate immune cells inflammation and antigen presentation; and alarmins may restore homeostasis by promoting the reconstruction of tissue [3].

The molecular characteristics of alarmins vary greatly depending on the damaged cell and tissue type that releases them. The majority of previously identified alarmins are intracellular proteins that do not initiate inflammatory responses until they are released into the extracellular milieu. Alarmins may be categorized as nuclear and cytosolic proteins such as the chromatin associated high-mobility group box 1 (HMGB1) and heat shock proteins; or they consist of antimicrobial peptides such as the cathelicidin-derived LL37 peptide, and defensins. Other, non-protein alarmins include DNA, uric acid, and ATP. Once released, alarmins also differ in their receptor binding and the mechanisms by which they induce release of proinflammatory cytokines or in their production of chemoattractant activities.

The best characterized protein alarmins include HMGB1 [4-10], heat shock proteins [11-15] and LL37 [47-53]. HMGB1 functions as a chemoattractant and activates antigen presentation following either passive release from necrotic cells [4] or secretion by activated mononuclear cells [5-7]. Several receptors have been implicated in these functions including the receptor of advanced glycation end products (RAGE) [6] and TLRs 4 and 9 [8-10]. Similar to HMGB1, heat shock proteins mediate inflammatory responses through various TLRs [11]. Notably, binding of HSPs to TLR2 and 4 induces the production of NO, TNFα [12], and IL-12 [13] as well as activation of DCs [13-14]. The involvement of HSPs in inflammatory responses is consistent with their abundant expression in synovial tissue of rheumatoid arthritis patients [15]. In contrast to HMGB1 and HSPs, LL37 belongs to the only human member of the cathelicidin family of antimicrobial peptides (AMPs) [16-22]. LL37 is the biologically active 18-kDa peptide cleaved from its immature form by serine proteases [17]. LL37 has similar activities to those described above in activation of the innate immune response by acting as a chemoattractant and inducing IL-1β secretion. While LL37 also appears to bind to TLR9, it has recently been shown that the receptor implicated in the role of LL37 induction of inflammation is the P2X7 nucleotide receptor [21].

The $P2X_7$ receptor ($P2X_7R$) is the seventh member of the family of purinergic ligand gated ion channels recognized by ATP and other non-protein alarmin ligands such as uric acid crystals and muramyl dipeptide [23]. It is predominantly expressed in cells of hematopoietic lineage, including monocytes, macrophages and lymphocytes [24]. Under conditions of homeostasis, ATP is present in the extracellular milieu at small concentrations but is rapidly released from cells damaged by hypoxia, ischemia, inflammation or even mechanical stress [25]. The ligation of $P2X_7R$ by ATP is followed by reversible pore formation, $Ca^{2+}$ influx, and

processing and secretion of IL-1β through inflammasome activation. IL-1β is a necessary cytokine for infection clearance, but high concentrations are responsible for pathological conditions [26]. Dysregulated production of IL-1β is associated with rheumatoid arthritis, inflammatory bowel disease, sleeping sickness, acute and chronic myelogenous leukemia, insulin-dependent mellitus, atherosclerosis, asthma, and septic shock [27]. Muckle-Wells syndrome (MWS), an inherited autoinflammatory disorder in the category of familial periodic fever syndromes, is linked to mutations in the *Nalp3* (*Cias1*) gene [28], that encodes a fundamental component of the inflammasome protein complex.

Although extensive work has been done to characterize functions of the $P2X_7R$ in humans and rodents, little has been done involving teleost fish species. One study found that endotoxin challenge led to a significant increase in IL-1β expression in giltehead seabream (*Sparus aurata*) macrophages that accumulated intracellularly [29]. Using an endotoxin-primed seabream fibroblast cell line (SAF-1 cells), addition of extracellular ATP promoted the release of IL-1β, consistent with stimulation of $P2X_7R$ [30]. However, use of the potent $P2X_7R$ agonist BzATP did not result in the release of IL-1β from seabream granulocytesprimed with different TLRs and Nod proteins as has been shown for ATP [30]. The zebrafish and seabream $P2X_7R$ were individually expressed in HEK293 cells to compare agonist and antagonist profiles to cells transfected with the rat $P2X_7R$ gene [31]. Responses to ATP and BzATP were greatly reduced in the cells expressing zebrafish and seabream $P2X_7R$ compared to the cells expressing the rat gene [31]. These studies highlight the need for in depth studies of the $P2X_7R$ in teleost species.

Our laboratory has recently characterized a cationic anti-microbial peptide, NCAMP-1 (nonspecific cytotoxic cell cationic antimicrobial peptide-1), was purified from catfish NCC [32-35]. Molecular analysis of NCAMP-1 indicated it is lysine-rich and shares 42.4% and 51.2%

identity with human and zebrafish histone family member H1X, respectively [32]. NCAMP-1 is expressed as a surface protein of catfish [32], zebrafish and mouse leukocytes [35] as well as on the human NK cell line YT-INDY [34]. On the cell surface NCAMP-1 binds GpC and CpG oligodeoxynucleotides (ODNs) as a pattern recognition receptor [32]. In soluble form, NCAMP-1 has direct antimicrobial activity. Both the full-length and truncated forms of recombinant NCAMP-1 killed *Escherichia coli* and *Streptococcus iniae* isolates in vitro [32] as well as the pathogen responsible for hemolytic septicemia in catfish, *Edwardsiella ictaluri* [33]. The anti-microbial activity of NCAMP-1 was also demonstrated from cells of higher vertebrates. Granule extracts from RAW 264.7 cells had anti-microbial activity, which was abrogated by anti-NCAMP-1 polyclonal antibody, corresponding to the dual role of the protein seen in catfish NCC [35]. Confocal analysis of RAW cells demonstrated that NCAMP-1 may be associated with a secretory exocytosis pathway rather than a constitutive component of a secondary granule/phagolysosome endocytic pathway. Recent observations demonstrated that serum levels of NCAMP-1 increased in catfish challenged with *E. ictaluri* and *Ichthyophthirius multifilis* (unpublished data). These results suggested a possible role of this molecule as an alarmin.

In the present study, the alarmin-like activities of NCAMP-1 were investigated utilizing cells lavaged and harvested from the coelomic cavity of the adult zebrafish. The effects of recombinant NCAMP-1 binding to zebrafish leukocytes were compared to those of a known endogenous danger signal molecule, ATP. The results show that NCAMP-1 and ATP bind to cells harvested from the coelomic cavity of zebrafish, induce pore-formation, and activate cells as measured by intracellular calcium mobilization and increased cell-mediated cytotoxicity of zebrafish cytotoxic cells. The varying degree to which this activity occured suggests that

NCAMP-1 may utilize a unique mechanism of cellular binding and activation in its role as an effector molecule and inflammatory mediator.

## Material and Methods

**Zebrafish care and maintenance.** Adult WIK strain zebrafish were maintained at 81-82$^{o}$F in a UV filtered fresh water flow through system [36] at the University of Georgia Zebrafish facility (UGA Animal Welfare Assurance #A3437-01). Average water quality parameters were pH average of 7.2 and conductivity 450uS, using Instant Ocean to adjust. Fish were fed brine shrimp twice daily. Adult fish used in experiments ranged from 3 to 8 months of age and were semi-syngeneic by single pair brother-sister matings through the F6 generation. Euthanasia of zebrafish was accomplished by immersion in MS-222 (#TRS1; Aquatic Eco-Systems Inc., Apopka, FL) at 300ng/ml.

**Preparation of zebrafish coelomic cavity cells (CC).** Female fish were euthanized with an overdose of MS222 and immediately lavaged intra-coelomically with 3ml trypsin-EDTA (Invitrogen, 15400-054) as previously described [37]. Briefly, trypsin-EDTA was injected into the coelomic cavity of zebrafish using a 10ml syringe and sterile 25 G polypropylene hub hypodermic needle (Kendall, 250321), and allowed to collect directly into a petri dish. Trypsin activity was inactivated with FCS after harvesting the cells. 10X trypsin-EDTA (0.5% Trypsin and 0.2% EDTA in saline) was diluted to 1X concentration before use.

**Histology and IHC.** Fish were euthanized with an overdose of MS222, and 10 minutes after cessation of movement the body cavity was injected with Dietrich's solution, prior to total fixation by Dietrich's solution [38]. An entire fish was placed into standard tissue cassettes for routine overnight processing. After the tissue was embedded in paraffin, 4 um-thick saggital

sections were cut. Sections were deparaffinized and rehydrated through graded ethanols. Sections were stained with Gill's hematoxylin and eosin (H&E) using standard protocols. Adjacent sections were used for immunohistochemistry (IHC). Antigen retrieval was performed using a pressure cooker with citrate buffer (pH 6.0) for 10 min at 120$^{o}$C. Power Block (Biogenex, San Ramon, CA) was applied to all sections for 5 min. Rabbit polyclonal anti-NCAMP-1 antibody was diluted 1:5,000 in 50mM Tris-buffered saline (TBS) (pH 7.4). Normal rabbit IgG was also diluted 1:5,000 in TBS. Diluted primary antibodies were applied to sections and incubated for 60 min at room temp. After multiple washes, a biotinylated anti-rabbit IgG (Biogenex, HK340-9K, San Ramon, CA), a Super Sensitive Multilink, was applied to all sections for 15 min. Next, sections were incubated with an alkaline phosphatase label conjugated to streptavidin (Biogenex, HK 331-9K, San Ramon CA) for 15 min. Lastly, the sections were incubated with Vulcan Fast Red chromogen (Biocare Medical, FR805, Concord, CA) for 5 min.

**Intracellular NCAMP-1 staining.** Zebrafish CC were harvested from adult females. At room temperature, CC were fixed with 4% paraformaldehyde in PBS with 0.1% $NaN_3$ (PBSN) for 20 min. CC were then permeabilized with 0.1% Saponin in PBSN (PBSNS) for 15 min. After 2x 5min washes, once with PBSN and once with BBS with 0.1% $NaN_3$ and 0.1% Saponin (BBSNS), cells were incubated for 1hr with either polyclonal rabbit anti-NCAMP-1 antibody (1-2ug) or normal rabbit IgG (isotype control) at identical concentrations. Samples were washed again, once with BBSNS and once with PBSNS, and suspended in goat anti-rabbit IgG conjugate, Texas red (Invitrogen, #), in PBSNS for 1h. Samples were washed with PBSN for flow cytometric analysis. A conjugate control using the same concentration as for test samples was included in analysis. Nonspecific staining (percent fluorescence) from the isotype control was subtracted from that obtained from anti-NCAMP-1 to represent positive NCAMP-1 staining.

**Fluorescence microscopy.** An eight well chamber slide (Lab-Tek II, Nunc) was coated with poly-L-lysine (0.01% sterile H20) at 37$^{o}$C for 1h prior to addition of lavaged cells. At room temperature, cells were allowed to settle for 30 min, then were fixed with 4% paraformaldehyde in PBS with 0.1% $NaN_3$ (PBSN) for another 20 min. The slide was washed 2x 5min with PBSN. Cells were permeabilized with 0.1% Saponin in PBSN for 20min. The slide was washed with PBSN and next incubated with Image-iT signal enhancer (Invitrogen, Carlsbad, CA, US) for 30min. One wash with PBSN was followed by BBS with 0.1% $NaN_3$ and 0.1% Saponin (BBSNS). The slide was blocked with 10% normal goat serum in BBSNS for 15 min. After one was in BBSNS, the slide was incubated with either polyclonal rabbit anti-NCAMP-1 or normal rabbit IgG (isotype control) for 1h. The slide was washed again, once with BBSNS and once with PBSNS. The slide was next incubated with goat anti-rabbit IgG conjugate, Texas red (Invitrogen, ), for 1h. After one was with PBSN, Phalliodin FITC at 25ug/ml (Sigma) was added for 30 min. The slide was washed again for 4x 5 min and was mounted with Prolong Gold Anti-fade with DAPI (Invitrogen, P36935). Images were captured with Zeiss Axiovert microscope, using a 63X 1.4NA objective. Once captured, images were manipulated with Axiovision software.

**Western blot analysis of NCAMP-1 secretion.** Zebrafish CC were harvested from the coelomic cavity of female fish, counted and suspended in non-FCS containing RPMI media. Cells were either treated with 5mM ATP or media. After 30 min the supernatant was collected for analysis. Each sample was TCA precipitated (20%) on ice for 1h. After several washes with ice cold acetone, SDS was added to each sample. Samples were run on 11% SDS gels and transferred to nitrocellulose. Filters were blocked for 15 min at room temperature (Superblock, Pierce Rockford, IL, USA, #37545, with 0.05% Tween 20). Primary antibodies include rabbit

polyclonal anti-NCAMP-1 (prepared in-house) and purified normal rabbit IgG (NRbIgG) as an isotype control. Primary antibodies were diluted in BBS (pH 8.5) supplemented with 1% BSA, and 0.05% Tween 20. Peroxidase-conjugated goat anti-RbIgG secondary antibody (Pierce, 31463, 1:50,000 dilution) was diluted in Super blocking buffer. Blots were developed ECL (Pierce Supersignal West Pico, 1856135).

**Ligand binding to zebrafish CC.** CC were harvested from the coelomic cavity of female zf, counted, and suspended in cold PBS with 0.1% $NaN_3$ and 1.0% BSA (PAB) for at least 1h for staining purposes. On ice, 200,000 cells were incubated with indicated concentrations of recombinant NCAMP-1 (NT) directly labeled with Cy3 (in house), washed 1X with PBSN and analyzed by flow cytometry. Inhibition of fluorescence constituted incubation with indicated concentrations of cold peptide prior to 0.5ug NCAMP-1 (NT). The same staining protocol was carried out with indicated concentrations of BODIPY FL labeled ATP (Invitrogen Cat. No. A-12410).

**Agonist induced YO-PRO-1 iodide uptake.** Zebrafish CC were harvested and suspended in RPMI at room temperature. CC were diluted to 100,000 cells per 100ul and suspended in 5uM YO-PRO-1 iodide (Invitrogen, Y3603), a green fluorescent nucleic acid dye used to measure pore formation before the uptake of Propidium Iodide (PI) indicates cell death. Recombinant NCAMP-1 NT (aa 1-60) and FL (aa 1-203) were expressed and purified as previously described [32] and diluted in 10mM phosphate buffer (pH 8.0). At indicated concentrations, soluble NCAMP-1 was added to the cell suspension and immediately analyzed for fluorescence as compared to YO-PRO-1 uptake in the absence of NCAMP-1. The same protocol was carried out with varying concentrations of ATP (Sigma) until an optimal

concentration was found. Toxicity of treatment was measured by addition of 2ug/ml PI (Invitrogen) along with the described analysis.

**Calcium mobilization assay.** Harvested CC were washed with RPMI and suspended in RPMI at $1x10^6$/ml. Cells were loaded with 4uM Fluo-4 AM (Invitrogen, F-1217) a high affinity calcium indicator, in the presence of Pluronic F-127 (Molecular Probes, Eugene, OR, USA, F-127) for 20min at 37°C. The cells were washed in media and resuspended at room temperature for analysis of mean fluorescence intensity (MFI) by flow cytometry. Dye-loaded cells were analyzed for 50 sec to generate a baseline. 50sec into analysis a calcium ionophore (A23187), 1ug NCAMP-1 (FL), or indicated concentrations of ATP were added and analysis was immediately continued. As a negative control, no agonist was added to show no MFI increase.

**Inhibition of ATP induced activity.** Inhibition of agonist-induced activity was performed using KN62 (Sigma, I2142), Comassie Brilliant Blue (CBB), and oxidized-ATP (oATP) (Sigma), an altered form of ATP with the 2'- and 3'-hydroxyl moieties oxidized to aldehydes by periodate treatment. Before analysis of ligand binding, YO-PRO-1 uptake, or $Ca^{2+}$ influx, inhibitors were added at the indicated concentrations at room temperature for 15 min.

**Cytotoxicity.** Cytotoxicity was measured using a CFSE-based nonradiometric flow cytometric assay [39-40]. Human promyelocytic leukemia cells HL-60 (ATCC CCL 240) were used as target cells (TC) while zebrafish CC served as effector cells (EC). HL-60 targets were suspended at 5mM CFSE (Sigma, 21888) in sterile PBS for 15min at 37°C. Cells were washed and suspended at $5 \times 10^4$ cells per 100ul. Before co-incubation, zebrafish EC were treated with 1ug NCAMP-1, 5mM ATP or media for 30min at 37°C, then washed 1X with media. Zebrafish EC and TC were combined at effector:target ratios of 1:1. 4:1, and 8:1. Control samples

contained only CFSE labeled target cells and media. Flow analysis was conducted after 1, 2, and 4hrs co-incubation at 37$^{o}$C and 5% $CO_2$. Cytotoxicity was quantified by measuring the time (45s counts) and loss of CFSE labeled targets (in the target cell gate) in the presence of unlabeled zebrafish effector cells.

**Flow cytometry.** Flow cytometry was performed using two different instruments. Analysis of YO-PRO-1 uptake and cytotoxicity assays was done using an EPICS XL-MCL four color analysis system (Beckman-Coulter Electronic Corp) equipped with a 15 mW air cooled argon-ion laser operating at 488nm wavelength. Data collection analysis was performed using Coulter System II software version 3.0. Zebrafish CC extracellular ligand staining and calcium influx assays were done using an LSRII (Becton Dickinson) equipped with a solid state 488nm coherent sapphire blue laser (50mW). Data collection analysis was performed using Facs DIVA software version X and FlowJo software version 7.5.

## Results

**NCAMP-1 is detected in different zebrafish tissues.** To demonstrate in vivo expression of NCAMP-1 in zebrafish, whole adult females were fixed, sectioned, and mounted onto one slide. A histopathological evaluation was performed on an entire fish. To view tissue localization of NCAMP-1, serial sections of individual fish were alternatively stained using H&E (Fig 1A); a rabbit polyclonal anti-NCAMP-1 antibody (Fig 1B); and a normal rabbit IgG antibody (isotype control) (Fig 1C). Positive staining for NCAMP-1 occurred in the head kidney along renal venules, compared to the isotype control which was negative. Positive NCAMP-1 staining localized within epithelial cells lining the intestine and was intense. Staining was also intense in the kidney, especially within the epithelial cells lining the proximal tubules. Less

intense staining occurred in the hematopoietic cells surrounding the tubules. Positive staining for NCAMP-1 in the liver was present in the cytoplasm of hepatocytes and was less intense and more diffuse compared to the staining of the kidney and intestine. There were centers of intense staining, however, in macrophage cell dense areas. The isotype control was negative for NCAMP-1 staining.

**Intracellular NCAMP-1 expression in cells of the zebrafish coelomic cavity.** Using fluorescence microscopy and flow cytometric analysis, intracellular expression of NCAMP-1 in zebrafish coelomic cavity cells (CC) was detected with polyclonal anti-NCAMP-1 antibodies as described in Materials and Methods. Very little non-specific binding was detected in staining with the isotype control antibody (Fig 2A-C) in a variety of cell types (identified by nuclear morphology). Positive punctate staining of NCAMP-1 (Fig 2D-I) was observed in the cytoplasm of a variety of cell types including polymorphonuclear cells (PMNs) (Fig 2E), mononuclear cells (Fig 2D, F-G), and lymphocyte-like cells (Fig 2E, H-I), as detected by nuclear morphology. Similar to fluorescence microscopic imaging, staining was observed in diverse cell types by flow cytometric analysis (data not shown). Overall staining of CC resulted in 35.6% NCAMP-1 positive for 1ug and 52.7% for 2ug anti-NCAMP-1 polyclonal antibody. Nonspecific isotype control binding was subtracted from the total. The positive staining of 1ug anit-NCAMP-1 antibody for the different cell populations found in the coelomic cavity were 72.3%, 73.5%, and 76% positive for mononuclear cells and PMNS, lymphocytes, and NCC, respectively (data not shown). Similar percentages were observed for anti-NCAMP-1 at a concentration of 2ug.

**NCAMP-1 is secreted by zebrafish CC in response to the alarmin, ATP.** To determine if NCAMP-1 is released by zebrafish leukocytes upon stress, in vitro treatment of CC with ATP was carried out at non-toxic concentrations. Supernatants were collected after 30 min

of 5mM ATP or media treated cells. A band of approximately 29kDa, corresponding to the size of NCAMP-1, appeared in the supernatant of ATP treated cells (Figure 3) that was comparable to the positive control. This band was absent in cells that were not treated with ATP.

**Soluble NCAMP-1 binds to zebrafish CC in a saturable dependent manner and is inhibited by cold peptide.** Using recombinant expressed, Cy3-labeled NCAMP-1 (generated in house), zebrafish CC were stained at indicated concentrations (Figure 4A) according to Materials and Methods. Binding of Cy3-labeled NCAMP-1 produced a titrating effect with optimal staining at 0.5ug NCAMP-1 (NT). When cells were stained with Cy3-labeled NCAMP-1, 0.125ug produced 15.26% fluorescence, 0.25ug produced 22.73% fluorescence, and 0.5ug produced 36.31% fluorescence. Binding was saturable and positive staining with 0.5ug of NCAMP-1 was next inhibited with cold peptide (Figure 4B). 0.5ug and 1ug cold NCAMP-1 reduced positive fluorescence produced by 0.5ug Cy3 NCAMP-1 (36.31%) to 5.41% and 2.75%, respectively.

**Pore formation is induced by binding of NCAMP-1 or ATP as measured by YO-PRO-1 uptake.** Soluble NCAMP-1 (FL) (Figure 5A) or ATP (Figure 5B) was added to zebrafish cells in the presence of 5uM YO-PRO-1, a common nucleic acid dye used to analyze $P2X_7R$ functions such as induced pore formation. YO-PRO-1 uptake, as measured by increase in fluorescence occurred immediately after addition of agonist. Optimal concentrations of NCAMP-1 (1ug) produced a 20-25% fluorescence compared to 5% fluorescence for media control. ATP induced YO-PRO-1 uptake was titratable with 30% fluorescence produced by the optimal concentration of 30mM ATP. Similar results were obtained when the N-terminal portion of NCAMP-1 was added (data not shown) compared to pore-formation induced by the full-length protein.

**Addition of multiple agonists augments YO-PRO-1 uptake.** To observe any additive effects on YO-PRO-1 uptake following the addition of multiple agonists the same assay as above was performed. When soluble NCAMP-1 (FL) was added to zebrafish CC in the presence of 5uM YO-PRO-1 followed by the addition of suboptimal concentrations of ATP, YO-PRO-1 uptake increased compared to addition of individual agonists, and media controls (Figure 6). Representative experiments show that compared to media control (5.5%), addition of 1ug of NCAMP-1 alone produced 12.1% fluorescence, 5mM ATP alone 8.77% fluorescence, and when added simultaneously produced 19.4% fluorescence (Fig 6A). 10mM ATP alone produced 12.7% fluorescence. When added simultaneously with NCAMP-1 the combination produced 21.8% fluorescence (Fig 6B). Addition of 20mM ATP alone produced 22.6% fluorescence. When this concentration of ATP was added to 1ug NCAMP-1 the combination induced 26.8% fluorescence in zebrafish CC (Fig 6C). 36% of zebrafish CC were positive for YO-PRO-1 fluorescence at the optimal concentration of ATP (30mM) alone. When this concentration was added simultaneously with NCAMP-1, 42.5% of the cells were positive for YO-PRO-1 fluorescence (Fig 6D).

In order to investigate whether the order in which the agonists are added affected YO-PRO-1 uptake, various concentrations of ATP were added to zebrafish CC in the presence of YO-PRO-1, then 1ug of NCAMP-1 was added (within seconds). There was no additive effect on the YO-PRO-1 fluorescence when ATP was added prior NCAMP-1 (data not shown).

**Agonist induced YO-PRO-1 uptake is not due to cell death.** To ensure that ATP and NCAMP-1 induced pore-formation was not due to nonspecific cellular toxicity, staining with PI was used during analysis of YO-PRO-1 uptake. Only 3-4% of the cells were PI+ following the addition of NCAMP-1 immediately after analysis and after 30min (Fig 7A), indicating that YO-

PRO-1 fluorescence induced by NCAMP-1 was not due to cellular damage. Percent PI+ cells did not increase after NCAMP-1 exposure intervals exceeding 1hr (data not shown). Immediate exposure to ATP (Fig 7B) produced 8-10% PI positive cells. After an hour of exposure to 30mM ATP damage to cells was apparent, as 15% cells become PI+. This number increased with longer exposure times to ATP. This indicated that short term exposure to ATP did not induce cellular damage while longer intervals of treatment were toxic.

**Intracellular $Ca^{2+}$ influx is induced by NCAMP-1 or ATP.** To determine if NCAMP-1 induced intracellular calcium mobilization upon binding, zebrafish CC were loaded with 4uM Fluo-4, a calcium chelator that fluoresces upon binding of calcium (described in Materials and Methods). After 50 seconds of flow cytometric analysis, NCAMP-1, A23187, or media control (no agonist) (Fig 8A) were added and the sample was immediately reanalyzed to detect mean fluorescence intensity (MFI). Similar to the effect observed with the calcium ionophore A23187 (positive control), NCAMP-1 induced a 2X increase in MFI. Media control (no agonist) did not produce an increase in fluorescence intensity. When ATP was added to Fluo-4 loaded zebrafish CC, a 2X increase in MFI was observed with 30mM ATP (Fig 8B). This effect was less pronounced with 20mM ATP (Fig 8B). In the plots (Fig 8) arrows indicate the addition of agonist. Results indicate that similar to the positive control, NCAMP-1 and ATP produced a calcium influx in zebrafish CC.

**Pre-treatment of zebrafish cytotoxic cells with NCAMP-1 or ATP increased cell-mediated killing of target (HL-60) cells.** To determine if NCAMP-1 affected effector functions of zebrafish coelomic cells, a CFSE based cell-mediated cytotoxicity assay was carried out as previously described [37]. Zebrafish CC (effector cells, EC) were treated with 1ug NCAMP-1 before co-incubation with CFSE labeled target cells (HL-60 cells). After 60, 120, and 240

minutes, cells were resuspended and analyzed for loss of fluorescence (Fig 9). Overall, treatment with NCAMP-1 produced higher percent cytotoxicity at lower effector: target (E:T) ratios and at shorter co-incubation periods than controls without NCAMP-1 pretreatment. 21.6% cytotoxicity was observed as soon as 60 minutes when ECs were exposed to NCAMP-1, while 25% cytotoxicity was not observed in the control until 120 minutes. Pretreatment of zebrafish effectors with NCAMP-1 induced higher cytotoxic functions.

ATP treatment of zebrafish effectors before co-incubation with targets had a similar effect on percent cytotoxicity. After 5mM ATP pretreatment of zebrafish CC, at 120 minutes E:T ratios of 4:1 and 8:1 had 25% and 41% cytotoxicity respectively, while controls had 20% and 30%. Pretreatment with ATP induced an increase in cytotoxic activity of zebrafish effectors compared to controls, but not to the same degree as NCAMP-1.

**ATP induced effects are blocked by $P2X_7R$ inhibitors.** To determine if agonist induced activity, such as pore formation and $Ca^{2+}$ influx, are specific to the $P2X_7R$, three different inhibitors were tested: oxidized-ATP (oATP), Commassie Brilliant Blue G (CBB), and KN62. Prior to exposure to 30mM ATP, zebrafish CC were incubated for 15 minutes with oATP (Fig 10A), KN62 (Fig 10B), or CBB (Fig 10C), and analyzed for YO-PRO-1 fluorescence. Each inhibitor abrogated fluorescence induced by ATP in a concentration dependent manner compared to no inhibitor controls. CBB and KN62 required low concentrations for inhibition at the nanomolar level, while micromolar concentrations were effective for oATP.

ATP-induced calcium influx was inhibited with oATP (Fig 10D). Zebrafish CC were incubated with oATP prior to 30mM ATP exposure. A calcium influx was not observed following ATP addition as detected by a lack of MFI increase, compared to cells not incubated

with the inhibitor. This effect was observed at 100 and 200uM oATP. NCAMP-1-induced pore formation and calcium influx, however, were not successfully blocked by these inhibitors. Increased cytotoxic activity of zebrafish effector cells due to ATP stimulation was also inhibited with oATP (data not shown).

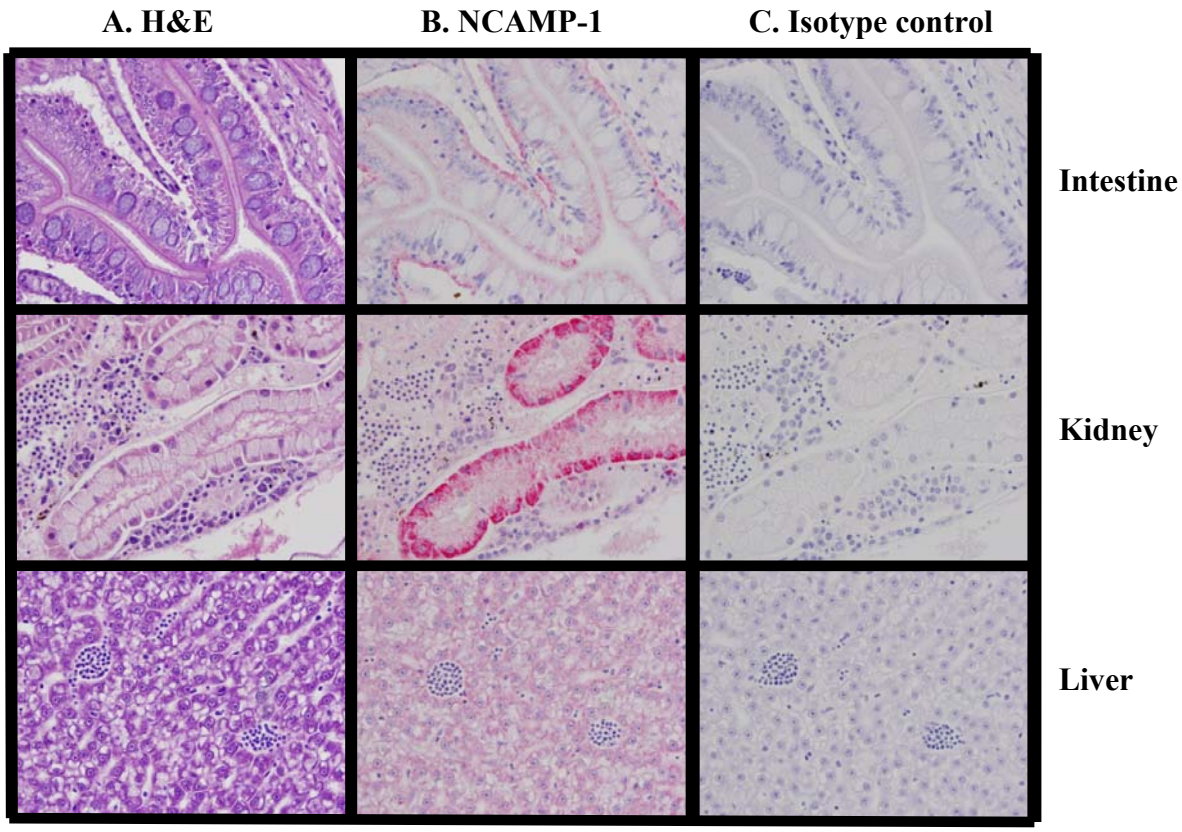


**Figure 1: NCAMP-1 expression in zebrafish tissues.** Representative slide of female zebrafish stained with H&E (A), rabbit anti-NCAMP-1 (B), or normal rabbit IgG (C) followed by a biotinylated anti- rabbit IgG conjugate, streptavidin-conjugated phosphatase, and Vulcan Fast Red chromogen.

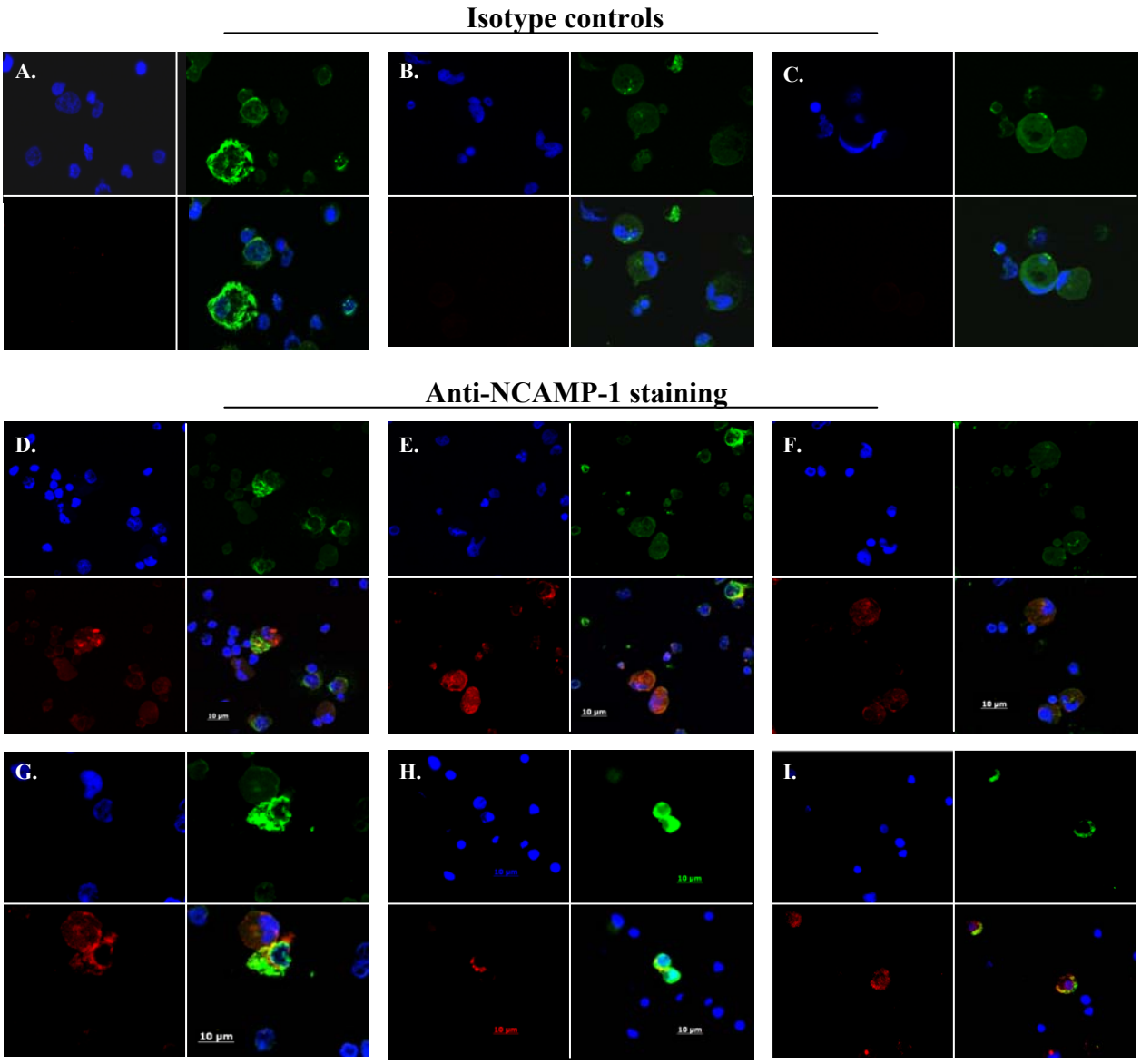


**Figure 2: Intracellular NCAMP-1 expression by zebrafish coelomic cavity cells (CC).**

Zebrafish CC were fixed, permeabilzed and stained for intracellular NCAMP-1 (red) with rabbit anti-NCAMP-1 polyclonal antibody or normal rabbit IgG. Actin was stained with phalloidin (green) and nuclear morphology indicated with DAPI staining (blue). (A-C) representive of cells stained with isotype control (IC). (D-I) representative of cells stained with anti-NCAMP-1 antibody. Matched frames for each image are single stain profiles. Note heterogeneity of nuclear morphologies of cells with intracellular NCAMP-1 expression.

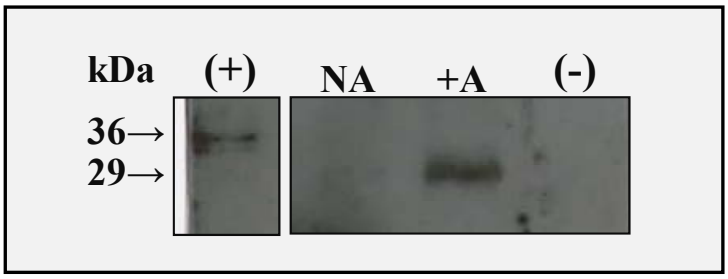


**Figure 3: Western blot analysis of NCAMP-1 release from ATP treated zebrafish CC.** Zebrafish CC ($3x10^5$ cells) were untreated (NA) or treated in vitro for 30 min with 5mM ATP (+A). Supernatants were harvested, TCA precipitated, and analyzed by Western blot using rabbit polyclonal anti-NCAMP-1 antibody. (+) Positive control of R-NCAMP-1-Histag. (-) Negative control had no primary antibody.

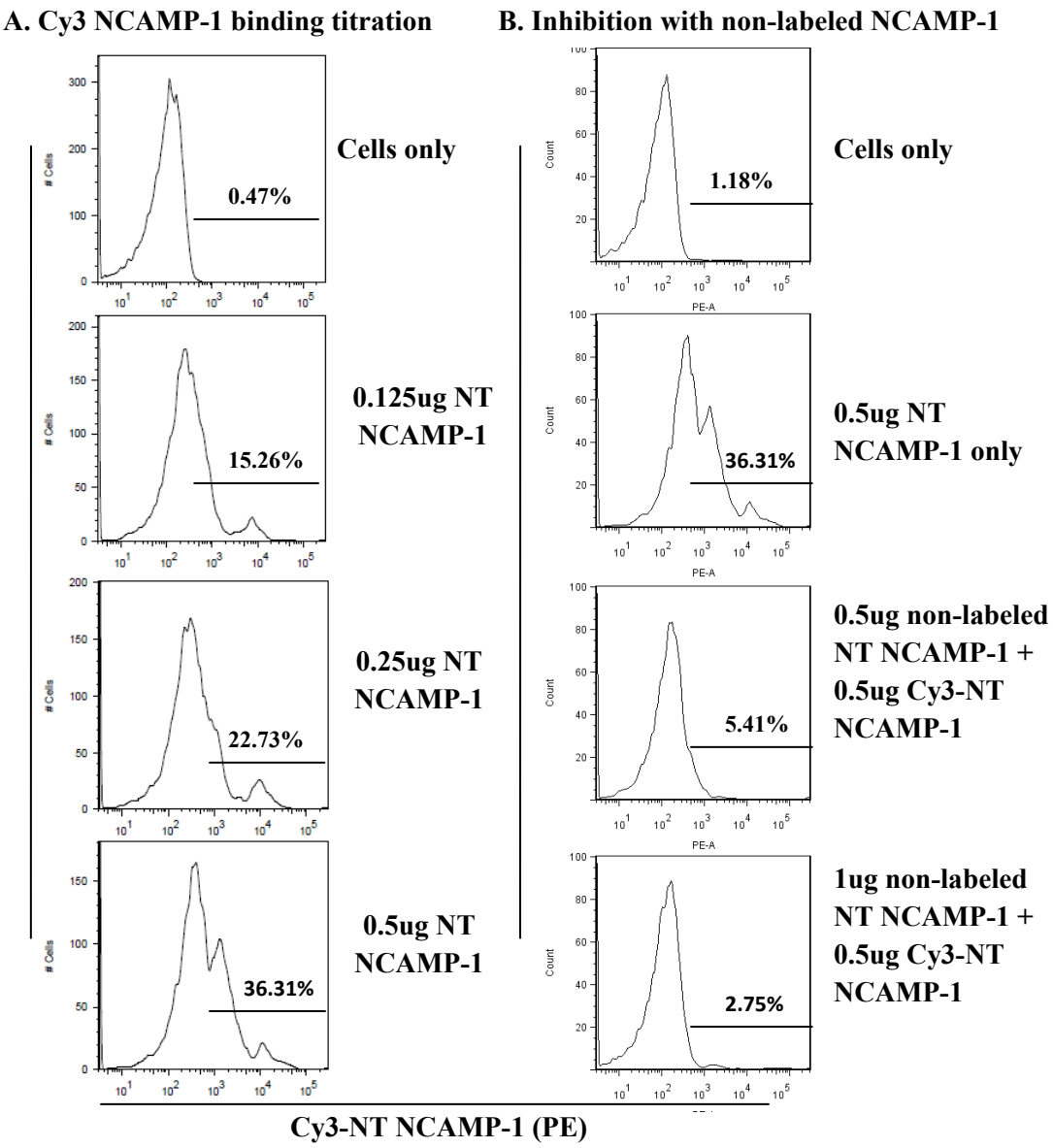


**Figure 4: NCAMP-1 (NT) binds to zebrafish CC.** (A) Harvested CC were stained for 30 minutes with varying concentrations of Cy3 labeled NCAMP-1 (NT), washed and analyzed by flow cytometry. (B) CC were incubated for 30 minutes with various concentrations of non-labeled NCAMP-1 (NT) prior to staining with 5ug of labeled NCAMP-1 (NT) for another 30 minutes.

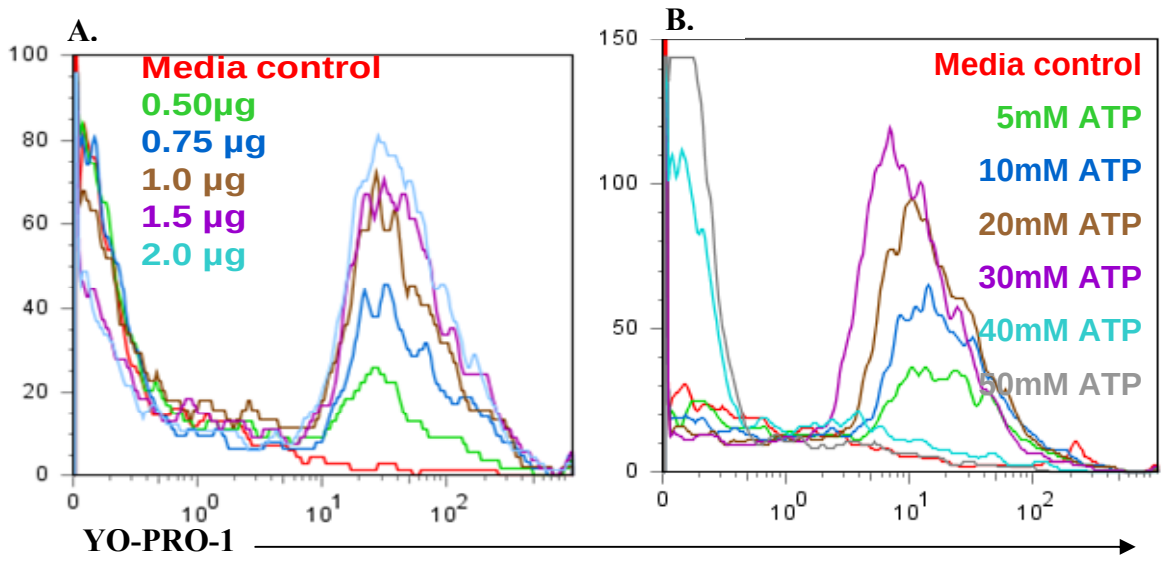


**Figure 5: Soluble NCAMP-1 and ATP bind to CC and induce a concentration dependent uptake of YO-PRO-1 dye.** Indicated concentrations of soluble NCAMP-1 (A) or ATP (B) were individually added to a suspension of CC ($2x10^5$ cells) at 5uM YO-PRO-1 and immediately analyzed (within 3sec) at room temperature by flow cytometry. Histograms are representative of at least three independent experiments per agonist.

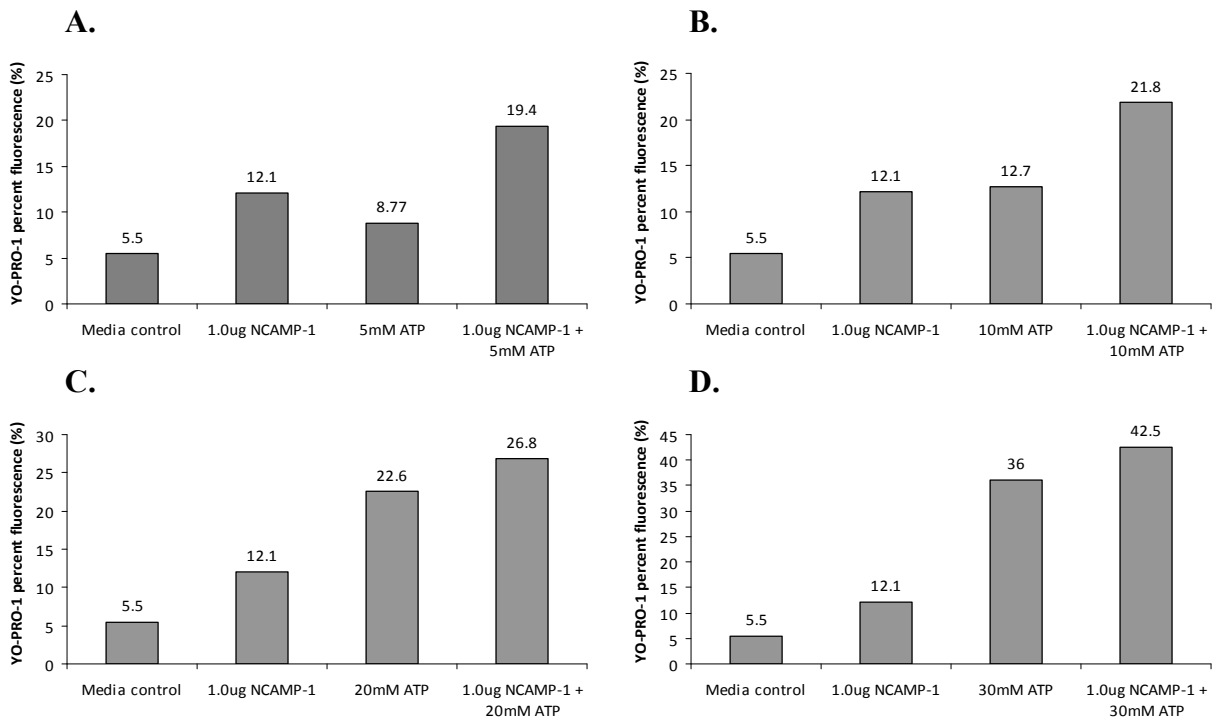


**Figure 6: Augmented YO-PRO-1 uptake with multiple agonists.** The combination of FL NCAMP-1 and ATP as agonists has an additive effect on YO-PRO-1 uptake. 1ug FL NCAMP-1 was added to a suspension of $2x10^5$ cells at 5uM YO-PRO-1 and analyzed immediately. Next, various concentrations of ATP, 5mM (A), 10mM (B), 20mM (C), and 30mM (D), were added and the YO-PRO-1 fluorescence was reanalyzed. An additive effect was observed especially at the lowest concentration of each agonist. Representative of at least three independent experiments.

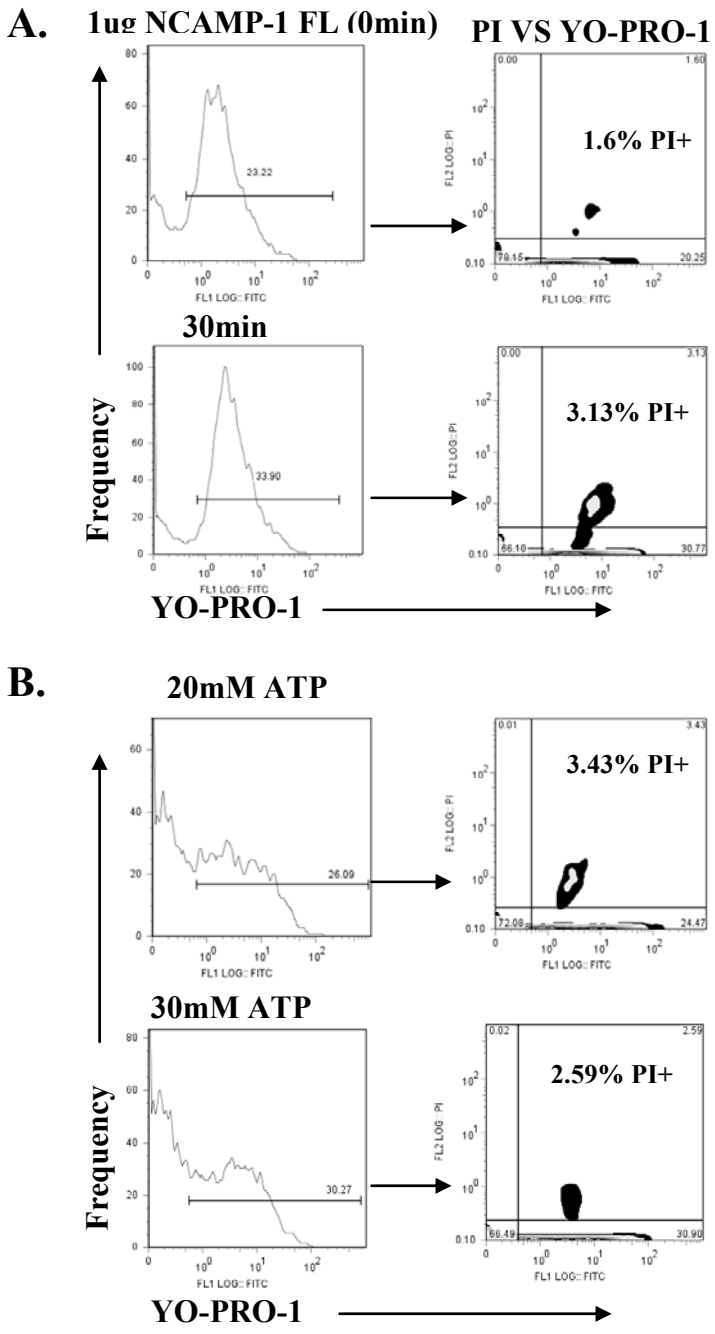


**Figure 7. Immediate exposure to NCAMP-1 nor ATP does not cause cellular damage.** Zebrafish CC were treated with 1ug NCAMP-1 FL (A) and analyzed immediately or after 30min for YO-PRO-1 and PI uptake to observe cellular damage. 20mM and 30mM ATP (B) were added in presence of YO-PRO-1 and PI and immediately analyzed (0min). Representative of at least 3 independent experiments.

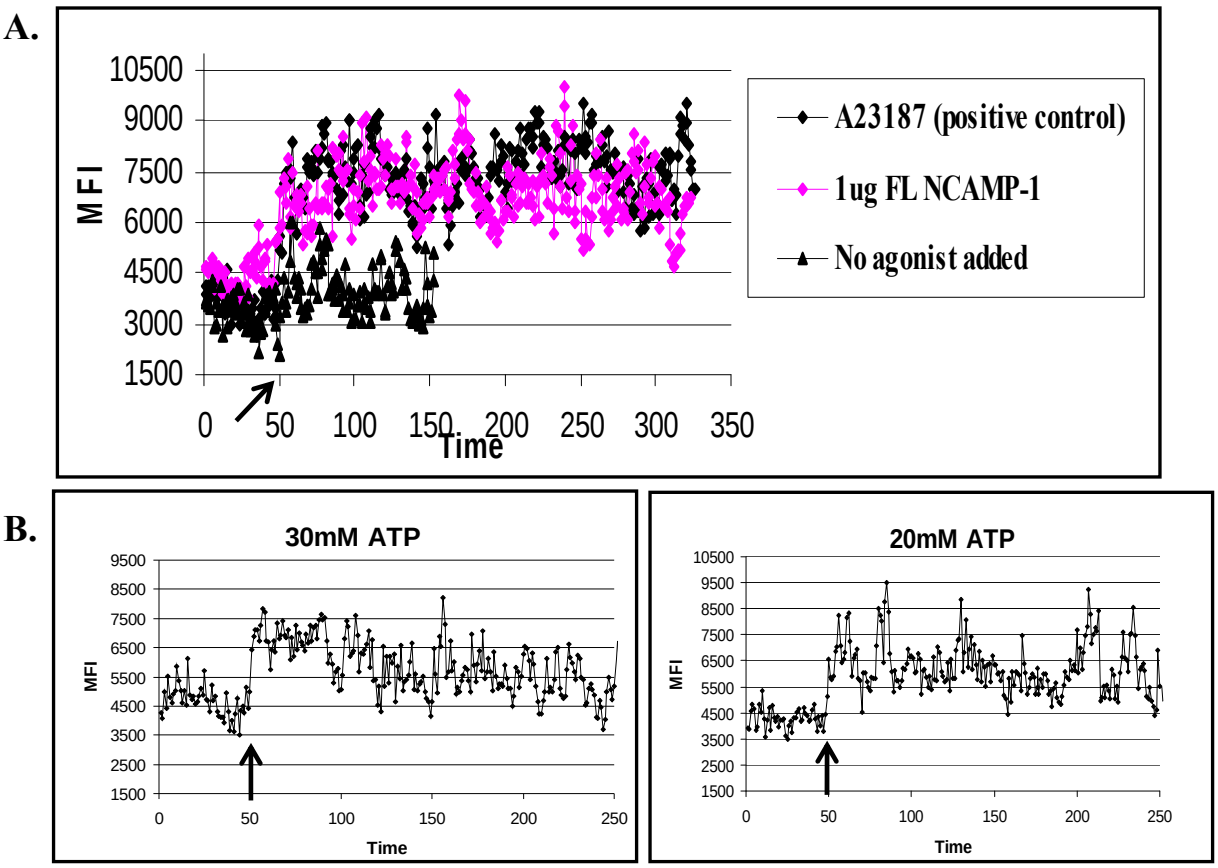


**Figure 8: NCAMP-1 and ATP bind to CC and induce an influx of $Ca^{2+}$ ions.** Zebrafish CC were loaded with 4uM Fluo-4 for 20 minutes at 37°C, washed with media, then analyzed for mean fluorescence intensity by flow cytometry. 50 seconds into analysis the calcium ionophore (positive control) or 1ug NCAMP-1 (FL) (A) or different concentrations of ATP (B) were added and analysis was continued. Arrows indicate the addition of the agonist. Representative of at least 3 independent experiments.

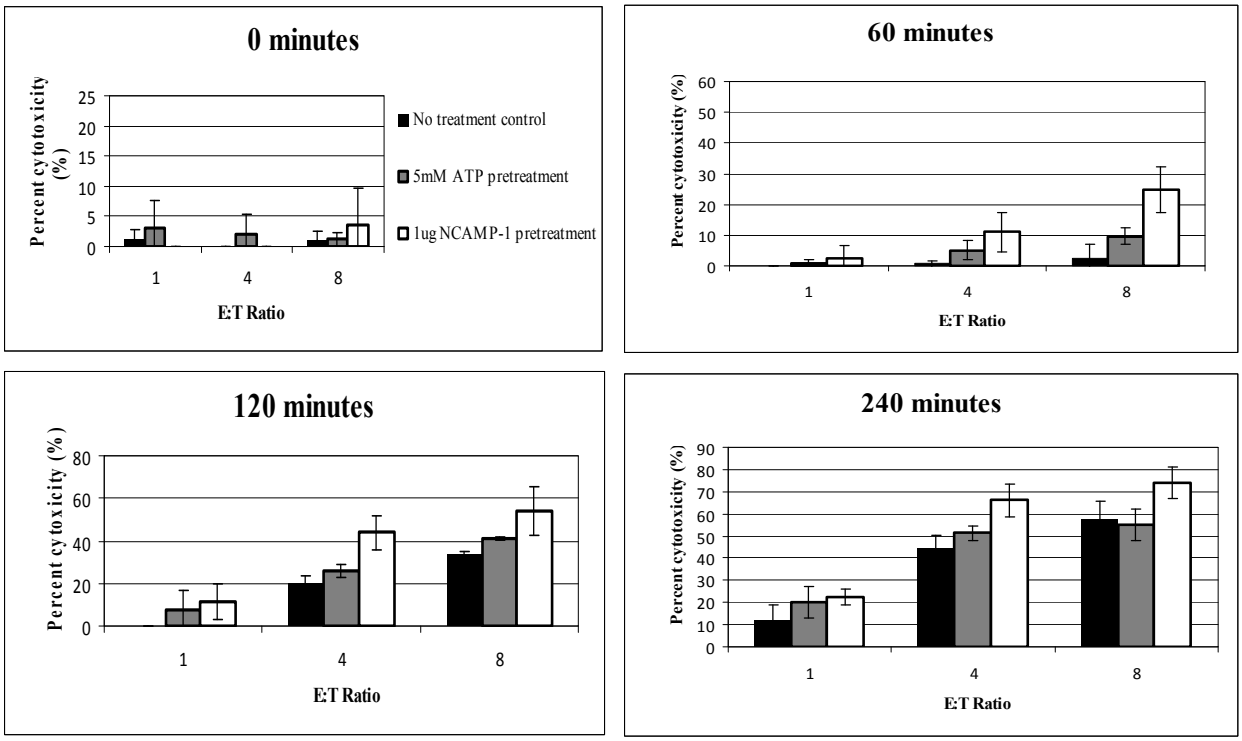


**Figure 9: Cytotoxic activity of zebrafish effectors is increased with pretreatment of NCAMP-1 or ATP.** HL-60 target cells were labeled with 5mM CFSE for 15 minutes at 37$^{o}$C and washed. Targets were added to zebrafish effectors, pretreated for 30 minutes with either 5mM ATP, 1ug NCAMP-1 (FL), or no treatment, at effector:target cell ratios of 1:1, 4:1 and 8:1. Flow analysis was conducted after 1, 2, and 4 hours of co-incubation. Data points are means and standard error of three independent experiments.

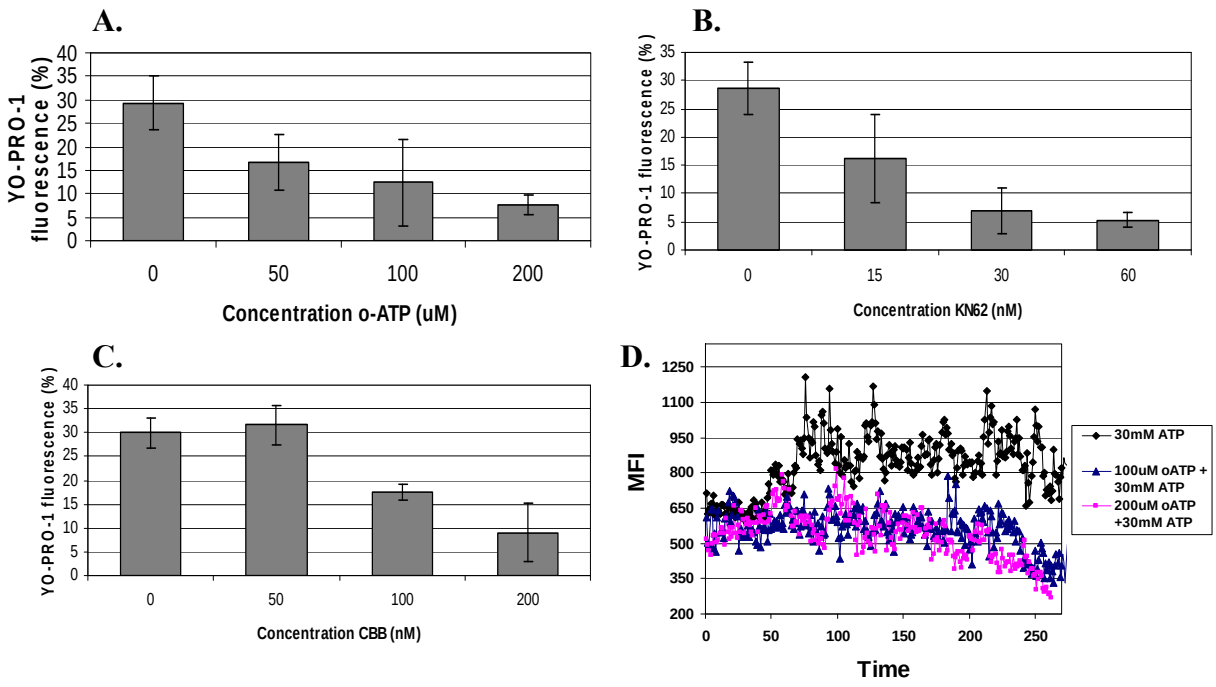


**Figure 10: ATP induced activation of CC is abrogated by P2X$_7$R inhibitors.** 30mM ATP was added to CC with 5mM YO-PRO-1 and analyzed for percent fluorescence immediately or incubated with oxidized-ATP (oATP) (A), KN62 (B), or CBB (C) for 15 minutes prior to addition of ATP. After incubation with 200uM or 100uM oATP (D) for 15 minutes, cells were loaded with Fluo-4 and analyzed for MFI. 50 seconds into analysis ATP was added. The arrow indicates the addition of agonist. Data displayed as mean and standard error.

## Discussion

Until now the alarmin properties of the innate immune system in adult zebrafish (Danio rerio) have not been addressed although it is an animal which is increasingly used as a model for immunity and infectious disease. The involvement and mechanisms of action by endogenous danger molecules in inflammatory diseases, protein and non-protein, have been investigated extensively in mammals.

As a vertebrate that is immersed in the environment, fish must have an efficient innate immune system that reacts appropriately to pathogens as well as host signals. Since zebrafish have a similar immune system to humans, it is important to take advantage of the many benefits that the zebrafish model provides. NCAMP-1, a novel phylogenetically conserved H1X-like protein with antimicrobial activity [32-33], is expressed in the cytoplasm of a variety of cell types in the coelomic cavity (Fig 2), as well as many tissues in the adult zebrafish (Fig 1). Many AMPs have been isolated in teleost fish species that are closely related to the zebrafish, primarily from skin and mucus, but also from the intestine and liver [41-43]. Since AMPs are important components of innate immunity it is reasonable that these proteins might serve as multifunctional effector molecules.

A recent study reported the ability of collagen and gelatin to prime the respiratory burst of phagocytes from the bony fish gilthead seabream, in vitro [44]. Both induced the expression of IL-1β, chemokine (C-C motif) ligand 4 and matrix metallopeptidases (MMP) 9 and 13 in acidophilic granulocytes and macrophages, indicating the ability of the innate immunes system of fish to efficiently respond to tissue injury [44]. Interestingly, pre-treatment of collagen and

gelatin by collagenase resulted in a higher stimulatory capacity compared to non-digested proteins [44].

Specifically, ATP induced activation of immune cells via the $P2X_7R$ has not been described in cells of teleost species. In a study using cDNA transcripts of seabream and zebrafish $P2X_7R$ tranfected into HEK 293 cells, it was found that responses to ATP and BzATP were greatly reduced in the cells expressing zebrafish and gilthead seabream (*Sparus Aurata*) $P2X_7R$ than cells expressing the rat transcript [31]. However, a previous study found that endotoxin challenge led to significant increase in IL-1β expression in giltehead seabream macrophages that accumulated intracellularly [29]. The same investigators found that extracellular ATP promoted the release of IL-1β from a seabream fibroblast cell line (SAF-1 cells) after endotoxin priming consistent with stimulation of the $P2X_7R$ [30]. When the potent agonist BzATP was used, permeabilization occured in a subset of acidophilic granulocytes and was completely reversible, but release of IL-1β did not result from these cells primed with different TLRs and Nod proteins as has been shown for ATP [30].

In contrast to the study using HEK 293 cells transfected with zebrafish $P2X_7R$, the present study demonstrates the ability of ATP to induce pore-formation as detected by YO-PRO-1 uptake that is concentration dependent, Fig 5B. Soluble NCAMP-1, full-length and N-terminal, was also able to induce pore-formation in zebrafish CC in a concentration dependent manner, Fig 5A, although not to the same extent as ATP. The concentration of ATP found to have an optimal effect, 30mM, produced a consistent 30% fluorescence in zebrafish CC, compared to a range of 18-25% YO-PRO-1 uptake observed in the cells treated with 1ug NCAMP-1. Secretion of IL-1β was not assayed here, so it is unknown whether LPS, or another PAMP, is necessary as a primary stimulus as is accepted in dogma of IL-1β secretion [45]. It is

important to note that LPS priming was not necessary to induce pore-formation in zebrafish CC by ATP or NCAMP-1.

Another characteristic of YO-PRO-1 uptake mediated by $P2X_7R$ activation is the augmentation by the addition of multiple agonists. In this study, the addition of ATP augmented pore-formation in zebrafish CC induced by NCAMP-1 treatment as observed increase in YO-PRO-1 fluorescence (Fig 6). The most dramatic responses of augmentation were seen at lower concentrations of ATP, such as 5 and 10mM. However, when NCAMP-1 was added after ATP the same augmented response was not observed (data not shown). These findings indicate that NCAMP-1 may not bind to the same region on the P2X7R as ATP and may bind an allosteric region to the ATP binding site. It is also possible that ATP may have a stronger affinity for this activity than NCAMP-1.

The $P2X_7R$ ortholog was identified in from zebrafish along with eight other identified P2X subunits [46]. Six of them are homologous to the mammalian counterpart, while 2 were identified as a paralog [46]. It remains unknown whether the last gene is a homolog, paralog or new subunit [46]. These findings demonstrate that zebrafish have the machinery necessary for $P2X_7R$ mediated activity. Also, multiple sequence alignment has shown that all the fish $P2X_7$ receptors sequenced maintain the five positively charged amino acids thought to be responsible for nucleotide binding (K64, K66, R294, R307, and K311) [47-49]. However, only three out of five mammalian amino acid residues of the intracellular domain (E496, I568, and R578) that have been implicated in receptor trafficking and function are also conserved in non-mammalian molecules [50-53].

ATP-induced processing and release of IL1β is reported to coincide cell death [54] and cell lysis has been a suggested mechanism of release and other $P2X_7R$ functions [55]. Propidium iodide (PI) was used in YO-PRO-1 uptake assays to ensure observations were derived from viable cells. Immediate analysis of short term exposure of ATP to zebrafish CC indicated that pore-formation did not correspond to cell lysis (Fig 7b). No more than 8-10% fluorescence of PI occurred in cells exposed to 20 and 30mM ATP. However, long term exposure (1hr) to 30mM ATP did induce high percentages of PI fluorescence. No toxicity was observed with the concentrations of NCAMP-1 used to zebrafish cells at any time after analysis (Fig 7A).

Calcium influx is also characteristic of $P2X_7R$ activation along with the activation of phospholipases. This is thought to be responsible for the subsequent secretion of IL-1β [56]. Although IL-1β secretion from zebrafish immune cells was not assayed in this study, both ATP and soluble NCAMP-1 were able to induce a $Ca^{2+}$ influx after stimulation as detected by Fluo-4 AM (Fig 8). NCAMP-1 stimulated a similar 2-fold increase in MFI, compared to the calcium ionophore, A23187. Although to a lesser degree, 30 and 20mM ATP were able to stimulate a near 2-fold increase in MFI.

Recently, cell-mediated cytotoxicity activity was reported in cells harvested from the coelomic cavity of zebrafish [37]. In theory, this immune function could be upregulated by inflammatory stimuli. With incubation of small concentrations of ATP and NCAMP-1 to prevent toxicity, cytotoxic activity of zebrafish CC against target HL-60 cells was increased (Fig 9) compared to media controls. NCAMP-1 treated cells, however, displayed higher percent cytotoxicity rates at lower E:T ratios and co-incubation time points than ATP and media treated cells.

P2X$_7$R specific activity of NCAMP-1 and ATP was tested using commonly used inhibitors, CBB, KN62 and oxidized ATP. CBB and oATP are selective for P2X$_7$R [56-58] while KN62 is able to inhibit a range of P2X receptors and thought to behave on an allosteric site to where ATP binds [59]. All of the ATP-induced activities observed in this study were able to be blocked by these inhibitors (Fig 10). Specifically, YO-PRO-1 uptake was blocked in a concentration-dependent manner by oATP, KN62, and CBB. oATP was chosen to demonstrate P2X$_7$R specific inhibition of $Ca^{2+}$ influx and increased cytotoxicity of zebrafish CC. NCAMP-1 induced activity, however, was not blocked by any of these inhibitors, at any concentration. These findings taken with the above observations, suggest that NCAMP-1 may bind to a different site on the P2X$_7$R than ATP, or a different receptor altogether with similar functions.

The human cathelicidin-derived peptide LL37, originally described as a potent antimicrobial peptide, is capable to stimulate IL-1β and caspase-1 activation via the P2X$_7$R in LPS-primed monocytes [21]. Furthermore, P2X$_7$R inhibitors, KN04, KN62 and oATP, suppressed pore-formation as measured by YO-PRO-1 uptake and IL-1β release [21]. It is important to note that LL37 is also involved in chemotactic activity mediated by the formyl peptide receptor family, formyl peptide receptor-like 1 (FPRL1) [20]. As an endogenous danger signal it is not well understood how NCAMP-1 is released from immune cells but it is important to demonstrate that this occurs from viable cells.

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# CHAPTER 4

## CONCLUSIONS

NCAMP-1 protein was detected in different tissues of the zebrafish by immunohistochemistry staining and in the cytoplasm of cells from the coelomic cavity, by confocal microscopy. NCAMP-1 bound to immune cells, induced pore formation and produced an influx of $Ca^{2+}$. These results are similar to the activities of ATP, an alarmin that acts through the $P2X_7R$. Pretreatment of zebrafish effector cells with NCAMP-1 or ATP increased target cell cytotoxicity at lower effector:target ratios and at shorter co-incubation time periods. Pretreatment of zebrafish effector cells with ATP, however, increased cytotoxicity to a lesser extent than NCAMP-1. These findings suggested that NCAMP-1 shared characteristics with ATP, a known host danger molecule. Although NCAMP-1 binding induced YO-PRO-1 uptake and $Ca^{2+}$ influx by zebrafish CC cells, it may not act through the $P2X_7R$ as many of the $P2X_7R$ specific inhibitors were unable to block either its binding or its activities. These inhibitors were successful at abrogating activity by ATP. It is possible that NCAMP-1 either may bind to a different portion of the $P2X_7R$ than ATP or to another receptor. Alarmins have been proposed to be responsible for aseptic inflammatory diseases. Knowledge gained from these studies may be applicable to better understand conditions such as autoimmunity and chronic inflammation.