Characterization of the Downstream Effects of the Rainbow Trout (*Oncorhynchus mykiss*) Chemokine, CK-2 on Macrophages

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Chemokines are **chemo**tactic cyto**kines** that have the ability to attract leukocytes and guide them via a concentration gradient to sites of injury/infection. These small, basic proteins are secreted when induced and act on their target cells through G-protein coupled receptors. The downstream effects of this family of immune molecules are vast and have not fully been characterized. These versatile molecules seem to be able to serve as a link between the innate and adaptive immune systems as they are capable of inducing the inflammatory response of the innate immune system as well as triggering the initiation of the adaptive immune system. There are currently 46 known chemokines in humans. Chemokines have also been isolated from mouse, chicken, frog and fish.

The rainbow trout chemokine CK-2 is the only known functional CC chemokine in possession of a mucin stalk. A previous study in this lab showed that stimulation by PHA causes a decrease in CK-2 transcript levels in rainbow trout head kidney and peripheral blood leukocytes (PBLs) as well as in the rainbow trout macrophage-like cell line RTS-11. CK-2 protein was found expressed in RTS-11 but not in the spleen tissues of stimulated fish. A chemotaxis assay was performed to determine the activity of recombinant CK-2. It was observed that recombinant CK-2 induces the migration of rainbow trout PBLs as well as RTS-11 cells at an optimal concentration of 100ng/mL when purified under native conditions. The migration of cells treated with pertussis toxin is significantly reduced, indicating that it relies on G-protein coupled receptors. Treatment of RTS-11 cells with recombinant CK-2 results in changes in the expression profiles of various immune response genes including those that are involved in the inflammatory response and the responses against both intracellular and extracellular pathogens. Interestingly, rCK-2 induced an upregulation in the expression of the surface molecule CD4 at the level of transcription. The increase in CD4 may suggest a possible role for CD4 in the regulation of the cell's response to chemokines, indicating a potential function for the molecule in macrophages which has yet to be determined. This study shows that CK-2 is a functional chemokine that has a role in the rainbow trout immune response involving, but not limited to, macrophages.

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Dedication

I would like to dedicate this thesis to my father, Dr. Francis C.Y. Tang, whose love has always and will forever push me to further my education.

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Chapter 1
Introduction

1.1 A General Overview of the Immune System

The immune system is a complex network of cells, tissues and molecules that have evolved in order to defend multicellular organisms against pathogens and non-self entities. All organisms have innate immunity which confers non-specific defense against foreign invaders. The innate immune system is always present and therefore relatively quick to respond. It is the first line of defense against intruders and is able to trigger the adaptive immune system if the innate response is not sufficient. The adaptive immune system is only present in jawed vertebrates and can be highly diverse across species (Matsunaga and Rahman, 1998; Zapata et al., 2006; Rolff, 2007). It provides specific protection against pathogens and as the name suggests, can adapt to the characteristics of each. The adaptive immune system can recognize different types of invaders and mount the appropriate response to eliminate them. In contrast to the innate immune system, the adaptive response has the ability to develop memory of intruders that it comes in contact with which allows it to initiate a quicker and stronger response upon subsequent encounters (Murphy et al., 2007). Furthermore, the adaptive response is highly regulated by an army of molecules which organize, activate and deactivate immune cell behaviour. Most adaptive immune responses require multiple signals from cytokines, chemokines, receptors and ligands in order to be fully functional. These signals can come from the pathogens themselves or the innate immune response. Therefore the innate and adaptive immune systems are tightly integrated in their response to foreign intruders.

1.2 Chemokines

Chemokines are a family of chemotactic cytokines that are responsible for directing the migration of target cells to points of interest (Laing and Secombes, 2004a). Traditionally, these points of interest are sites of injury or infection (Laing and Secombes, 2004a). However more recently, evidence has been reported that chemokines also guide cells to their proper positions during development (Doitsidou et al., 2002; Molyneaux et al., 2003). The

intricacies of how these proteins work within the immune system is of great interest due to their role in intercellular communication. The network of immune cells depends on chemokines to relay messages of attack on the organism so that they can be effective in defending against disease. Therefore, the potential for the use of chemokines to assist in the treatment of many diseases may have a huge impact on medicine, both human and veterinary. It is for this reason that the downstream effects of individual chemokines should be investigated.

The chemokine family is a group of basic, low-molecular weight proteins that range from 7 to 14 kDa (Graves and Jiang, 1995). Structurally, they are characterized by three antiparallel β-strands, a C-terminal α-helix, and two essential disulfide bonds that are formed by four conserved cysteine residues. The positions of these essential cysteine residues are used to classify the members of the family into α, β, γ and δ subfamilies. For example, the α-subfamily includes chemokines that have an amino acid residue sandwiched between the first two cysteine residues. Thus, they are also referred to as CXC chemokines. Chemokines in which cysteines one and two are adjacent to each other are designated as CC chemokines or as part of the β-subfamily. Gamma-chemokines are also known as XC chemokines because they lack cysteine one in addition to cysteine three and can only form one disulphide bond (Nomiyama et al., 2008). The fourth chemokine class is the CX3C or δ-subfamily and consists of one member, CX3CL1, commonly known as fractalkine (Bazan et al., 1997). This chemokine is distinctive due to the presence of its mucin stalk. A novel subfamily of chemokines has recently been proposed to classify four chemokines identified in zebrafish. These molecules are missing one of the first two cysteines while retaining cysteines three and four (Nomiyama et al., 2008). These new chemokines have been termed CX chemokines (Nomiyama et al., 2008). Although the current chemokine nomenclature reflects the classification system, a previous format identified the proteins as *small inducible cytokines* (SCY). Thus, the old system catalogued the CC, CXC, XC and CX3C chemokines as SCYA, SCYB, SCYC and SCYD, respectively (Zlotnik and Yoshie, 2000).

In addition to the cysteine classification system, chemokines can be organized into "inflammatory" and "homeostatic" classes (Zlotnik et al., 2006). Those that belong to the inflammatory class are only expressed when induced while homeostatic chemokines are expressed constitutively (Zlotnik et al., 2006). Chromosomal location of the genes encoding chemokines can also be used to categorize the molecular family (Zlotnik et al., 2006). Chemokines that are encoded on a chromosome with many others are known as "major-cluster" chemokines, while those that appear with only one or two others are called "mini-cluster" chemokines (Zlotnik et al., 2006). Chemokines that appear in unique locations are known as "non-cluster" chemokines (Zlotnik et al., 2006).

1.3 Chemokine Families

There are currently 46 known chemokines in humans, of which 17 are CXC, 26 are CC, 2 are XC and 1 is CX3C (Zlotnik et al., 2006). The first chemokine discovered and characterized was a CXC chemokine that is commonly referred to as interleukin-8 (IL-8) and officially as CXCL8 (Baggiolini et al., 1989; Zlotnik et al., 2006). CXCL8 is the prototypical CXC chemokine and apart from mammals, has been found in many species amongst birds, amphibians, and fish (Laing and Secombes, 2004a). This chemokine is known for being produced early in the inflammatory response and for a prolonged period of time (Remick, 2005). It activates neutrophils as well as weakly stimulating monocytes and basophils (Graves and Jiang, 1995). CXCL8 is one of the CXC chemokines that includes the ELR motif, which confers specificity to neutrophils. Thus, CXC chemokines can be further subdivided into ELR+ and ELR- groups. The ELR+ chemokines in humans include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 (Laing and Secombes, 2004a). These chemokines cause neutrophils to adhere to endothelial cells and to migrate towards sites of inflammation (Laing and Secombes, 2004a). CXC chemokines that do not possess the ELR motif are poor chemoattractants of neutrophils and instead attract lymphocytes and monocytes (Laing and Secombes, 2004a). Most CXC chemokines are found clustered on chromosome four, either in the GRO or the IP10 regions, but a few individual CXC chemokines are encoded singly on chromosomes five, 10, 17 and 19 (Zlotnik et al., 2006). In

addition, the majority of CXC chemokines belong to the inflammatory group, except for CXCL12 and CXCL13 which are homeostatic (Zlotnik et al., 2006).

The chemokines of the CC subfamily target the mononuclear cells of the immune system and can be further classified based on activity into allergenic, pro-inflammatory, haemofiltrate CC chemokine (HCC), developmental, and homeostatic subgroups (Laing and Secombes, 2004a). The HCC, allergenic, and pro-inflammatory chemokines are usually expressed when induced while the developmental and homeostatic chemokines are constitutively expressed (Laing and Secombes, 2004a). It should be noted that the inducible CC chemokines are clustered on chromosome 17 while the constitutive ones are scattered on chromosomes two, nine, 16 and 19 (Zlotnik et al., 2006; Laing and Secombes, 2004a). In addition, there are two allergenic chemokines, CCL24 and CCL26, which are found on chromosome seven (Zlotnik et al., 2006; Laing and Secombes, 2004a).

However, not all CC chemokines are created equal in terms of gene structure and typical amino acid residue conservation. An interesting feature of the gene structure of both CCL14 and CCL20 is that unlike most CC chemokines which are encoded by three exons and two introns, CCL14 and CCL20 are encoded by four exons and three introns (Pardigol, 1998; Nelson et al., 2001). Also of note is the evidence that CCL18 may have evolved from the fusion of two duplicates of the gene encoding CCL3 (Tasaki et al., 1999). It seems that there are mutated copies of this gene surrounding the locus and it has been proposed that this can possibly constitute a “hotspot” for the generation of new chemokine genes (Tasaki et al., 1999). This may be of considerable importance since it may form the basis of how protein superfamilies, in particular the chemokine superfamily, expanded. In addition, there are some CC chemokines that, instead of having the usual four cysteine residues, have five or six and are therefore designated as C5 or C6-CC chemokines (Laing and Secombes, 2004b). The C5-CC chemokines include CCL1 and CCL24 while CCL21 and CCL28 are C6-CC chemokines (Laing and Secombes, 2004b; Laing and Secombes, 2004a). These seemingly odd chemokines may provide answers to how chemokines evolve.

The XC subfamily has two known members in humans, XCL1 and XCL2 (Laing and Secombes, 2004a). XCL1 is commonly known as lymphotactin and is chemotactic for lymphocytes (Kelner et al., 1994). The gene encoding XCL1 is found on chromosome one in a mini-cluster with the gene for XCL2 (Kelner et al., 1994; Zlotnik et al., 2006). XCL2 was initially thought to be an allele of XCL1 because their nucleotide sequences have 97% identity (Yoshida et al., 1996). However, after additional analysis of the sequences encoding XCL1 and XCL2 it was decided that they were separate genes (Yoshida et al., 1996). It has been suggested that XCL1 evolved from a CC chemokine and was duplicated with an insertion in the first intron (Yoshida et al., 1996). It was then proposed that part of the first intron was deleted from one copy of the gene, resulting in XCL2 (Yoshida et al., 1996). Since the nucleotide sequences of XCL1 and XCL2 are so similar that the chemokines only differ by a single amino acid residue, the gene duplication must have occurred quite recently (Zlotnik et al., 2006). This provides further evidence that the expansion of the chemokine superfamily is driven by gene duplication events.

The only known member of the CX3C subfamily in human is CX3CL1, also known as fractalkine (Bazan et al., 1997). This chemokine possesses a mucin stalk as well as a transmembrane domain and has been seen to exist in both anchored and soluble forms (Bazan et al., 1997). Soluble CX3CL1 is chemotactic for T-lymphocytes and monocytes while when expressed on endothelial cell surfaces, it aids in leukocyte adhesion (Bazan et al., 1997).

1.4 Evolution of Chemokines

Comparison of the mouse and human genomes show that immune system genes evolve more rapidly than any other and is probably due to high diversifying selection pressures (Zlotnik et al., 2006; Peatman and Liu, 2007). The mouse proteome includes homologs of most chemokines found in humans. The clustering of CXC and CC chemokines is similar in mouse and human with homologs even appearing in roughly the same order (Zlotnik et al., 2006). Again, it is proposed that the clustering of chemokine genes is due to multiple tandem gene duplication events and the subsequent divergence of the gene copies (Zlotnik et al., 2006). This is reflected in the low sequence identities of cluster homologs between species as

well as the promiscuity in receptor-ligand relationships (Zlotnik et al., 2006). The gene duplication probably occurred recently in evolutionary history due to the observation that in a phylogenetic tree of human and mouse chemokines, cluster chemokines form groups including chemokines from both species (Zlotnik et al., 2006). That is, the multitude of mouse and human cluster chemokines were probably derived from a few common ancestral genes that existed right before the divergence of the two species (Nomiyama et al., 2001).

On the other hand, mini-cluster and non-cluster chemokines tend to be more conserved between species and have monogamous receptor-ligand relationships (Zlotnik et al., 2006). It is hypothesized that these chemokines maintain essential functions that constrain them from diverging too much (Zlotnik et al., 2006). Therefore mini- and non-cluster chemokines must reflect ancient, ancestral chemokines more closely than cluster chemokines (Nomiyama et al., 2001; Zlotnik et al., 2006).

Fish represent an important period in evolution because they occur at the tipping point between invertebrates that have only an innate immune system and tetrapods that rely on adaptive immunity (Peatman and Liu, 2006). They include, in order of divergence, jawless fish (lamprey), cartilaginous fish (shark), ray-finned fish (zebrafish) and teleost fish (rainbow trout, cichlid) (Volff, 2005). Fish make for an interesting subject of study due to evidence of tetraploidization and rediploidization events (Volff, 2005). These events have resulted in multiple gene duplications which have a great role in the perpetration of molecular evolution (Volff, 2005). The first chemokine found in fish was a CC chemokine found in rainbow trout and named CK-1 (Dixon et al., 1998). CK-1 includes six cysteine residues, making it a member of the C6 group of CC chemokines (Dixon et al., 1998; Lally et al., 2003). The protein structure of CK-1 classifies it as a CC chemokine; however its gene organization is more akin to that of a CXC chemokine, having four exons and three introns (Dixon et al., 1998). It has been suggested that the presence of traits from both CC and CXC subfamilies in CK-1 is indication that the rainbow trout chemokine is a modern version of the common ancestor of these subgroups (Dixon et al., 1998). As mentioned previously, the human CC chemokines CCL14 and CCL20 are also in possession of a four exon, three intron gene

structure. This could indicate that CCL14 and CCL20 are mammalian representatives of the ancestral chemokine. CK-1 shows 33-35% sequence identity to human CCL20 and shares a similar gene structure that is not common in CC chemokines (Lally et al., 2003). As well, in a phylogenetic tree constructed from known mammalian, chicken and fish CC chemokine sequences, CK-1 grouped with human and chicken CCL20 (Laing and Secombes, 2004b). Thus it is believed that CK-1 is most closely related to human CCL20 (Lally et al., 2003).

A sequence encoding a CXCL8-like peptide was isolated from a stimulated rainbow trout leukocyte cDNA library (Laing et al., 2002). The amino acid sequence of the molecule has 35%, 38% and 31% identity with human CXCL8, chicken cCAF, and lamprey CXCL8, respectively (Laing et al., 2002). Rainbow trout CXCL8 has an even higher identity with the flounder CXCL8, at 56%, which may reflect the closer temporal relationship trout shares with flounder than with lamprey (Laing et al., 2002). However, rainbow trout CXCL8 has a low sequence identity with CK-1, of 18% (Laing et al., 2002). The rainbow trout homolog lacks the ELR motif that is essential for human CXCL8 function, which appears to be characteristic of fish CXCL8-like chemokines (Laing et al., 2002).

A second CC chemokine was identified in a cDNA library made from activated rainbow trout haematopoietic cells and named CK-2 (Liu et al., 2002). The protein shows 44% amino acid identity with a carp CC chemokine and about 20% identity with mammalian CC chemokines (Liu et al., 2002). An interesting feature of CK-2 is the presence of a glycosylated mucin stalk that is similar to the ones seen in the mammalian chemokines, CX3CL1 and CXCL16 (Liu et al., 2002). CK-2 has 25% identity with CX3CL1 and is not similar to CXCL16 (Liu et al., 2002). The stalk in CK-2 is half the length of the CX3CL1 stalk and does not include a transmembrane domain (Liu et al., 2002). However, an allele of the CK-2 gene known as CK-2.1 includes a longer mucin stalk. It has been suggested that the mucin stalk was present in a common ancestor of modern chemokines, and then lost during the divergence of the protein since it is present in a rainbow trout CC chemokine, a mammalian CXC chemokine, and mammalian CX3C chemokines (Liu et al., 2002).

A search for chemokine-like sequences in rainbow trout ESTs yielded 15 CC chemokines, named CK-4 to CK-12, each having ‘a’ and ‘b’ forms (Laing and Secombes, 2004b). It is suggested that each ‘a’ and ‘b’ pair are closely related genes and that they may have even duplicated from each other (Laing and Secombes, 2004b). However it is also possible that the two sequences are alleles of the same gene (Laing and Secombes, 2004b). CK-5a and b have 37-39% sequence identities to human CCL8 and chicken AH294 and incidentally, are grouped with these chemokines along with a *Xenopus* CCL5-like protein, in a phylogenetic tree constructed from known CC chemokines (Laing and Secombes, 2004b). The same tree grouped both CK-7a and b proteins with the human MCP subgroup, while CK-9 groups with human CCL25 as well as cichlid SCYA102 and SCYA103 (Laing and Secombes, 2004b). Trout CK-11 groups with zebrafish CCL1, cichlid SCYA101 and human CCL27 and CCL28 (Laing and Secombes, 2004b). This new set of trout chemokines shows that extra cysteine residues have been conserved throughout the evolution of chemokines (Laing and Secombes, 2004b). CK-7a and b have a fifth cysteine residue near their C-terminals and are most closely related to mammalian CCL1 and CCL24 which also have C-terminal cysteines (Laing and Secombes, 2004b). Likewise, both CK-10 and human CCL21 have fifth and sixth cysteine residues at their C-terminals (Laing and Secombes, 2004b).

The lamprey is a jawless fish and is thought to be one of the earliest vertebrates, estimated to have appeared about 560 MYA (Najakshin et al., 1999; Laing and Secombes, 2004a; Volff, 2005). Adaptive immunity was thought to be a “unique trait of jawed vertebrates” but it has recently been reported that the most ancient antibodies have been discovered to exist in lamprey (Rolff, 2007; Pancer and Mariuzza, 2008). Therefore, the lamprey makes for an interesting subject to study in an attempt to elucidate the evolution of the immune system.

A CXCL8-like chemokine was identified from a cDNA library created from lamprey leukocytes and termed LFCA-1 (Najakshin et al., 1999). The protein shows 32% sequence identity to human CXCL8, 40% identity to the chicken homolog, cCAF and 41% identity to the dogfish version (Inoue et al., 2005; Najakshin et al., 1999; Laing and Secombes, 2004a; Inoue et al., 2003). However, LFCA-1 does not include the ELR motif responsible for the

ability of CXCL8 to recruit neutrophils (Najakshin et al., 1999). Again, the lack of an ELR motif in CXCL8-like molecules is common in fish. This indicates that ELR-directed neutrophil specificity probably evolved after the split between fish and amphibians. The lamprey homolog of CXCL8 is the most ancient known example of a chemokine (Najakshin et al., 1999). A phylogenetic analysis of LFCA-1 with mammalian and chicken chemokines showed that CXC chemokines form a monophyletic cluster and probably arose from a common ancestor (Najakshin et al., 1999).

Invertebrate species have very primitive immune systems and lack the genes necessary to carry out adaptive immunity (Matsunaga and Rahman, 1998). However, because it is believed that all vertebrates possess the chemokine system, chemokines must have evolved before the divergence of vertebrates and invertebrates. It would therefore be interesting to determine which of the invertebrates that are more closely related to fish, encode chemokine-like proteins in their genome.

Although the sea squirt is an invertebrate, it does have a dorsal notochord and a primitive multiple organ system (DeVries et al., 2006). Since chemokines have an important role in cell migration during development, it is reasonable to predict that they would be present in sea squirt. However, a search of the draft genome of the sea squirt did not reveal any possible chemokine encoding sequences (DeVries et al., 2006). It is interesting that the sea squirt does not appear to possess the chemokine system because it is one of earliest organisms to possess a multiple organ system but does not have an adaptive immune system (DeVries et al., 2006). Therefore, one may infer that chemokines evolved for the function of directing leukocytes to sites of infection before acquiring the role of directing the migration of cells during development.

Chemokines have been found in many organisms on the evolutionary timeline ranging from 560 MYA (lamprey) to present (mammals). If chemokines exist in all vertebrates, then it stands to reason that chemokines also existed before vertebrates. However, chemokines have not yet been isolated from hagfish, a vertebrate species that diverged before lamprey. If they do exist in hagfish, the question of when chemokines appeared on the molecular scene

remains. Which invertebrate first started to produce a chemokine-like protein and what gene did it evolve from? These questions may start being answered as the genomes of more and more species are being sequenced. However the extinction of key species in the evolutionary path of the chemokine may prove to be an insurmountable challenge in the search for its origin

Another glaring question asks if chemokines evolved to facilitate cell migration during the development of the multiple organ system and then later took on the role of reconnaissance to alert leukocytes to sites of infection? Or did the reverse happen? Zlotnik et al. suggests that the role of the CXCR4-CXCL12 pair in primordial germ cell homing in zebrafish is an indication that the chemokine system evolved in order to control cell movement (2006). However, chemokine-like molecules are curiously absent in the sea squirt, which has the need for chemotactic proteins to aid in the development of its multiple organ system. This seems to be evidence that chemokines evolved for the function of signaling leukocytes to migrate towards sites of infection before it was hijacked for its chemotactic abilities by development.

Although the knowledge of non-mammalian chemokines is limited, recent studies have elucidated many novel chemokines, especially in fish species. The study of chemokines has been seen to be very important due to the extensive role played by the molecular superfamily in the progression and physiological responses to so many diseases. Thus, it is also important to determine the differences and similarities of the system between species in order to infer functions and responses from model organisms. Furthermore, understanding the evolution of chemokines will provide major insight on not only the immune system, but also development and physiology (Zlotnik et al., 2006). The search for putative chemokine-encoding genes in the zebrafish genome resulted in over a hundred sequences, indicating that gene expansion of the chemokine system occurred in fish species as well as mammalian. The sequencing of the genomes and ESTs of more species that diverged before mammals should result in the identification of many more chemokines. It has also been suggested that the localization of chemokine sequences can shed light on how the chemokine family emerged by looking at

possible gene duplications, translocations and divergence (Peatman and Liu, 2007). Through extensive comparisons of chemokine sequences and genome localization, we should then be able to decipher exactly how chemokines arose in vertebrates. As well, by looking at the functions of different classes of chemokines and deciphering the elements required for each specific function, we can determine how function-element relationships correlate across species. This can lead to the elucidation of how the chemokine sub-families evolved from each other.

1.5 CK-2

As discussed previously, the rainbow trout chemokine CK-2 is a CC chemokine with the interesting possession of a glycosylated mucin stalk. It has been shown by a Southern blot that there are at least two copies of the CK-2 gene in the trout genome (Liu et al., 2002). The mature protein consists of 163 amino acids with the mucin stalk comprising 88 amino acids, 43 of which are potential O-linked glycosylation sites (Liu et al., 2002). Originally thought to be a novel chemokine, CK-2.1 has been determined to be an allele of CK-2 (Eshaque et al., unpublished). CK-2.1 has 98% nucleotide sequence identity with CK-2 and a longer mucin stalk (Eshaque et al., unpublished). Several putative CC chemokines that also include a potential glycosylated mucin stalk have been identified in zebrafish by searching within the zebrafish genome and EST databases (Nomiyama et al., 2008). However it has yet to be reported whether any of these chemokines are expressed and/or functional. It is interesting to note that mucin-stalk containing CC chemokines have only been found in fish to date and may possibly be unique to fish species. The function of the mucin stalk in chemokines has yet to be determined.

CK-2 mRNA transcripts were seen in brain, head kidney, spleen and peripheral blood leukocytes (PBL) in phytohemaglutinin (PHA)-stimulated fish (Eshaque et al., unpublished). However CK-2 protein was only detected in head kidney, liver and brain tissues (Eshaque et al., unpublished). The rainbow trout spleen cell line RTS-11 expressed CK-2 at the protein level regardless of treatment with PHA (Eshaque et al., unpublished). Chemotaxis assays using recombinantly produced protein, showed that CK-2 is capable of inducing chemotaxis

in rainbow trout PBLs as well as RTS-11 (Eshaque et al., unpublished). The migration was blocked by the treatment of cells with pertussis toxin, anti-CK-2 antibody or excess CK-2, indicating that it was indeed the action of the chemokine (Eshaque et al., unpublished).

1.6 Chemokine Signaling

Chemokines produce their chemotactic effects by triggering intracellular signal transduction through pertussis toxin sensitive G-protein coupled receptors (GPCRs) (Kelvin et al., 1993). The relationship between chemokines and their receptors can be mutually promiscuous, with the ligands being capable of binding multiple receptors and vice versa (Peatman and Liu, 2007). The chemokine receptor is part of the rhodopsin family of seven-transmembrane domain serpentine receptors (Kelvin et al., 1993). Chemokine receptors can usually be distinguished by the conserved DRYLAIV amino acid motif, found in the second intracellular loop (Murphy, 1994). Upon contact with its ligand, the receptor activates its associated G-protein such that it changes in conformation to release its βγ-subunit from the α-subunit (Bokoch, 1995). The released βγ-subunit is subsequently able to activate the effector protein phospholipase C (PLC), which proceeds to cleaving phosphoinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Bokoch, 1995). IP_3 open IP_3-gated calcium channels in the endoplasmic reticulum to release to the intracellular stores of Ca^{2+}. The spike in intracellular Ca^{2+} can then trigger many signaling pathways. It can cause DAG to activate protein kinase C (PKC), which goes on to phosphorylate a variety of proteins involved in leukocyte activation (Bokoch, 1995). On the other hand, the α-subunit can activate Src tyrosine kinases which proceed to activate the MAP kinase signaling cascade, resulting in gene transcription (Soldevila and Garcia-Zepeda, 2007). Src can also activate PI3 kinase which helps initiate the molecular events that lead to chemotaxis (Soldevila and Garcia-Zepeda, 2007).

In addition, chemokine receptors are capable of mediating their effects through the JAK/STAT signaling pathway (Vila-Coro et al., 1999). The ligand-bound receptor dimerizes causing the recruitment and activation of Jak, which facilitates phosphorylation of the receptor (Soldevila and Garcia-Zepeda, 2007). This allows for Stat to be engaged and

activated so that it can translocate to the nucleus and induce gene transcription (Soldevila and Garcia-Zepeda, 2007).

Although we know many of the physiological effects that chemokines have on immune system cells, there is little comprehensive information on how these molecules affect the overall immune-related gene expression that perpetrates these end results. There have been few studies over the past twenty years on how chemokines affect the gene expression profiles of the cells that they act on. Jiang et al., showed that the CC chemokine MCP-1, or CCL2, can regulate the expression of adhesion molecules as well as cytokines in human monocytes (Jiang et al., 1992). It was seen in this study that CCL2 induces interleukin (IL)-1 and IL-6 while having no effect on tumour necrosis factor-alpha (TNFα) (Jiang et al., 1992). It was proposed on the basis of this study that CCL2 modulates the chemotaxis of monocytes through the regulation of adhesion molecules and that its effect on the inflammatory cytokines points to a role in inflammation (Jiang et al., 1992). It was reported by Taub et al., that incubation of activated type 1 and type 2 helper T-cells with various CC chemokines induced IL-2 production and proliferation of clones (Taub et al., 1996). However, a study on the effects of CC chemokines on $CD4^+$ helper T-cells showed that MIP-1α, or CCL3, does not cause an increase in IL-2 in naive T-cells but instead induces the expression of interferon (IFN) 2, also known as IFNγ, resulting in type 1 helper T-cell activation (Karpus et al., 1997). In addition, this group saw that CCL2 exposure of these cells caused an increase in IL-4 production indicating a push towards type 2 immune T-cell response (Karpus et al., 1997). In addition, a study by Gu et al., reported that CCL2 induces IL-4 production in mice, giving further indication that it is responsible for directing a T_H2 immune response (Gu et al., 2000). The gene expression profile of monocytes exposed to CCL5 was investigated by an Italian research group (Locati et al., 2002). They reported that CCL5 does not induce TNFα or IL-6 and only stimulates IL-1β at the mRNA transcript level (Locati et al., 2002). On the other hand, it was seen in this study that CCL5 induced the production of genes that affect the extracellular matrix and may be the mediators of CCL5-driven chemotaxis (Locati et al., 2002). It was seen in a study on cultured rainbow trout monocytes the rainbow trout version of CXC chemokine IL-8, or CXCL8, invokes the expression of pro-inflammatory cytokines

IL-1β and TNFα (Montero et al., 2008). In addition, IL-8 can induce a positive-feedback loop in which it stimulates its own expression (Montero et al., 2008). Conversely, the rainbow trout CC chemokine CK-6 does not induce the expression of pro-inflammatory genes but does increase expression levels of iNOS and integrin molecule CD-18 (Montero et al., 2008). The induction of iNOS possibly indicates that CK-6 has a role in the activation of macrophages while the increased expression of CD-18 could be responsible for its chemotactic effect (Montero et al., 2008). These studies show that not only do chemokines have an effect on gene expression; they also use their ability to regulate gene expression to mediate their effects on leukocytes. In light of this, we can use the gene expression profiles of immune system cells to determine chemokines functions other than the induction of chemotaxis.

1.7 Research Objective

The objective of this research was to elucidate the downstream effects that CK-2 has on the immune system. Specifically, the endeavour was to determine how CK-2 affects the expression of immune-related genes using the macrophage and monocyte-like cells of the rainbow trout spleen cell line, RTS-11. Recombinant CK-2 was produced and purified under native conditions then tested for functional capability by testing its chemotactic capacity towards RTS-11 and peripheral blood leukocytes isolated from rainbow trout. The active protein was then used to treat RTS-11 in time course and dose response experiments. RNA was collected from each time point and dose response sample and converted to cDNA in order to analyze how various immune-related genes were affected by rCK-2. The expression data was subsequently used to assemble a gene expression profile which may be able to afford us the ability of predicting the immune response that would occur upon the induction of CK-2 by different types of pathogens. Knowing the types of infection that CK-2 is able to stimulate an immune response against can assist in the design of vaccines towards these pathogens to be used in the aquaculture industry.

Chapter 2
Materials and Methods

2.1 Expression, Purification, and Analysis of Recombinant Protein

2.1.1 Pilot Expression of Recombinant CK-2

The full-length cDNA sequence of rainbow trout chemokine CK-2 was cloned into the pRSET A vector (Invitrogen, CA, USA) by a previous student. This vector was used to transform competent BL21(DE3)pLysS *Escherichia coli* cells (Novagen, WI, USA) according to the pRSET A, B, C transformation protocol detailed in the technical manual provided by Invitrogen. The transformed bacteria were plated on SOB plates containing 50μg/mL of ampicillin (Sigma, MO, USA) and 35μg/mL of chloramphenicol (Sigma, MO, USA) and grown at 37°C. A colony was used to inoculate 2mL SOB including ampicillin and chloramphenicol and incubated in a 37°C shaking water bath overnight. The culture was used to make a glycerol stock, by adding 200μL of sterile 50% glycerol to 800μL of culture, and then stored at -80°C. A loopful of the glycerol stock was used to inoculate 2mL overnight cultures with antibiotics. A volume of 750μL of the 2mL overnight culture was used to inoculate a 25mL overnight culture with antibiotics. The 25mL culture was incubated in a 37°C shaking water bath until it reached an OD_{600} of 0.52, then induced with isopropyl β-D-1 thiogalactopyranoside (IPTG) (Fermentas, ON, Canada) to a final concentration of 1mM. Starting at time zero, 1mL samples were taken at each hour for an additional eight hours. Samples were then taken every three hours until the culture had been grown for 24 hours. All samples were centrifuged at 15000rpm in a benchtop microcentrifuge for 5mins immediately after being taken. The supernantants were discarded and the pellets were stored at -20°C.

2.1.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

The pilot expression study samples were thawed on ice and run on a sodium dodecyl sulfate (SDS) polyacrylamide gel for protein analysis. Samples were diluted 1:2 with sample buffer

(24mM Tris-HCl, 10% glycerol, 0.8% SDS (Fisher Scientific, ON, Canada), 5.76mM β-mercaptoethanol (Sigma, MO, USA), 0.04% bromophenol blue). The 12% resolving gel with 1.5M Tris-HCl (pH 8.8), 30% Acrylamide/Bis-acrylamide 37.5:1 solution (Bioshop, ON, Canada), 10% SDS (Fisher Scientific, ON, Canada), 10% ammonium persulphate, and TEMED (Biorad, CA, USA). The 4% separating gel was made with all the same ingredients except for 1.5M Tris-HCl (pH 8.8), which was substituted with 0.5M Tris-HCl (pH 6.8). The gels were cast and run using the Mini-Protein® II gel aparatus (Bio-Rad, CA, USA). Samples were run through the separating gel at 76V until they passed into the resolving gel and then run at 170V. Gels were stained in a 0.1% Coomassie blue G-250 (MP Biomedicals, OH, USA) staining solution and destained in a solution containing 5% methanol and 7.5% acetic acid.

2.1.3 Bulk Expression of Recombinant CK-2

A loopful of the CK-2 glycerol stock was used to inoculate 2mL overnight cultures with antibiotics. A 50mL overnight culture with antibiotics was inoculated with 100µL of the 2mL overnight culture. From the 50mL overnight culture, 24mL was pelleted, resuspended in fresh SOB media, and subsequently used to inoculate 300mL of SOB in a 1L Erlenmyer flask. The 300mL culture was incubated in a 37°C shaking water bath until it reached an OD_{600} of 0.4-0.6. Cultures were induced to make recombinant CK-2 by IPTG to a final concentration of 0.1mM, then allowed to grow for an additional five hours, as determined by the pilot study to be the optimal growth time (Section 2.1.2). Cultures were harvested by pelleting bacterial cells at 10,000rpm for fifteen minutes in a Sorvall floor model centrifuge with an SLA-1500 rotor (Thermo Scientific, MA, USA). Cell pellets were stored at -80°C until pellets from at least 8 cultures were collected.

2.1.4 Optimization of Recombinant Protein Production

In order to optimize aeration conditions for rCK-2 production, 50mL overnight cultures of BL21(DE3)pLyspRSETA-CK-2 was used to inoculate 300mL and 500mL of SOB in 1L Erlenmeyer as well as 1L of media in 2.8L Fernbach flasks. Cultures were grown in a 37°C water bath with shaking until they reached an OD_{600} of 0.5. Cultures were induced with IPTG

to a final concentration of 0.1mM then returned to the water bath for an additional 5hrs. Cells were pelleted at 10,000rpm for 15 mins in the Sorvall centrifuge with an SLA-1500 rotor and stored at -80°C before purification (described below). In order to determine the optimal temperature for recombinant CK-2 production 300mL of SOB in 1L flasks were inoculated with the cell pellet from a 50mL overnight culture of BL21(DE3)pLyspRSETA-CK-2 and grown in a water bath at 37°C until it reached an OD_{600} of 0.5. Cultures were induced with IPTG to a final concentration of 0.1mM and incubated at temperatures of 23°C, 30°C and 37°C for an additional 5hrs. Cells were pelleted at 10,000 rpm for 15 mins in the Sorvall centrifuge fitted with an SLA-1500 rotor. Pellets were stored at -80°C until protein purification was performed (described below).

2.1.5 Purification of Recombinant CK-2 under Native Conditions

Cell pellets were thawed on ice for ten minutes and resuspended in 8mL phosphate-buffered saline with protease inhibitor cocktail (Roche, ON, Canada). Cells were lysed by subjecting the cell suspensions to six freeze-thaw cycles in dry ice and a 42°C water bath. Cell lysates were centrifuged in a floor model Sorvall centrifuge with a SS-24 rotor (Thermo Scientific, MA, USA) at 21000rpm at 4°C until a solid pellet formed, approximately 25 min. The supernatant was collected and purified using Ni-Nitrilotriacetic acid (NTA) resin (Qiagen, ON, Canada). The 50% resin slurry was added to the supernatant in a 1:4mL ratio and incubated on an end-over-end shaker for for 1hr at 4°C. The resin was washed eight times with 10mL of wash buffer containing. Recombinant protein was eluted from the resin in three 1mL elutions with elution buffer containing. Samples of the flow-through, washes and elutions were stored at 4°C for SDS-PAGE. Elutions were transferred into dialysis tubing and dialyzed in phosphate-buffered saline for 1hr at 4°C to remove imidazole from the samples.

2.1.6 Quantification of Protein

Protein concentrations of the elutions were determined via the Bradford Assay (Sambrook et al., 1989). A standard curve was constructed using known concentrations of bovine serum albumin (BSA) (Sigma, MO, USA), ranging from 0mg/mL to 120mg/mL in a 96-well plate.

All standards and samples were diluted to 25% 1N NaOH and 20% Bradford reagent (Bio-Rad, CA, USA), in triplicate. The plate was shaken for 1min, incubated at room temperature for 1hr, then read at 595nm using the Versamax micro-plate reader (Molecular Devices, CA, USA). The standard curve was plotted with protein concentrations on the x-axis and absorbance at 595nm on the y-axis. Protein concentrations of the samples were determined by extrapolation from the standard curve. Dialyzed elutions were diluted to protein concentrations of 250µg/mL and stored in 100µL aliquots at -20°C.

2.1.7 Western Blotting and Immunodetection for the Confirmation of the Presence of Recombinant CK-2

Samples from the flow-through, washes and elutions of the recombinant protein purification were run on an SDS-polyacrylamide gel as described in section 2.1.2. Instead of staining gels in Coomassie Blue, some were transferred to nitrocellulose membranes (Bio-Rad, CA, USA) for specific detection of bands with the use of antibodies in Western Blots. Nitrocellulose membranes, gels and extra thick blot papers (Bio-Rad, CA, USA) were soaked in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 30min, 15min and 40sec, respectively. Transfer sandwiches were assembled as follows: blotting paper, membrane, gel, blotting paper. Sandwiches were placed in a semi-dry transfer unit (Bio-Rad, CA, USA) and transfer buffer was pooled on top. Transfer of proteins from the gels to the nitrocellulose membranes occurred at 20V for 25mins. Membranes were blocked in fetal bovine serum (FBS) (Sigma, MO, USA) for 48hrs at 4°C. Membranes were allowed to equilibriate to room temperature for 30mins then rinsed with Tris-Buffered Saline-Tween-20 (TBST) (20mM Tris, 300mM NaCl, 0.1% Tween-20). Rabbit anti-CK-2 antibody was used to probe the membranes at a final dilution of 1:3000 at room temperature. After 1hr, membranes were subjected to two 10sec washes, a 15min wash and two 10min washes in TBST. Membranes were further probed with mouse anti-rabbit secondary antibody (Sigma, MO, USA) diluted 1:10000 in FBS for 1hr at room temperature. They were then washed in TBST for two 10sec washes, a 10min wash, and two 5min washes. Bands were detected with alkaline phophatase detection solution (52.4mM Tris, 52.4mM NaCl, 26.2mM $MgCl_2$, 1.1% 4-nitro blue tetrazolium chloride (NBT) (Roche, ON, Canada), 0.17% 5-bromo-4-chloro-3-indolyl

phosphate (BCIP) (Roche, ON, Canada) for 2min. Membranes were rinsed in MilliQ water and allowed to sit in fresh MilliQ water for 2min to intensify the bands.

2.2 Chemotaxis Assays

2.2.1 Maintenance of RTS-11 Cells

The rainbow trout spleen cell line RTS-11 was generously provided by Dr. Niels Bols. The cells were maintained in L-15 media (Thermo Scientific, ON, Canada) including 15% fetal bovine serum (Sigma, MO, USA) and 1% penicillin + streptomycin (Sigma, MO, USA) in $25cm^2$ flasks (BD Biosciences, CA, USA). The cells were passaged 1:2 every 3-4 weeks and topped up with media, then stored in a 20°C incubator.

2.2.2 Preparation of RTS-11 Cells

RTS-11 cells were harvested by scraping the bottom of cell culture flasks with the use of a sterile cell scraper (BD Biosciences, ON, Canada). The growth media was transferred into a 15mL falcon tube (VWR, ON, Canada) and cells were pelleted at 440xg for 5min in a benchtop centrifuge (Eppendorf, ON, Canada). The supernatant was aspirated and the cell pellet was resuspended in fresh L-15 media, including 15% FBS and 1% penicillin/streptomycin. Cells were counted using a haemocytometer and diluted in media to 10 million cells per mL.

2.2.3 Collection of Blood Samples

Rainbow trout were kept in tanks in the University of Waterloo Department of Biology WetLab facility. Fish were anaesthetized in a 0.001% 2-phenoxyethanol (Sigma-Aldrich, ON, Canada) water bath. 5mL of blood was drawn from the caudal vein with a 23G needle (Fisher Scientific, ON, Canada) heparinized in 200units/mL heparin-saline solution (Sigma-Aldrich, ON, Canada) and kept on ice until peripheral blood leukocyte isolation.

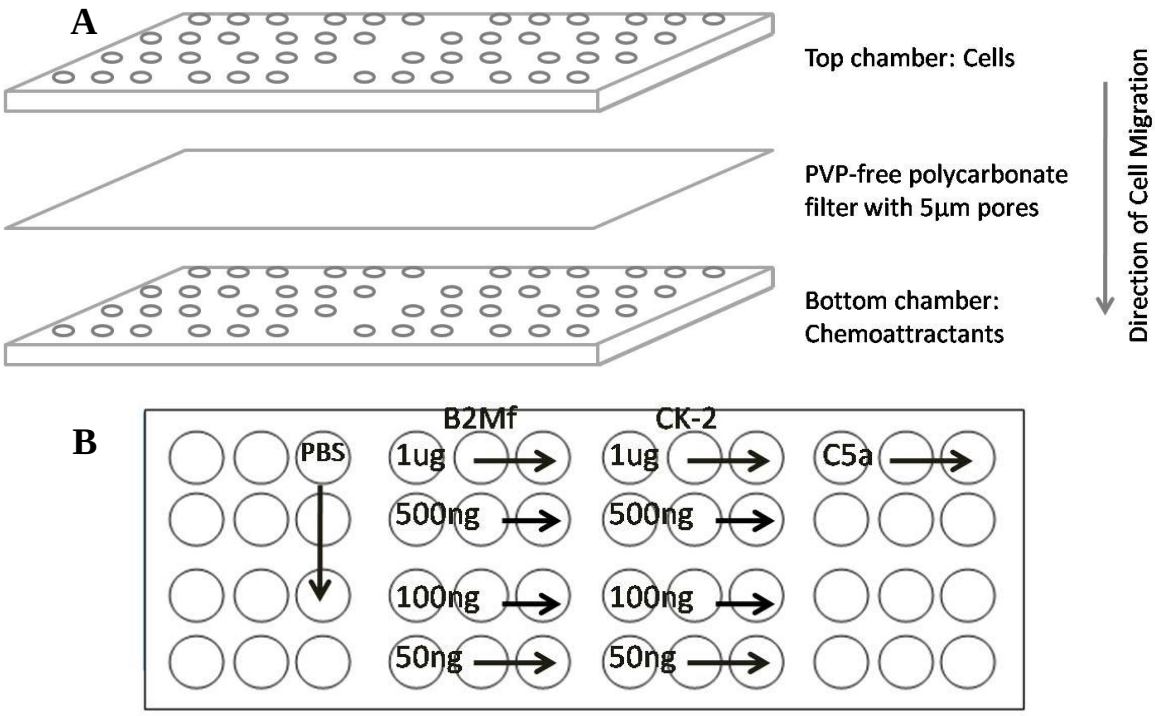
2.2.4 Preparation of Protein Samples

Recombinant β2M fragment (β2Mf) was expressed and purified as described in sections 2.1.3 and 2.1.4 for the production of recombinant CK-2. Protein aliquots were thawed on ice and diluted to appropriate concentrations with sterile MilliQ water.

2.2.5 Chemotaxis Assay

The chemotaxis assay was performed using a 48-well micro chemotaxis assembly (Neuro Probe, MA, USA) (Figure 2.1). Recombinant CK-2 was administered to the lower chambers of the apparatus in concentrations of 1µg/mL, 500ng/mL, 100ng/mL and 50ng/mL, in triplicate. Recombinant human C5a (Cedarlane, ON, Canada) at a concentration of 100ng/mL and 1X PBS were used as positive and negative controls, respectively. Recombinant β2Mf was used as a negative control to rule out the possibility of chemotaxis due to a component of the recombinant protein production. A polyvinylpyrrolidone (PVP)-free polycarbonate filter (Neuro Probe, MA, USA) with 5µm pores was placed in between the upper and lower compartments. The upper wells were filled with 50µL of the RTS-11 cell suspension. The assembly was covered and incubated at room temperature for 2hrs. The cell suspensions were then aspirated from the upper wells. The membrane was allowed to dry upside-down until all liquid was evaporated, fixed in methanol for 1min, and stained in 0.1% toluidine blue O (TBO) (Sigma, ON, Canada) for 5min. The membrane was rinsed in deionized water and mounted on a microscope slide. Cells that had migrated across the membrane towards the chemoattractant were visualized at 400x magnification using a light microscope that was equipped with a digital camera. Pictures were taken of three fields of view for each well. The average of the number of cells in each field of view in all three wells of the same dose of CK-2 was taken. A chemotactic index was determined by calculating the ratio of the number of cells that showed chemotaxis to the number of cells that randomly migrated towards the negative control.

Figure 2.1: Set-up of the Chemotaxis Assay. (A) Schematic of the 48-well chemotaxis chamber used in the chemotaxis assay. Substances to be tested for chemotactic ability are placed in the wells of the bottom chamber. A PVP-free polycarbonate membrane with 5μm pores is sandwiched between the top and bottom chambers. Cells are placed in the wells of the top chamber. The assembly is incubated for 2hrs before staining the cells on the membrane. Chemotaxis is defined in this assay as the migration of cells across the membrane. **(B)** The triplicate set-up of the chemoattractants in the dose-response chemotaxis assay. PBS was used as the negative control while human C5a was used as the positive control.


A
Top chamber: Cells
PVP-free polycarbonate filter with 5µm pores
Bottom chamber: Chemoattractants
Direction of Cell Migration
B
B2Mf
CK-2
PBS
1ug
1ug
C5a
500ng
500ng
100ng
100ng
50ng
50ng

2.3 *In vitro* Expression Study

2.3.1 Preparation of RTS-11 Cells

RTS-11 cells were harvested by pelleting cells at 440xg for five minutes. Cells were resuspended in fresh media and counted using a hemocytometer. Cell suspension was used to seed 12.5cm^2 flasks (BD Biosciences, CA, USA) with 5 million cells and topped up with media to 3mL each. Cells were allowed to settle overnight at 20°C.

2.3.2 Treatment of RTS-11 Cells with rCK-2

The next day, each flask was treated with varying doses of recombinant CK-2 for varying lengths of time, according to Table 2.1, designed to observe time course and dose-response effects of the chemokine on the cell line. All flasks were dosed at the same time and then returned to 20°C until it was time to harvest. Cells were harvested by pelleting at 440xg for three minutes and washed in 600µL 1X PBS.

2.3.3 RNA Extraction

Total RNA extraction was performed using the Qiagen RNeasy Mini Kit (Qiagen, Germany), as per the manufacturer's protocol. RNA was aliquotted and flash-frozen in dry ice and stored at -80°C. RNA concentration was determined by measuring absorbance of each sample at a light wavelength of 260nm using a NanoDrop ND-1000 (Thermo Scientific, ON, Canada).

2.3.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA samples were thawed on ice and 500ng was used to synthesize cDNA using the Fermentas RevertAid H-minus First Strand cDNA Synthesis Kit (Fermentas, ON, Canada), according to the manufacturer's instructions.

Table 2.1: CK-2 Treatments on RTS-11

	Sample	[CK-2] (ng/mL)	Time (mins)
Time Course	1	100	**0**
	2	100	**30**
	3	100	**60**
	4	100	**75**
	5	100	**90**
	6	100	**105**
	7	100	**120**
	8	100	**150**
	9	100	**180**
	10	100	**240**
Dose Response	11	**0**	120
	12	**5**	120
	13	**10**	120
	14	**50**	120
	15	**100**	120
	16	**500**	120
	17	**1000**	120

2.3.5 Amplification of Expressed Genes via PCR

cDNA samples were used as template DNA to amplify various immune genes via PCR. Each reaction consisted of 1X Incubation Buffer, 1.5 mM $MgCl_2$, 200µM dNTPs (Fermentas, ON, Canada), 0.4µM forward primer, 0.4µM reverse primer, 0.2µL Taq Polymerase (Fisher Sceintific, ON, Canada) and 1µL of cDNA. PCR amplification of each gene was carried out using the primers, annealing temperatures (T_a) and elongation times (t_e) listed in Table 2.2. The thermal cycler was programmed for 45 sec at 95°C, followed by cycles of 30 sec at 95°C; 1 min at the appropriate T_a; t_e at 72°C, then a final elongation time of 5 min at 72°C. PCR products were store at 4°C. Expression profiles of each gene were compared to an

internal control, 28S rRNA (Denz and Dube, 2005). PCR products for each gene were run on a 1.5% agarose gel in 0.5X TAE with 0.003% GelRed (Biotium, CA, USA) at 50V for 55 minutes, with internal controls. Gels were visualized with an Alpha Innotech UV transilluminator and EtBr filter (Cell Biosciences, CA, USA). Densitometry analysis on the bands was performed using ImageJ, a program developed at the National Institute of Health (NIH). The bands of each gene for every sample were compared to the 28S amplified band of the corresponding sample, run on the same gel.

Table 2.2: Expression Study PCR Conditions

Gene	Primers	Expected Size (bp)	T_a (°C)	t_e (s)	Cycles
IL-1β	F: AGGGAGGCAGCAGCTACCACAA	353	67	30	35
	R: GGGGGCTGCCTTCTGACACAT				
TNFα	F: CCACACACTGGGCTCTTCTT	111	59.5	30	35
	R: GTCCGAATAGCGCCAAATAA				
IL-8	F: ATTGAGACGGAAAGCAGACG	105	61	45	35
	R: CTTGCTCAGAGTGGCAATGA				
MHIα	F: CATTCCAGCTGATGTATGGC	258	59.5	30	35
	R: CCAAAGTGTCTTTGCCGTAG				
IFN1	F: AAAACTGTTTGATGGGAATATGAAA	141	62	30	32
	R: CGTTTCAGTCTCCTCTCAGGTT				
IFN2	F: GAAGGCTCTGTCCGAGTTCA	119	59.5	30	35
	R: TGTGTGATTTGAGCCTCTGG				
MHIIα	F: ACACCCTTATCTGCCACGTC	160	59.5	30	35
	R: TCTGGGGTGAAGCTCAGACT				
S25-7	F: GGAGAAGCCCCCTGCACCCA	270	67	30	29
	R: ATCATCCTGGGGAAAGCTGC				
CD4	F: CCTGCTCATCCACAGCCTAT	111	61	45	35
	R: CTTCTCCTGGCTGTCTGACC				
28s rRNA	F: CATTTCCTCTGTCGGTGGTG	343	59.5	30	26
	R: CGTCTTGAAACACGGACCAA				

2.3.6 Data Analysis

The relative expression level of each gene in each sample was determined by calculating the ratio of the band density of the gene at that sample to the density of the corresponding 28s rRNA band. Time course and dose response graphs were plotted using the relative expression levels with error bars representing standard deviation between two independent trials. Trendlines were constructed from the moving averages of the relative expression level of the gene in a particular sample and the sample preceding it. In order to identify significant changes in the expression levels of each gene during the time course and the dose response, time course points and doses were divided into two groups that represented lower and higher expression levels or vice versa, depending on the response of the gene to rCK-2. The average gene expression level at each time point or dose in either group was calculated for each gene. Multiple time point and dose divisions were tested to determine the time point or dose at which there was the most significant change in average expression level between groups. The Student's T-test was performed to identify significant changes. The time point or dose that provided the point of division of the two groups that showed the most significant change in average expression levels was used as the time point or dose at which treatment with rCK-2 affected the expression of the gene.

Chapter 3
Results

3.1 Expression of Recombinant CK-2

The pRSET A vector containing the CK-2 cDNA sequence was transformed into the BL21(DE3)pLysS strain of *Escherichia coli*. A time course assay revealed that the optimal induction time for recombinant CK-2 expression is 5hrs, displayed in Figure 3.1. Once the induction time was established, more trials were conducted to determine the most advantageous conditions for protein production. Aeration conditions were optimized for rCK-2 expression by comparing different volumes of media in two types of flasks (Figure 3.2). The production of rCK-2 in cultures that were grown in 300mL and 500mL of media in 1L Erlenmeyer flasks, as well as 1L of media in 2.8L Fernbach flasks, was examined and comparisons were made. It appeared that cultures that were grown in 300mL of media in 1L Erlenmeyer flasks produced the most protein. In order to determine the temperature that yielded the highest concentration of recombinant protein, there was an assessment of the incubation of cultures after induction at three different temperatures: 23°C, 30°C and 37°C (Figure 3.3). It was found that the culture that had been induced in a 37°C water bath gave the highest concentration of rCK-2 compared to the other incubation temperatures. Two 300mL cultures induced at each temperature of 23°C, 30°C and 37°C, cumulatively produced recombinant protein concentrations of 118µg/mL, 93µg/mL and 141µg/mL, respectively. It was therefore concluded that the conditions favouring rCK-2 production included growing cultures in 300mL of media in a 1L Erlenmeyer flask at 37°C and inducing expression for 5hrs.

Figure 3.1: Time course of recombinant CK-2 expression in BL21(DE3)pLysS *E. coli* cells. The *E. coli* strain BL21(DE3)pLysS was transformed with the expression vector pRSETA that had the coding sequence of CK-2 cloned into it. An overnight culture of the bacteria was used to inoculate fresh media and allowed to grow until it reached an optical density of 0.52 at 600nm. A t=0 sample was taken at this point in order to determine baseline expression. Bacteria were then induced to produce recombinant CK-2 with IPTG. Samples of the culture were taken at every hour for 8hrs, after which they were taken every 3hrs until hour 23. A final sample was taken at 24hrs. All samples were pelleted and frozen immediately after collection. Pellets were then resuspended and put through six freeze-thaw cycles. Samples were centrifuged and the supernatants were run on a 12% SDS-PAGE gel, which was stained in Coomassie Blue. The expected size of His-tagged recombinant CK-2 was approximately 30kDa. * denotes a band of interest.

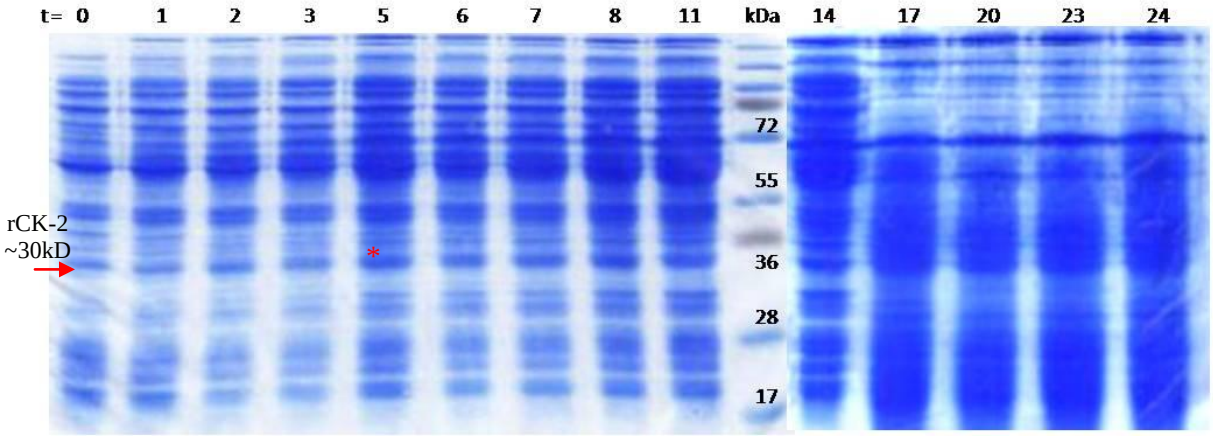

t= 0
1
2
3
5
6
7
8
11
kDa
14
17
20
23
24
72
55
36
28
17
rCK-2
~30kD
*

Figure 3.2: Determination of optimal aeration conditions for the production of recombinant CK-2. Overnight cultures of *E.coli* strain BL21(DE3)pLysS carrying the RPA-cloned pRSET A expression vector were used to inoculate three scale-up conditions: **(A)** 300mL of media in a 1L Erlenmeyer flask, **(B)** 500mL of media in a 1L Erlenmeyer flask and **(C)** 1L of media in a 2.8L Fernbach flask. Cultures were grown to an optical density at 600nm of 0.5. rCK-2 expression was induced with IPTG at a final concentration of 0.1mM. Cultures were incubated in a 37°C water bath with 225rpm of shaking for 5hrs. Bacterial cells were pelleted, resuspended in PBS and freeze-thawed six times. Recombinant protein was isolated via its 6X His-tag using Ni^{2+}-column chromatography. Samples from each stage of the purification were run on a 12% SDS-PAGE gel and stained with Coomassie Blue. FT = flow-through, W = Wash, E = Elution.

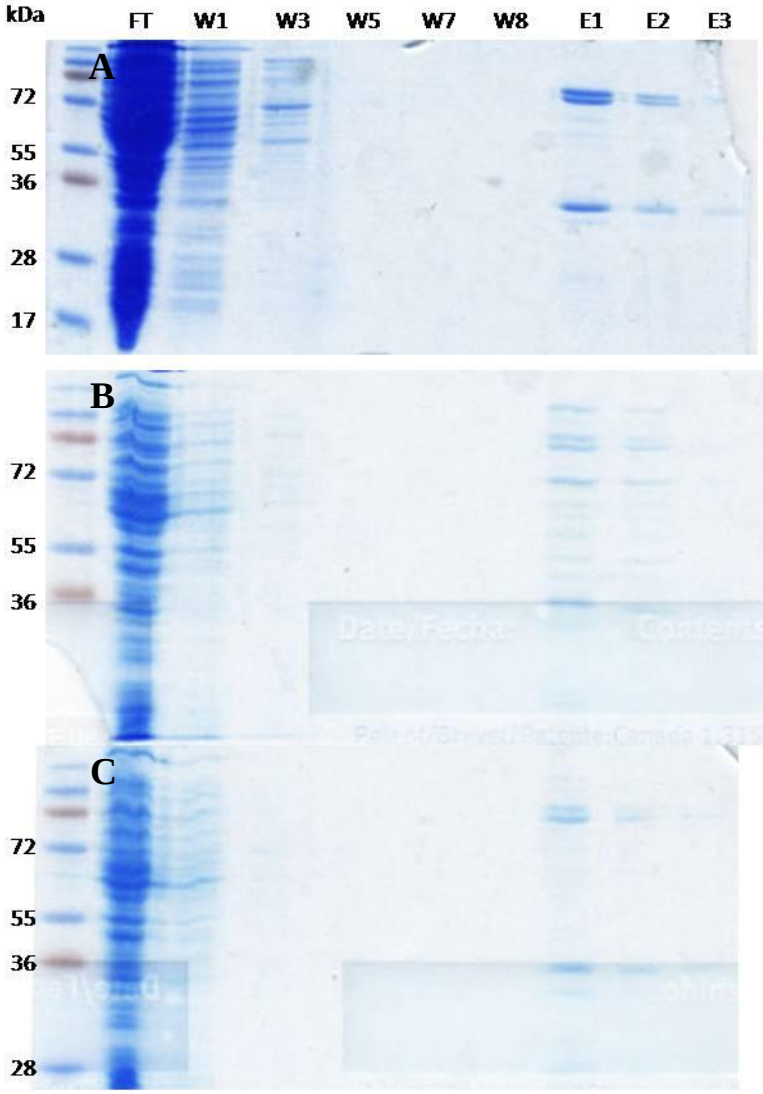
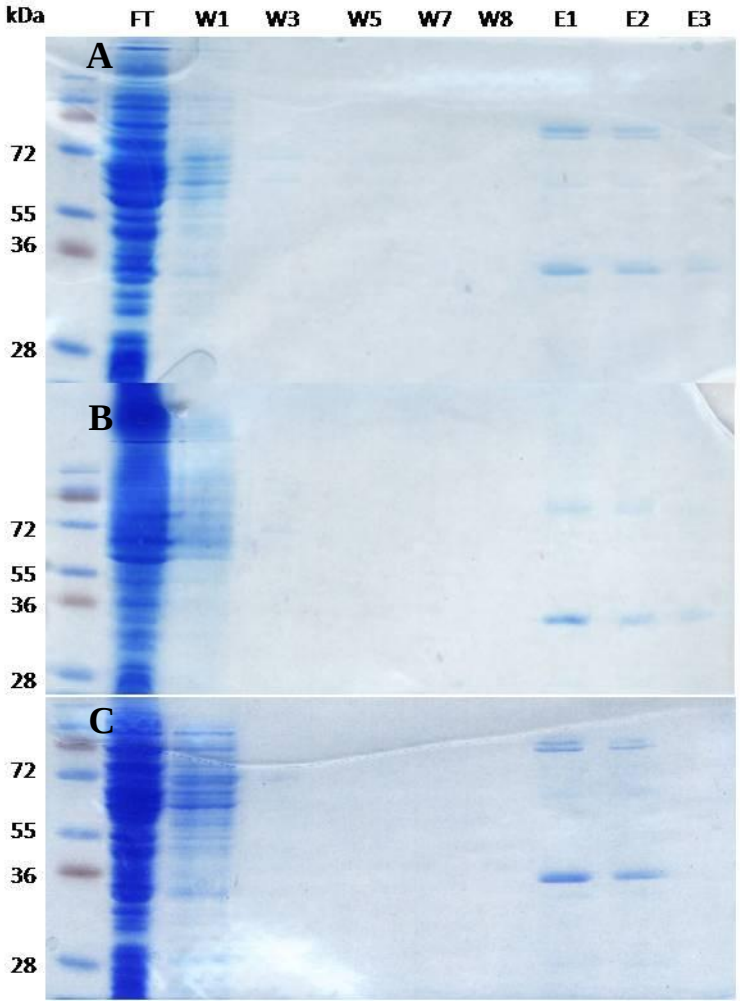

kDa
FT
W1
W3
W5
W7
W8
E1
E2
E3
A
72
55
36
28
17
rCK-2
~30kDa
B
72
55
36
rCK-2
~30kDa
C
72
55
36
28
rCK-2
~30kDa

Figure 3.3: Optimization of temperature for the production of recombinant CK-2. 500mL cultures of BL21(DE3) pLysS pRSETA-CK-2 in 1L Erlenmeyer flasks were grown at 37°C until an optical density of 0.53 at 600nm was reached. Cultures were then induced with IPTG and incubated at 23°C, 30°C or 37°C for an additional 5hrs, then harvested and stored. Cell pellets were resuspended in PBS and lysed by six freeze-thaw cycles. The His-tagged recombinant CK-2 was isolated by nickel column chromatography. Samples from each stage of the purification were run on 12% SDS-PAGE gels to ensure purity. Elution fractions that were seen to contain amounts of purified protein were analysed by the Bradford Assay to determine their protein concentrations. **(A)** Two cultures incubated at 23°C cumulatively produced 118µg/mL of purified protein. **(B)** A concentration of 93µg/mL of purified protein was attained from the first elution fraction of two cultures that were incubated at 30°C. **(C)** The highest concentration of protein was produced by two cultures that were incubated at 37°C, which gave 141µg/mL. FT = flow-through, W = Wash, E = Elution.


kDa
FT
W1
W3
W5
W7
W8
E1
E2
E3
A
72
55
36
28
rCK-2
~30kDa
B
72
55
36
28
rCK-2
~30kDa
C
72
55
36
28
rCK-2
~30kDa

3.2 Purification of Native Recombinant CK-2

The first step in protein isolation is to lyse the cells and so various methods of lysing BL21(DE3)pLysS pRSETA-rCK-2 were assessed in order to determine which method allowed for the cleanest purification as well as the highest yield. Methods of cell lysis that were tested included incubation with lysozyme followed by vortexing or sonication, incubation with urea in conjunction with either vortexing or sonication, incubation with guanidine-HCl followed by vortexing, and a method of six freeze-thaw cycles in dry ice and a 42°C water bath (Figure 3.4). It was found that the cleanest elutions were produced when cell pellets were resuspended in PBS and subjected to the freeze-thaw treatment.

3.3 Confirmation of the Presence of rCK-2

In order to ensure that the recombinant protein that has been produced was in fact recombinant CK-2, a Western blot using specific antibodies against CK-2 was performed (Figure 3.5). Samples from each fraction of the rCK-2 purification protocol were run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The blot was probed with rabbit anti-rainbow trout rCK-2 that had been produced previously. The antibody has previously been confirmed to be specific to rainbow trout recombinant CK-2 as it is able to block the action of rCK-2 in chemotaxis assays (Eshaque et al., unpublished). The secondary antibody was conjugated to alkaline phosphatase which reacted with 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt to produce a coloured product. It was shown that the band of interest in the Coomassie-stained gels was indeed recombinant CK-2. In addition, none of the desired product was being lost in the flow-through or wash fractions of the purification.

Figure 3.4: Determination of the optimal method of lysing cells to obtain the cleanest elution in rCK-2 purification. 25mL cultures of recombinant CK-2 producing *E.coli* were grown to an optical density at 600nm of 0.5. They were induced with IPTG and grown for 3hrs then harvested and stored at -80°C. The cell pellets were thawed on ice for 15 mins, then tested for the best method of lysis. Six different methods were assessed to determine which would retain the most rCK-2 in the cleanest fractions. **(A)** Lysis by lysozyme in conjunction with vortexing provided clean elutions but very little of the protein of interest. **(B)** A thick band was seen at the expected molecular weight of rCK-2 along with many other undetermined bands in the elution samples when cells were treated with lysozyme and sonication. In addition, it appeared that recombinant CK-2 was being lost in the washes. Interestingly, a band at the expected molecular weight was only seen in the third elution when cells were treated with urea and either **(C)** vortexing or **(D)** sonication. However in both cases, elutions were not as clean as desired. **(E)** The treatment of guanidine hydrochloride produced the least amount protein. **(F)** The cleanest elution with a considerable band of interest was produced by the cell pellet that had been resuspended in PBS then put through six freeze-thaw cycles on dry ice and in a 42°C water bath. FT = flow-through, W = Wash, E = Elution.

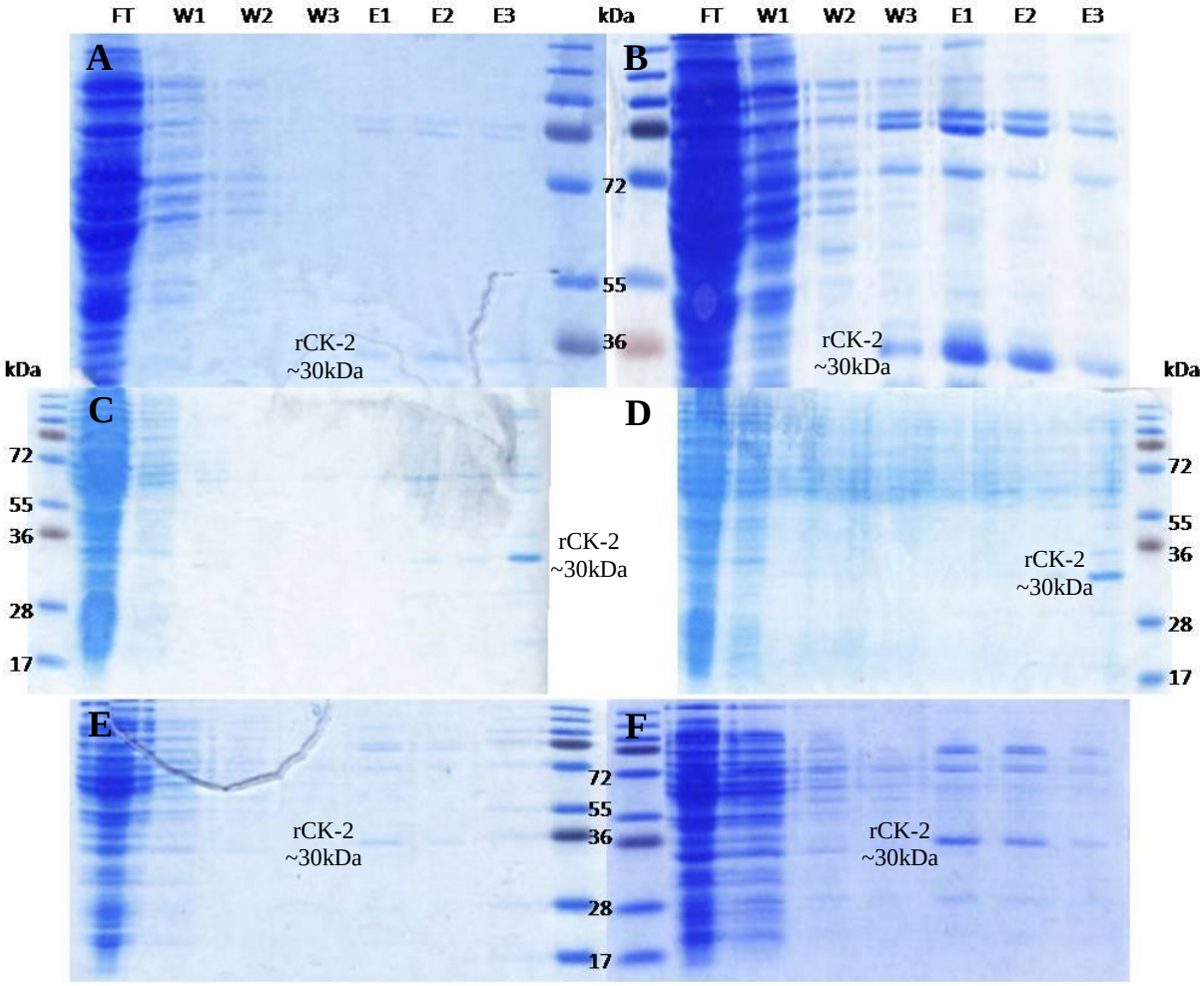
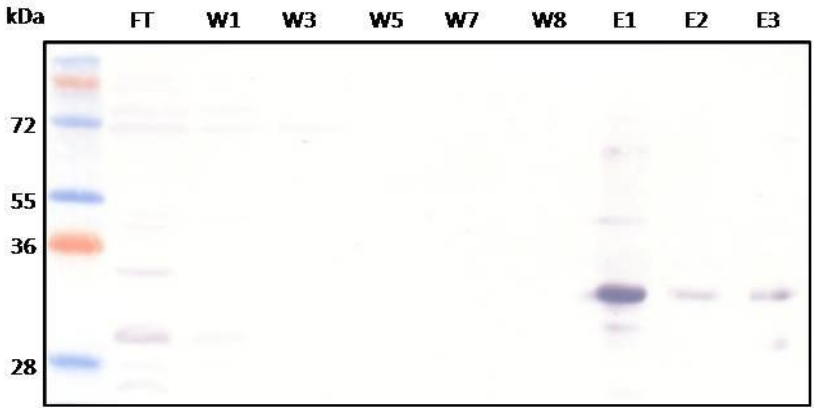

FT
W1
W2
W3
E1
E2
E3
kDa
A
B
C
D
E
F
72
55
36
28
17
rCK-2
~30kDa

Figure 3.5: Verification of rCK-2 production by a Western blot probed with specific antibodies made against rCK-2. Samples were taken from each stage of the purification protocol and run on a 12% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane. The membrane was blocked with fetal bovine serum and probed with a 1:3000 dilution of rabbit anti-rainbow trout CK-2. It was then incubated with a 1:10000 dilution of alkaline phosphatase-conjugated mouse anti-rabbit IgG. rCK-2 was detected by the coloured reaction of alkaline phosphatase with BCIP. FT = flow-through, W = Wash, E = Elution.

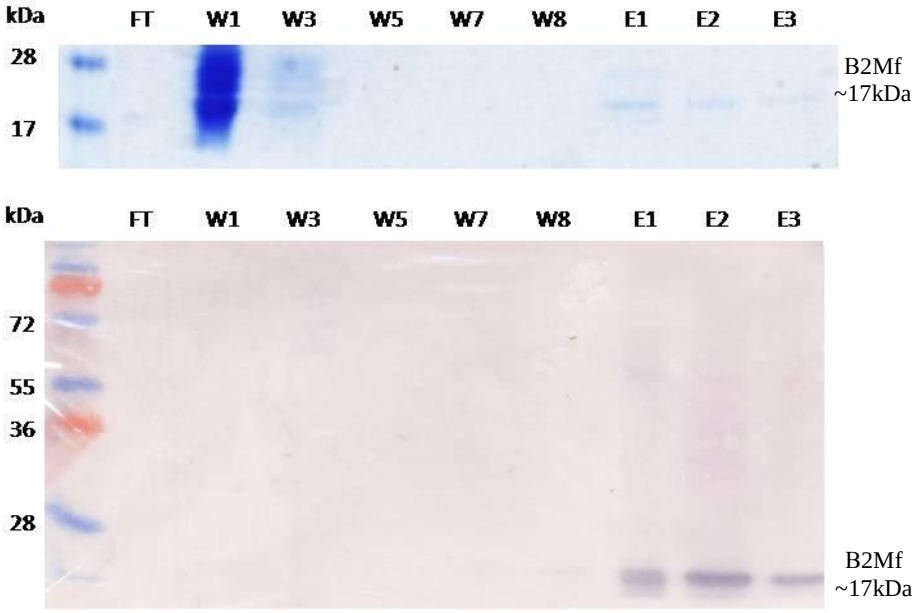


rCK-2
~30kDa

3.4 Production of Recombinant β2M fragment

Recombinant β2Mf was made to use as a negative control in the chemotaxis assay. This was to control for the presence of chemotactic agents that may occur in the recombinant protein production and purification protocols, since β2M has no inherent chemotactic ability (Eshaque et al., Unpublished). BL21(DE3)pLysS *E.coli* was transformed with pRSET A expression vector carrying the partial coding sequence of β2M. Cultures of this strain were grown to an optical density at 600nm of 0.47. They were then induced to a final concentration of 1mM of IPTG and incubated for 5hrs in a 37°C shaking water bath. Bacterial cells were harvested then lysed through six freeze-thaw cycles, as described previously. rβ2Mf was isolated from the lysate by nickel column chromatography via its 6x His-tag. To confirm the presence of rβ2Mf in the elutions, samples from each step in the purification were run on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. rβ2Mf was detected in all three elutions on the Western blot by rabbit anti-rainbow trout β2Mf (Figure 3.6B). In addition, a similar gel that was stained with Coomassie Blue, showed that the elutions were fairly clean (Figure 3.6A).

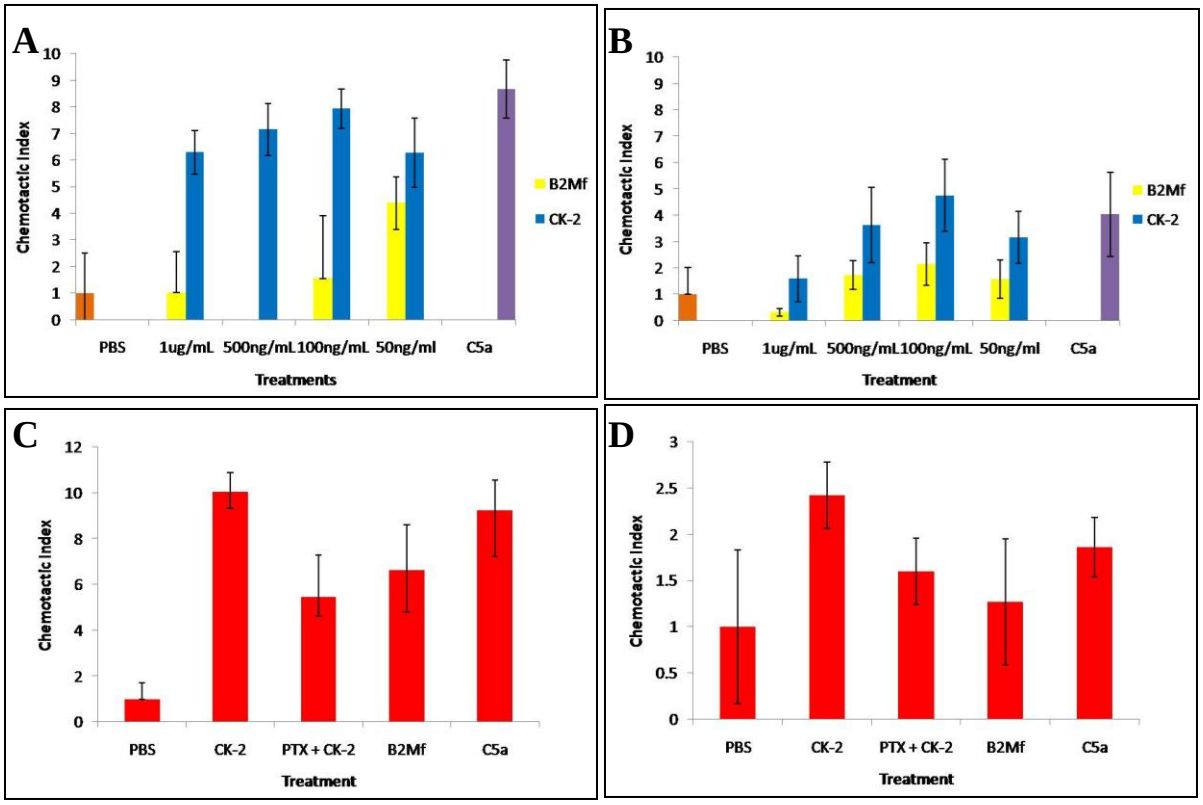
Figure 3.6: Confirmation of recombinant β2M fragment production and purification. Two 500mL cultures of BL21(DE3)pLysS transformed with pRSETA-β2Mf were grown to an optical density at 600nm of 0.47. Cultures were induced with IPTG to a final concentration of 1mM, and incubated at 37°C for 5hrs. Cells were harvested and stored at -80°C. Cell pellets were lysed by six freeze-thaw cycles and recombinant protein was isolated from the lysate via its 6x His-tag, using nickel column chromatography. Samples from each stage of the purification were run on 12% SDS-PAGE gels and either **(A)** stained with Coomassie Blue or **(B)** transferred to a nitrocellulose membrane. The blot was blocked with fetal bovine serum and probed with rabbit anti-rainbow trout β2Mf. It was then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase, which reacted with BCIP and NBT to form a coloured product indicating the presence of rβ2Mf. FT = flow-through, W = Wash, E = Elution.


kDa
FT
W1
W3
W5
W7
W8
E1
E2
E3
28
17
B2Mf
~17kDa
kDa
FT
W1
W3
W5
W7
W8
E1
E2
E3
72
55
36
28
B2Mf
~17kDa

3.5 Determination of the functionality of recombinant CK-2 purified under native conditions

Chemotaxis assays were conducted in order to determine whether the recombinant CK-2 that was produced and purified under native conditions was functional (Figure 3.7). The rainbow trout spleen cell line, RTS-11, is known to respond to CK-2 and was used to test the chemotactic ability of the recombinant protein (Eshaque et al., unpublished). The chemotactic index of rCK-2 was determined by examining the ratio of the number of cells that migrated across a membrane with 5μm pores in response to the chemokine, to the number of cells that migrated towards a negative control. There was a significant increase in the migration of RTS-11 cells when treated with rCK-2. Its chemotactic index peaked at 7.9 when exposed to 500ng/mL of rCK-2 (Figure 3.7A). This was significantly different from the chemotactic indices of the two negative controls, PBS and recombinant β2M fragment. rβ2Mf was used as a negative control to ensure that chemotaxis was not induced by any element used in the recombinant protein production or purification protocols. rCK-2 was also significantly chemotactic towards RTS-11 at the other concentrations of 1000ng/mL, 100ng/mL and 50ng/mL with chemotactic indices of 6.49, 7.18, and 6.76, respectively. Interestingly, rCK-2 was not as chemotactic towards isolated rainbow trout peripheral blood leukocytes (Figure 3.7B). However, it was still able to attract significantly more cells across the synthetic membrane than the negative controls. The recombinant chemokine had a maximum chemotactic index of 4.75 when used at a concentration of 100ng/mL. In order to reinforce the hypothesis that cells were migrating due to the chemokine, RTS-11 was blocked with pertussis toxin (PTX) in order to inhibit their G-protein coupled receptors. As expected, these cells had a significantly lower migration rate towards rCK-2 than cells that had not been treated with the toxin (Figure 3.7C). In fact, there was a 43% decrease in movement across the membrane. A similar effect was seen when PBLs were inhibited by PTX (Figure 3.7D).

Figure 3.7: Confirmation of the functionality of the recombinant CK-2 purified under native conditions. Chemotaxis assays were conducted in order to determine whether or not our rCK-2 was chemotactic. We assessed how effective it was in attracting rainbow trout leukocytes and inducing them to migrate across a membrane. This was measured by a chemotactic index which is ratio of the number of cells that migrate towards a chemoattractant to the number of cells that migrate towards a negative control. **(A)** When used as the chemoattractant in a chemotaxis assay with RTS-11 cells, rCK-2 had a high chemotactic index in comparison to PBS ($p < 5x10^{-6}$) as well as the recombinant β2M fragment ($p < 10^{-6}$) (n=9). In addition, there was no significant difference in chemotactic index between the positive control, C5a, and rCK-2 at 100ng/mL and 500ng/mL (n=9). **(B)** The chemoattractant properties of rCK-2 were also tested against rainbow trout PBLs. In this case, its chemotactic index at concentrations of 500, 100 and 50ng/mL was also significantly higher than that of PBS ($p < 5x10^{-4}$) and β2M fragment ($p < 5x10^{-4}$) (n=9). Conversely, there was no significant difference in chemotactic index at concentrations of 500, 100 and 50ng/mL of rCK-2 and the positive control ($p > 0.1$). **(C)** RTS-11 that had been blocked with pertussis toxin to a final concentration of 100ng/mL, showed significantly less migration towards rCK-2 than untreated cells ($p = 2.3x\ 10^{-12}$). **(D)** PTX-treated rainbow trout PBLs also migrated significantly less than untreated cells ($p = 0.0001$).


A
Chemotactic Index
10
9
8
7
6
5
4
3
2
1
0
PBS
1ug/mL
500ng/mL
100ng/mL
50ng/ml
C5a
Treatments
B2Mf
CK-2
B
Chemotactic Index
Treatment
C
Chemotactic Index
12
10
8
6
4
2
0
PBS
CK-2
PTX + CK-2
B2Mf
C5a
Treatment
D
Chemotactic Index
3
2.5
2
1.5
1
0.5
0
Treatment

3.6 *In vitro* Expression Study

In order to determine the function of CK-2 in the immune system, its effects on the expression of immune-related genes in RTS-11 were examined. Cells were exposed to native recombinant CK-2 in time course and dose response experiments. A final exposure concentration of 100ng/mL was chosen for the time course and dose response samples were incubated for 2rhs. Total RNA was extracted from each time course and dose response sample and used to synthesize cDNA. Polymerase chain reaction was then conducted on each cDNA sample to amplify various immune-related genes. Gels for each gene were run with their PCR products amplified from cDNA of all time course and dose response samples, as well as products from the PCR amplification of the internal control, 28s rRNA (Figures 3.8, 3.11, and 3.14). Band intensities were measured by densitometry and used to calculate relative expression levels by taking the ratio of gene band density to the band density of 28s for the same time or dose sample. The relative expression levels were used to construct time course and dose response curves. These allowed us to examine how each gene was affected by rCK-2.

The expression of pro-inflammatory genes IL-1β, and TNFα showed 33.3% and 23.6% increases, respectively, after 90 mins of exposure to rCK-2 (Figure 3.9). The relative expression level of IL-8 in response to rCK-2 increased 27.3% after 105 mins of exposure (Fiugre 3.9). There was a significant 64.5% increase in IL-1β transcript level and a 12.6% increase in TNFα expression when cells were exposed to 1000ng/mL of recombinant protein (Figure 3.10A and B). However, IL-8 expression decreased by 45% when treated with 5-500ng/mL of CK-2. Interestingly, expression of IL-8 returned to basal level at 1000ng/mL (Figure 3.10C).

Firstly, the expression levels of MHIα, IFN1 and IFN2 were examined in order to determine the role of CK-2 in the immune response against intracellular pathogens. Increases in the transcript levels of all three genes were seen after 105 mins of exposure (Figure 3.12). The 26.9 and 31.5% increases in MHIα and IFN2 expression were significant at the 0.01 level. IFN1 increased by 32%, which was significant at the 0.05 level. There seemed to be no

statistically significant dose response effect of CK-2 on MHIα but expression levels of the interferons were seen to decrease at higher concentrations of the chemokine (Figure 3.13). IFN2 only decreased by 11.5% at concentrations of 10-1000 ng/mL but IFN1 expression decreased by 47.4% at 5-500ng/mL and returned to basal levels at 1000ng/mL of rCK-2.

The effect of CK-2 on the immune response to extracellular pathogens was also assessed, by looking at the time courses and dose responses of MHIIα, S25-7 and CD4 expression. Transcription of MHIIα and CD4 increased by 36.6 and 25.8% respectively, after 90 min of incubation and were both significant at the 0.01 level (Figure 3.15A and C). Interestingly, rCK-2 did not appear to have a significant effect on S25-7 (Figure 3.15B). However, there was no significant dose response effect on any of the three genes (Figure 3.16).

Since RTS-11 is a heterogeneous cell line consisting of both monocyte-like and macrophage-like cells, it was important to determine if the effects on immune-related gene expression was being seen in one or the other, or both cell populations. A separate flask of RTS-11 that was seeded at the same time from the same culture as the samples used in the time course and dose response experiments, were sorted by flow cytometry. Cell sorting was conducted based on size and granularity since the monocyte-like cells are small and non-granular while the macrophage-like cells are larger and contain granules. It was seen that the cell population of RTS-11 used in the expression experiments mostly consisted of monocyte-like cells (Figure 3.17).

Figure 3.8: RTS-11 expression profiles of pro-inflammatory genes in response to rCK-2. 10^6 cells were seeded in 6mL of L-15 media with 10% FBS and 1% penicillin/streptomycin and allowed to equilibrate at 20°C overnight. Each flask of cells in the time course was treated with 100ng/mL of rCK-2 then incubated at 20°C for varying times, while the dose response samples were incubated for 120 mins with the indicated doses of recombinant protein. Total RNA was collected from each flask of cells and converted to cDNA. Time point and dose cDNA samples were used as template DNA for PCR amplification of pro-inflammatory genes IL-1β, TNFα and IL-8, as well as an internal control gene, 28s rRNA. Products for all **(A)** time course and **(B)** dose response samples of a gene were run on a 1.5% agarose gel stained with GelRed, in 0.5X TBE for 55mins at 35V, along with the products of 28s. Band densities of each sample were measured by densitometry and compared to their corresponding control band to give relative expression levels.

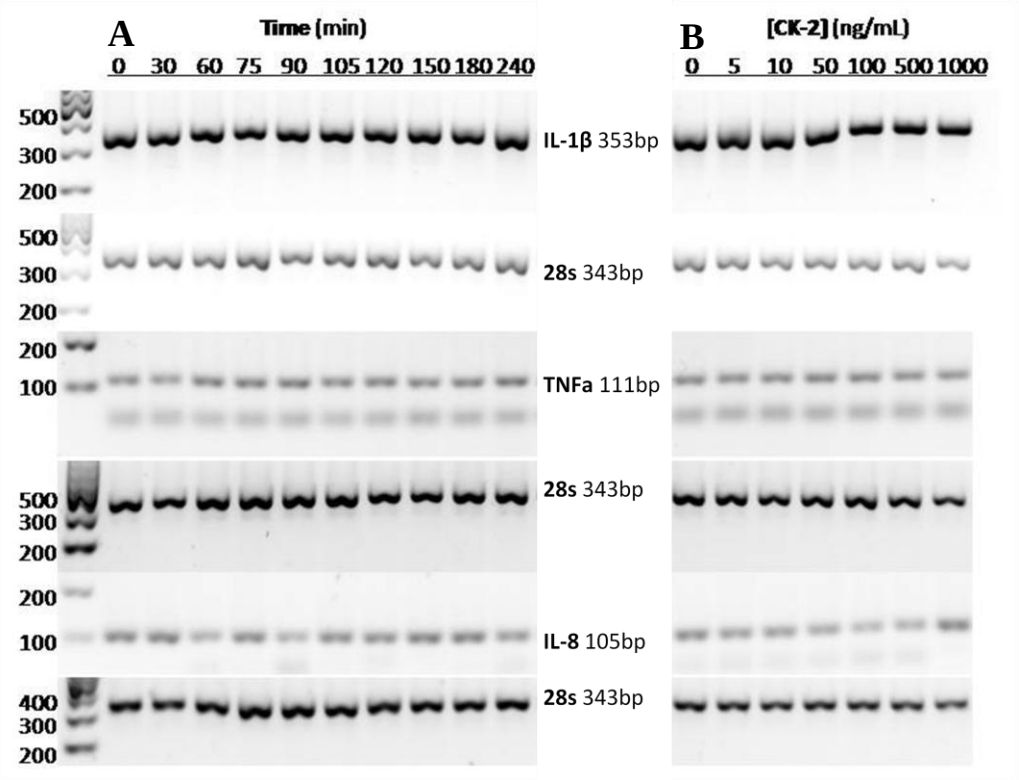

A
Time (min)
0 30 60 75 90 105 120 150 180 240
B
[CK-2] (ng/mL)
0 5 10 50 100 500 1000
500
300
200
IL-1β 353bp
28s 343bp
200
100
TNFa 111bp
28s 343bp
IL-8 105bp
400
28s 343bp

Figure 3.9: Time course of relative pro-inflammatory gene expression in RTS-11 treated with recombinant CK-2. The relative expression levels of IL-1β, TNFα and IL-8 over 240 mins from two trials were averaged. Error bars represent the standard deviation between trials. A trendline (red) was constructed from moving averages calculated from two time points. Based on the trendline, samples with baseline expression versus induced expression levels could be clearly distinguished (purple boxes). The average expression levels of each category (yellow) were taken in order to ascertain the degree to which expression was affected by rCK-2. Student's two-sample t-tests were used to determine significant differences in expression levels. **(A)** Relative IL-1β expression time course showed a significant increase after 90 mins of exposure to rCK-2 ($p=0.0023$). **(B)** TNFα expression relative to 28s, increased significantly after 90 mins ($p=0.000921$). **(C)** A Student's t-test showed that 105 mins of treatment with rCK-2 was sufficient to cause a significant increase in relative IL-8 expression in RTS-11 ($p=0.0055$).

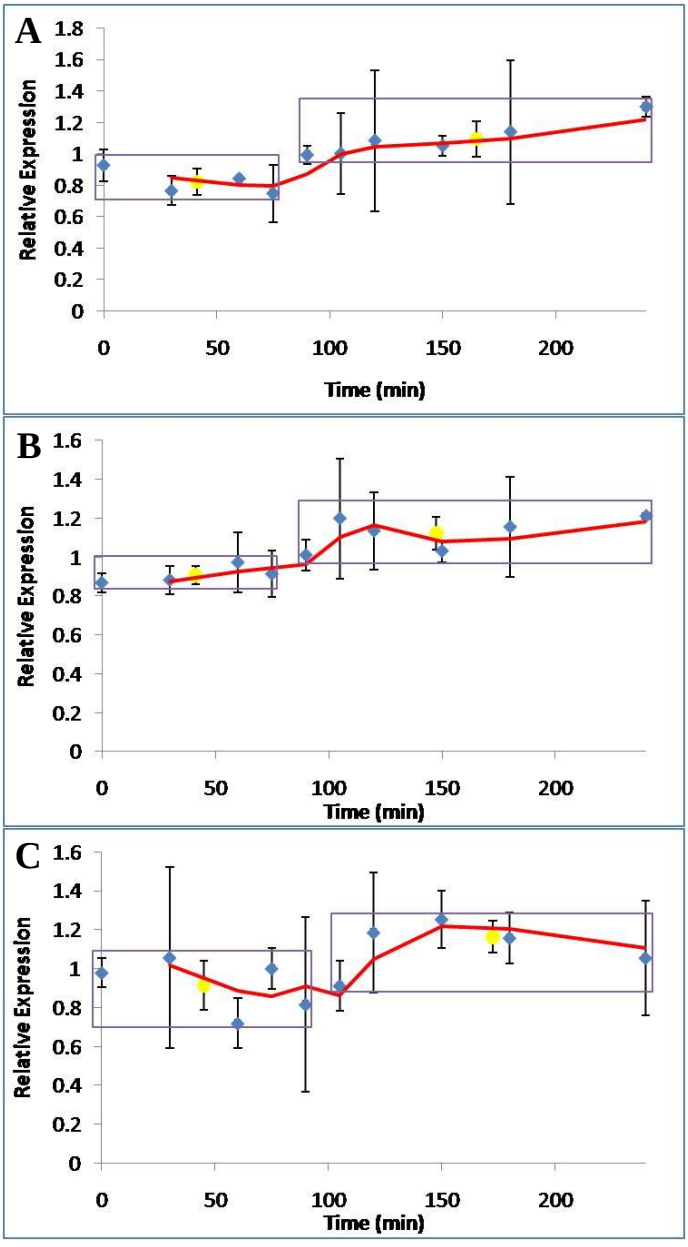

A
Relative Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (min)
B
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (min)
C
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (min)

Figure 3.10: Dose response of pro-inflammatory genes to rCK-2 in RTS-11. The average relative expression levels IL-1β, TNFα and IL-8 in response to 0, 5, 10, 50, 100, 500 and 1000 ng/mL of recombinant chemokine are shown (blue), along with standard deviations as error bars (n=2). Trendlines (red) were constructed from moving averages between two doses and were used to determine baseline and induced expression (purple). The average baseline and induced expression levels (yellow) were used to assess how pro-inflammatory gene expression was affected by rCK-2. One sample t-tests were used to detect significant changes. **(A)** Relative IL-1β expression at various doses of recombinant CK-2, which was seen to increase significantly at 1000ng/mL of CK-2 ($p=0.00066$). **(B)** The small increase in TNFα expression level in response to 1000ng/mL of rCK-2 was significant at the 0.05 level ($p=0.016$). **(C)** IL-8 expression relative to 28s decreased significantly at doses of rCK-2 of 5-500ng/mL ($p= 0.0044$).

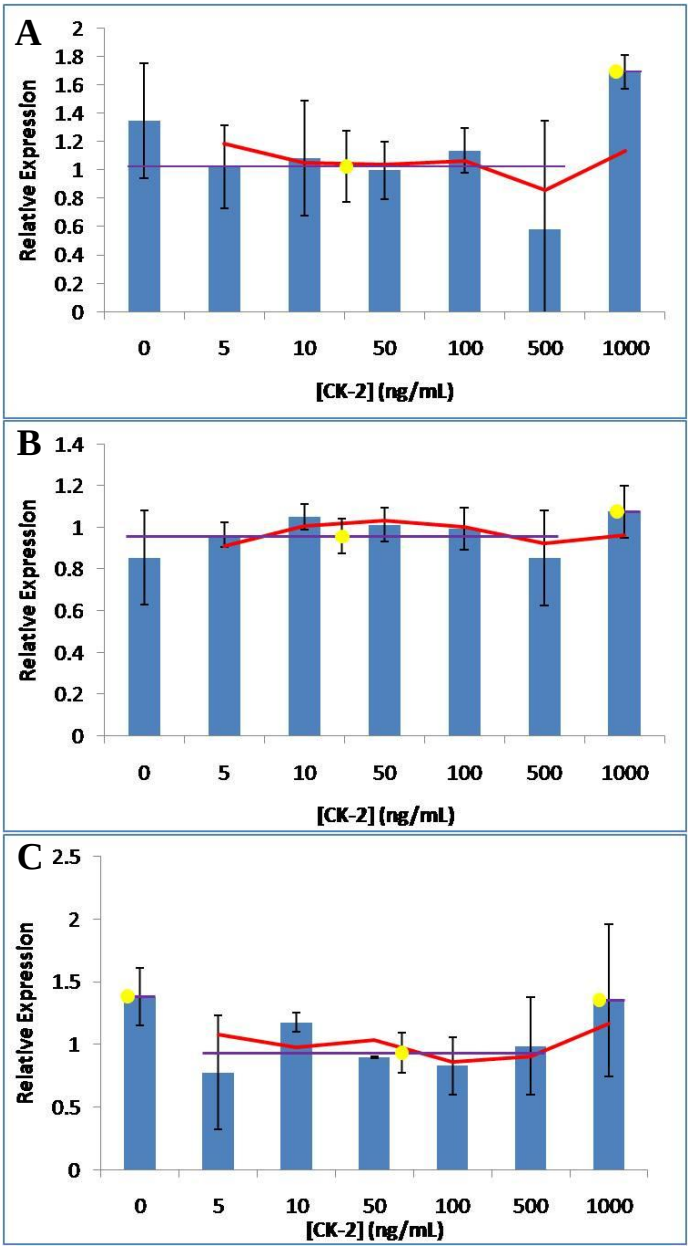

A
Relative Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)
B
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)
C
Relative Expression
2.5
2
1.5
1
0.5
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)

Figure 3.11: Expression profiles of intracellular pathogen immune response genes in RTS-11 treated with rCK-2. Ten million cells were seeded at a cell density of 1.67 million cells per mL in L-15 media enhanced with 10% FBS and 1% penicillin/streptomycin for each sample. Cells were allowed to settle overnight in a 20°C incubator. Time course samples were treated with 100ng/mL of rCK-2 then incubated at 20°C for varying times, while the dose response samples were treated with the indicated doses of protein and incubated for 120 mins. cDNA was synthesized from 500ng of total RNA that was harvested from each sample, which served as template for PCR amplification of MHIα, IFN1 and IFN2 and the internal control, 28s. PCR products of all samples from **(A)** the time course and **(B)** the dose response were run on a GelRed-stained 1.5% agarose gel in 0.5X TBE at 34V for 55 mins for each gene, along with those of the internal control. Densitometry analysis was performed for each band. Relative gene expression levels were determined by comparing the band densities of each sample for each gene to their 28s counterpart.

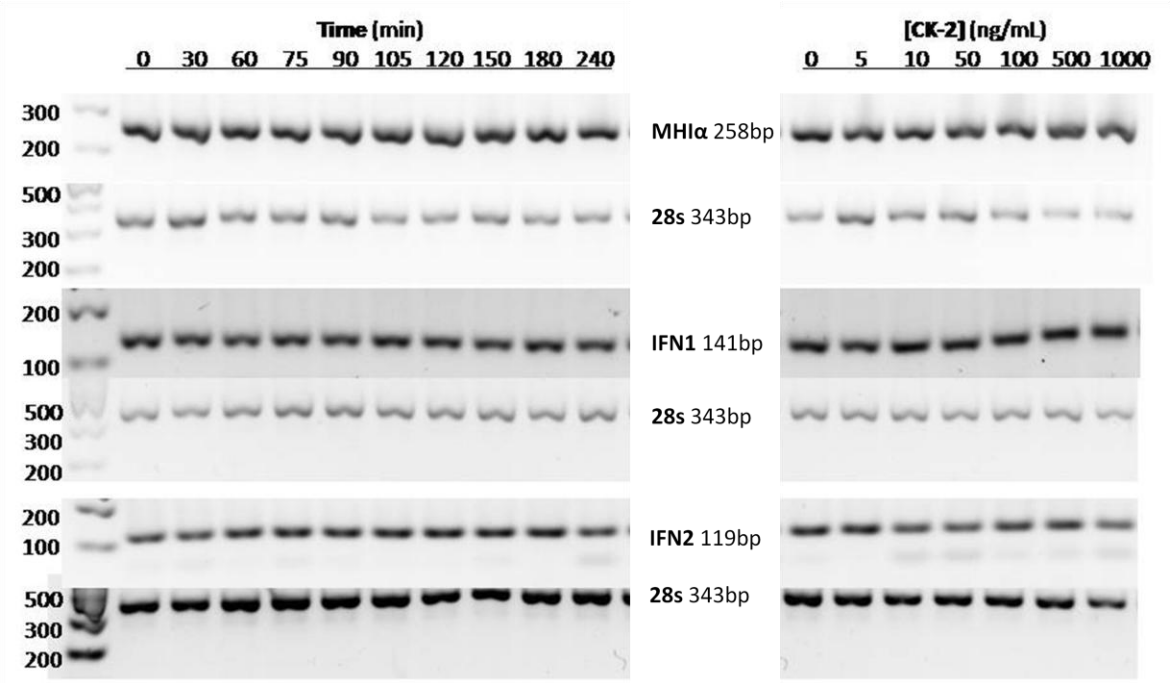

Time (min)
0 30 60 75 90 105 120 150 180 240
[CK-2] (ng/mL)
0 5 10 50 100 500 1000
300
200
MHIα 258bp
500
300
200
28s 343bp
200
100
IFN1 141bp
500
300
200
28s 343bp
200
100
IFN2 119bp
500
300
200
28s 343bp

Figure 3.12: Time course of relative expression of immune-related genes associated with intracellular pathogens in RTS-11 treated with rCK-2. The ratios of the band densities of MHIα, IFN1 and IFN2 to 28s for each sample were averaged between two trials and plotted with standard deviations. Moving averages using two time points were used to construct a trendline (red). From this, baseline and induced expression levels could be distinguished (purple boxes) and averaged in order to determine the degree to which rCK-2 affected gene expression (yellow). **(A)** The Student's two-sample t-test showed that there was a significant increase in MHIα expression after being treated with rCK-2 for 105 mins ($p=0.0044$). **(B)** The increase in IFN1 at 105 mins of rCK-2 exposure was significant at the 0.05 level ($p=0.012$). **(C)** IFN2 transcript level showed a significant increase after 90 mins of incubation with the recombinant protein ($p=0.0028$).

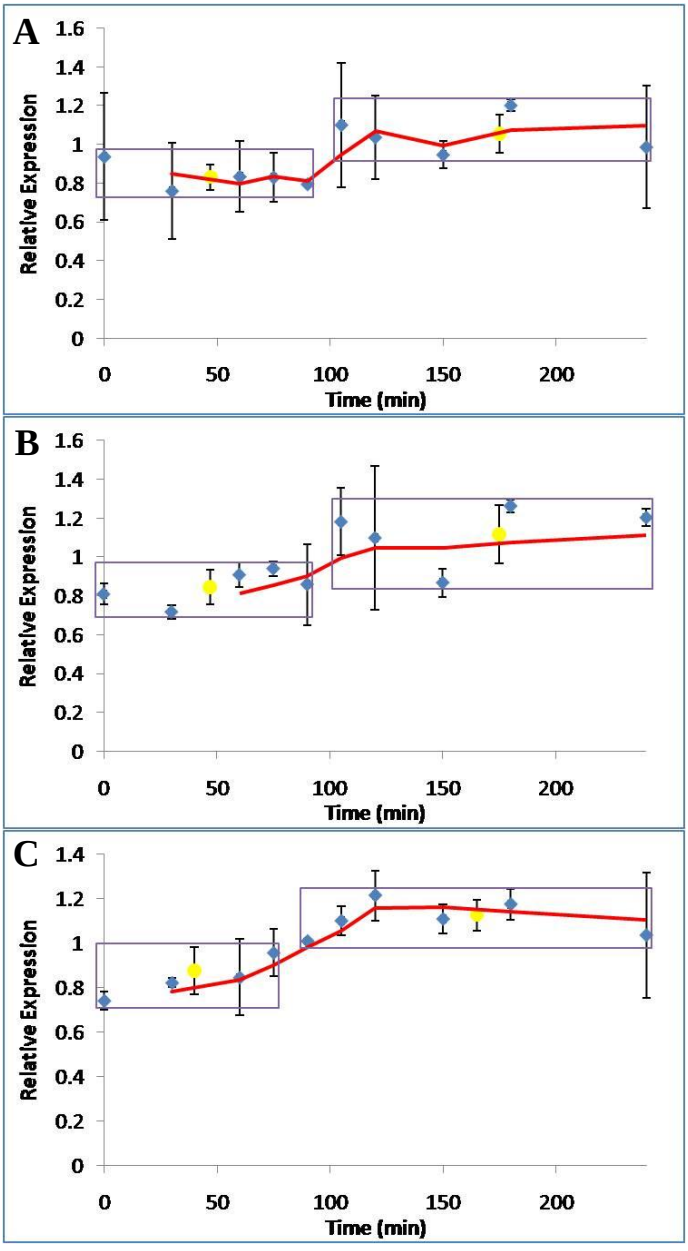

A
B
C
Relative Expression
Time (min)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
50
100
150
200

Figure 3.13: Dose-response expression of intracellular pathogen immune response-related genes in RTS-11 exposed to rCK-2. The relative expression levels of MHIα, IFN1 and IFN2 in RTS-11 treated with increasing doses of recombinant CK-2 from two trials were averaged. Error bars represent standard deviations. A trendline was constructed based on moving averages between two samples (red) and from this, baseline and induced expression levels were determined (purple). **(A)** A Student's two-sample t-test showed that the apparent increase in relative MHIα expression level was not significant ($p=0.12$). **(B)** IFN1 was seen to decrease significantly when treated with doses of 5-500ng/mL of rCK-2 ($p=0.0011$). **(C)** IFN2 also showed a slight decrease in expression that was significant with doses of rCK-2 of at least 10ng/mL ($p=0.026$).

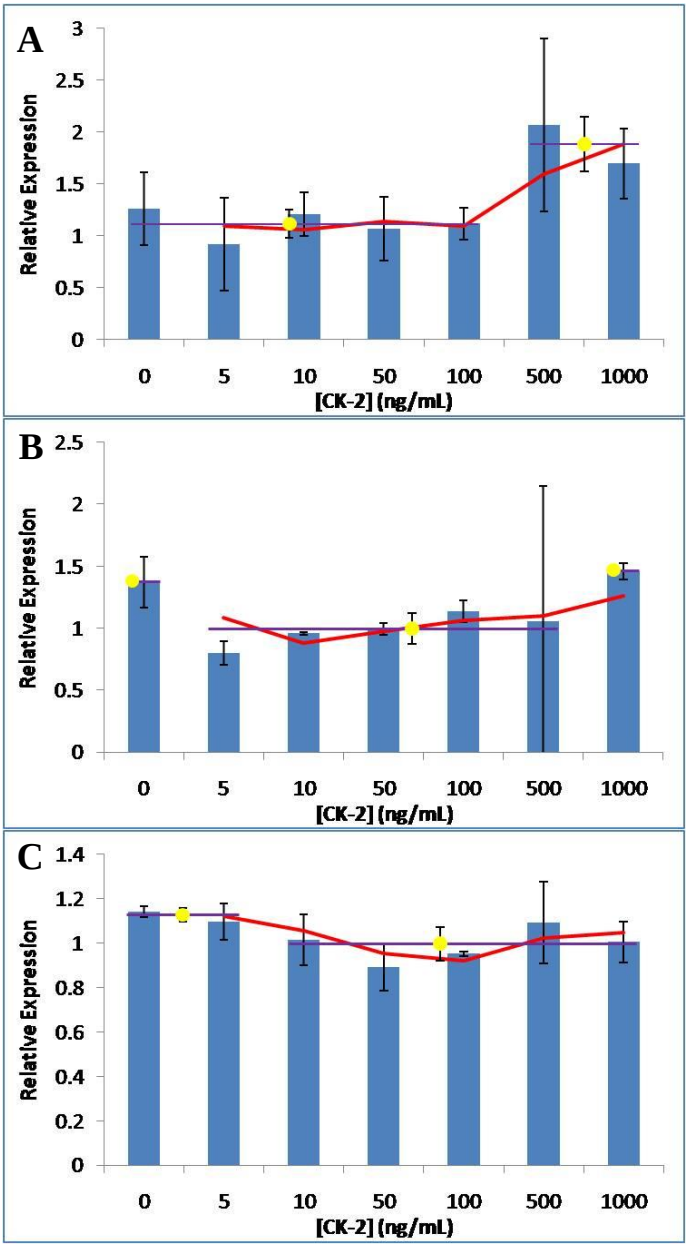

A
Relative Expression
3
2.5
2
1.5
1
0.5
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)
B
Relative Expression
2.5
2
1.5
1
0.5
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)
C
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)

Figure 3.14: Extracellular pathogen immune-response related gene expression profile of RTS-11 exposed to rCK-2. RTS-11 cells were seeded in L-15 media with 10% FBS and 1% penicillin/streptomycin at a cell density of 1.67 million cells/mL and allowed to rest overnight at 20°C. Each flask contained 10^6 cells and was treated with 100ng/mL of rCK-2 for each time point in the time course and for 120 mins with each dose in the dose response. Total RNA was collected from each sample and 500ng of each RNA sample was converted into cDNA. Amplification of MHIIα, S25-7 and CD4 were performed via PCR with each cDNA sample. GelRed stained 1.5% agarose gels were run with the products of these PCR reactions of each gene for each **(A)** time point and **(B)** dose, with the products of 28s amplification. Densitometry analysis was used to quantitate the expression levels of the genes in each sample. Expression levels were compared to the internal control to obtain relative expression profiles of MHIIα, S25-7 and CD4.

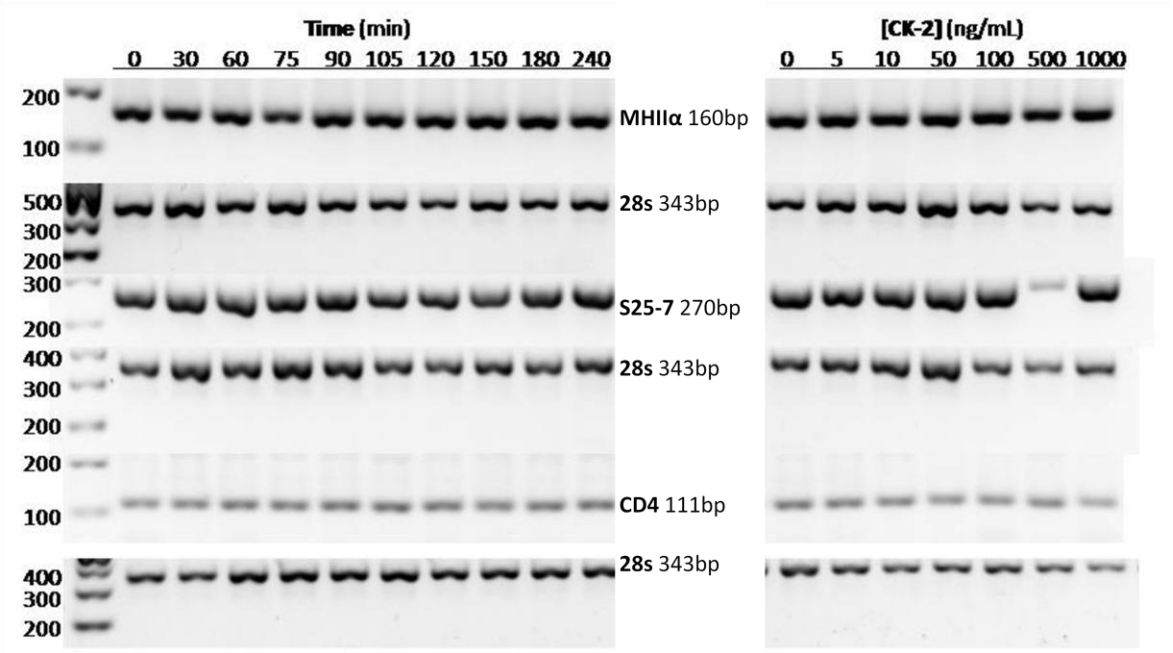

Time (min)
0 30 60 75 90 105 120 150 180 240
[CK-2] (ng/mL)
0 5 10 50 100 500 1000
200
100
500
300
200
300
200
400
300
200
200
100
400
300
200
MHIIα 160bp
28s 343bp
S25-7 270bp
28s 343bp
CD4 111bp
28s 343bp

Figure 3.15: Relative expression time course of genes related to extracellular pathogen immune response in RTS-11 cells dosed with rCK-2. The average expression levels of MHIIα, S25-7 and CD4 relative to 28s expression in RTS-11 exposed to recombinant CK-2 are displayed with standard deviations as error bars (n=2). The average expression level between two time points was used to create a moving average trendline (red). Expression levels were designated as baseline or induced (purple boxes) based on the trendline. Baseline and induced expression levels were averaged (yellow) in order to determine how the immune response to intracellular pathogens was affected by rCK-2. **(A)** A Student's two-sample t-test showed that rCK-2 caused an increase of significance in relative MHIIα expression after 90 mins of exposure ($p=0.0034$). **(B)** There appeared to be no significant change in S25-7 expression over 240 mins of treatment with the recombinant protein ($p=0.082$). **(C)** The increase in relative CD4 expression after 90 mins was significant at the 0.01 level ($p=0.0084$).

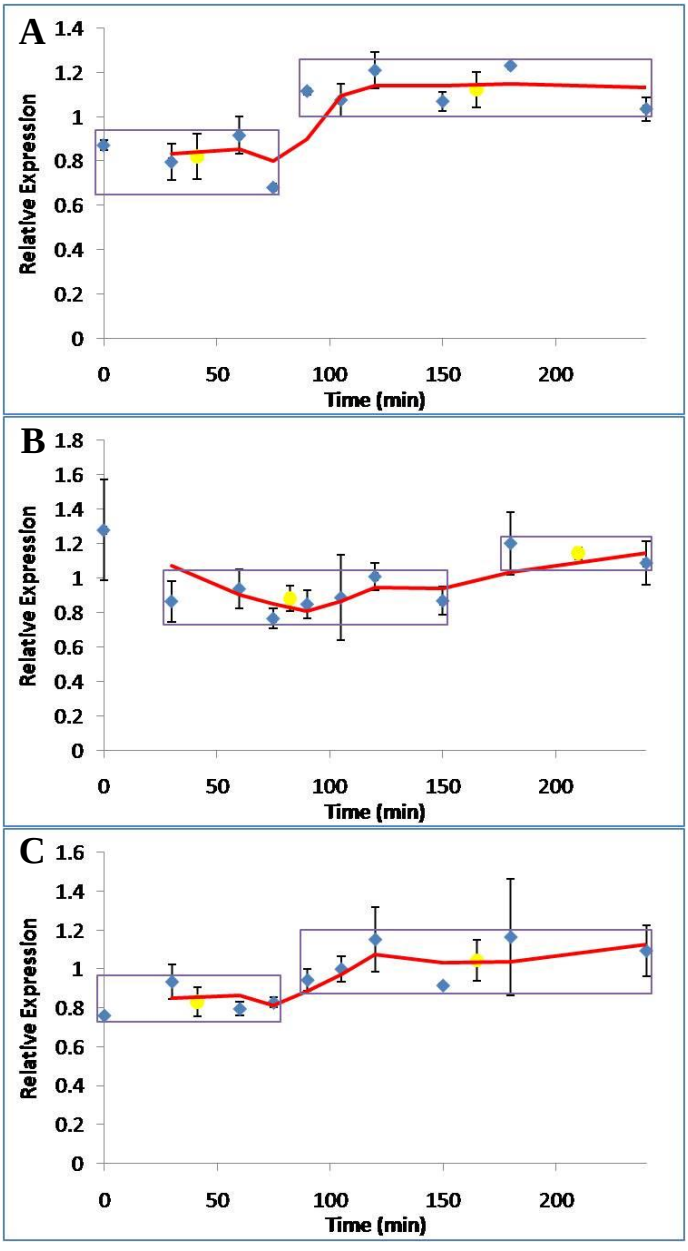

A
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (min)
B
Relative Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (min)
C
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
200
Time (min)

Figure 3.16: Dose response effects of rCK-2 on the relative expression levels of genes involved in the extracellular pathogen immune response in RTS-11. Relative expression levels of MHIIα, S25-7 and CD4 in RTS-11 cells dosed with 0, 5, 10, 50, 100, 500 or 1000ng/mL of recombinant CK-2 for 120 mins. The average expression levels of two trials are depicted with standard deviations as error bars. Moving averages were used to construct a trendline (red) of the dose response, which was used to determine baseline and induced expression levels (purple). The average expression levels of the baseline and induced samples (yellow) were used to examine significant changes using two-sample t-tests. **(A)** MHIIα expression did not show any significant dose response effect in response to rCK-2 ($p=0.23$). **(B)** There were no significant changes in relative S25-7 expression levels in any of the doses tested ($p=0.67$). **(C)** The perceived increase in CD4 transcript at doses of 500 and 1000ng/mL was shown to be insignificant by the two-sample t-test ($p=0.12$).

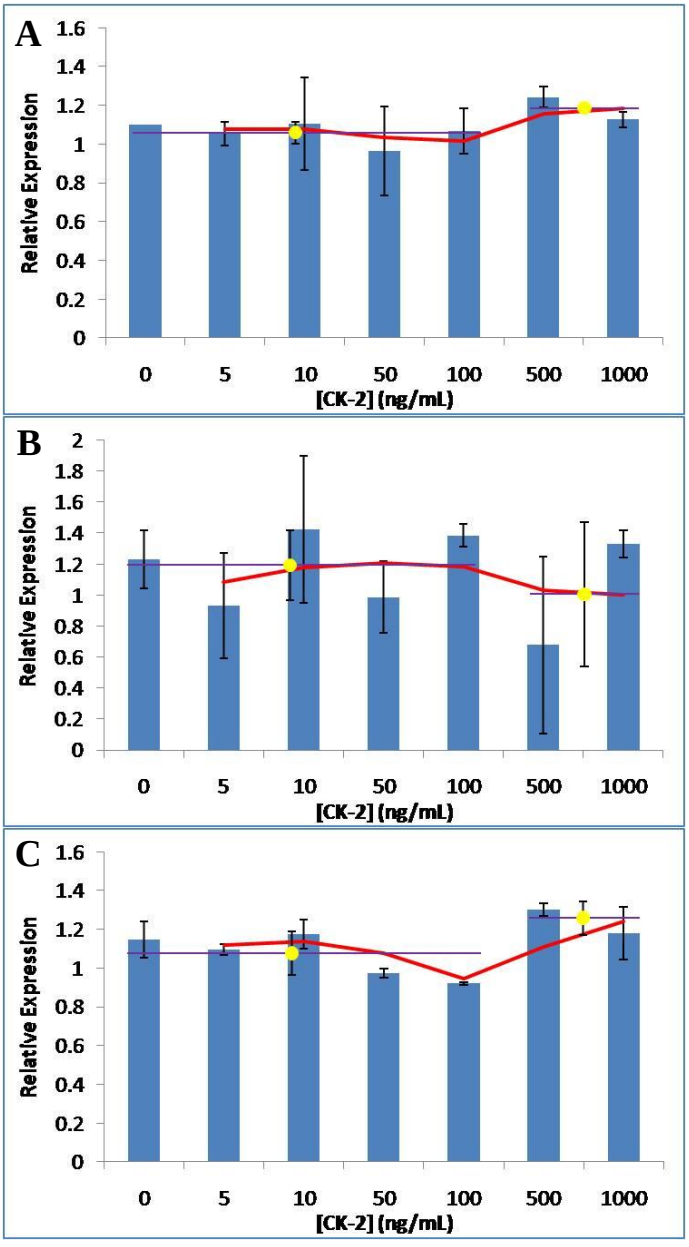
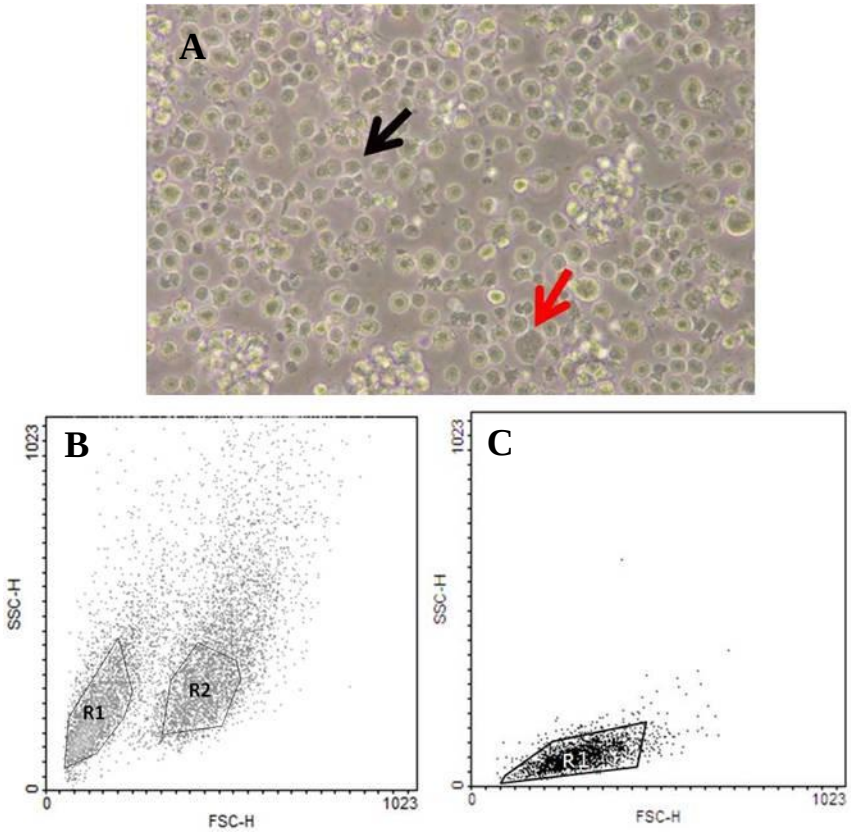

A
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)
B
Relative Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)
C
Relative Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
50
100
500
1000
[CK-2] (ng/mL)

Figure 3.17: Flow cytometry analysis of the heterogeneous cell line, RTS-11. (A) Two distinct cell types can clearly be seen under the microscope in a culture of the rainbow trout spleen cell line, RTS-11. The black arrow points out a typical small, round monocyte-like cell while the red arrow indicates a typical larger, granular macrophage-like cell. **(B)** In addition, flow cytometry can sort the cells based on size and granularity using forward and side scatter parameters. The two cell types are present in equal amounts in this particular culture of cells, which had been passaged three weeks prior to flow cytometry. **(C)** A flask of cells that had been seeded in the same density and at the same time as the time course and dose response samples was harvested and fixed in 70% ethanol. These cells were analyzed by flow cytometry to determine the demographics of the cell population used in the expression experiments.


A
B
1023
SSC-H
0
R1
R2
0
FSC-H
1023
C
1023
SSC-H
0
R1
0
FSC-H
1023

Chapter 4
Discussion

4.1 Functional activity of recombinant CK-2 purified under native conditions

As expected, the chemotaxis assays showed the functional activity of rCK-2 purified under native conditions. In addition, this native protein had a higher chemotactic index at lower concentrations than rCK-2 produced previously but purified under denaturing conditions (Eshaque et al., unpublished). The rCK-2 purified under native conditions had an optimal chemotactic index at a concentration of 100ng/mL when used in the chemotaxis assay to attract RTS-11 cells as well as peripheral blood leukocytes (PBLs) isolated from rainbow trout blood (Figure 3.7A and B). The concentration of 100ng/mL is the typical concentration at which most chemokines are active *in vitro* (Lally et al., 2003). For example, this concentration is also the dose at which cell migration of human peripheral blood mononuclear cells induced by recombinant CCL22 peaks in the chemotaxis assay (Godiska et al., 1997). Cells from the human osteosarcoma-derived cell line (HOS) transfected with the CC chemokine receptor CCR1 were also reported to migrate optimally towards recombinant CCL5 and recombinant CCL15 at the concentration of 100ng/mL (Kim et al., 2005). Interestingly, concentrations of 1ng/mL and 1000ng/mL of CCL3 and CCL16, respectively, were seen to provide optimal conditions for the migration of these cells (Kim et al., 2005). However, a study by Durig et al., showed that the optimal concentration of CCL3 was 150ng/mL for the induction of calcium mobilization indicating chemokine stimulation in human peripheral blood mononuclear cells (Durig et al., 1999). The discrepancy in optimal concentrations of CCL3 for leukocyte stimulation may be due to the expression levels of CC chemokine receptors on each type of cell used in each study. The cells from the HOS cell line that were used in the Kim et al. study were transfected to specifically express CCR1 whereas Durig et al. used mononuclear cells isolated from human blood which may express less CCR1 than the HOS/CCR1 cells and would require a higher concentration of CCL3 for optimal stimulation. It was shown previously in the lab that the highest chemotactic index of recombinant CK-2 that had been purified under denaturing conditions was achieved at a

concentrations of 500ng/mL when used in the chemotaxis assay with RTS-11 cells as wells as PBLs (Eshaque et al., unpublished). In addition, it has been previously reported that in RTS-11, recombinant IL-8, a CXC chemokine, induced chemotaxis at an optimal concentration of 150ng/mL while cell migration peaked at a dose of 1500ng/mL of recombinant CC chemokine CK-6 purified under denaturing conditions (Montero et al., 2008). It was also seen that peripheral blood leukocytes isolated from Japanese flounder (*Paralichthys olivaceus*) were attracted to recombinant CC chemokine *Paol*-SCYA104 that was purified under denaturing conditions, at an optimal dose of 1000ng/mL (Khattiya et al., 2004). Thus, the recombinant CK-2 that was produced for this study under native conditions is acting within the range that has been previously reported for recombinant CC chemokines, including teleost chemokines, by other research groups.

The chemotactic index of rCK-2 for PBLs was significantly lower than it was for RTS-11 (Figure 3.7A and B). This is most likely due to the presence of other cell types in the isolated leukocyte fraction that were not monocytes/macrophages. Thus although the total number of cells used in either assay was the same, many of the cells in the PBL sample may not have been attracted by rCK-2. rβ2Mf was used in the chemotaxis assay as a negative control in order to control for the presence of chemotactic agents that may have come from the protein production procedure. The chemotactic index of rβ2Mf was not significantly different from the PBS negative control except for at the 50ng/mL concentration in RTS-11 (Figure 3.7A). The relatively high chemotactic index at this low concentration may be due to the presence of chemotactic endotoxins that came from the bacterial host, such as lipopolysaccharide, as a by-product of protein purifcation. It is possible that at the higher concentrations of rβ2Mf, the endotoxins would be bound by the protein leaving little free to stimulate chemotaxis (Nakamura et al., 2001). However at the lower concentration of β2Mf there is less protein to bind LPS, allowing more of the unbound form to cause chemotaxis. This explanation can be verified by testing for the presence of endotoxins in the protein samples. Chemotaxis was also tested in the presence of pertussis toxin, which is known to block chemokine receptor activity, in order to ensure that it was occurring due to rCK-2 (Figure 3.7B and C) (Viola and Luster, 2008; Baggiolini et al., 1997). In both assays with RTS-11 and PBLs, chemotaxis was

significantly lower when cells were blocked with pertussis toxin indicating that it was indeed occurring in response to the recombinant protein interacting with a serpentine family receptor. Based on the results of the chemotaxis assays performed, it can be concluded with confidence that the recombinant chemokine that was produced under native conditions can be used to examine the function and downstream effects of CK-2 in the rainbow trout immune system.

4.2 Downstream Effects of rCK-2 on the Inflammatory Response

As macrophages are among the first responders to invading pathogens and the initiators of the inflammatory response, the role of CK-2 in this process was examined. When encountered with a bacterial cell, macrophages present in the infected tissue become activated and release inflammatory mediators to recruit cells of the innate immune response. Among these are the cytokines IL-1β, TNFα and IL-8. These molecules are important in promoting the extravasation of neutrophils and later, monocytes, from the bloodstream. From the expression experiment, it was seen that the incubation of the macrophage and monocyte-like cell line with rCK-2 led to an increase in transcript levels of IL-1β and TNFα after 90 mins and IL-8 after 105 mins (Figure 3.9). Human monocytes induced with bacterial lipopolysaccharide (LPS) have previously been seen to upregulate their expression of IL-1β, TNFα and IL-8 mRNA in a similar pattern, beginning to increase significantly starting at 60-90 mins of exposure (Agarwal et al., 1995). IL-1β and TNFα were seen to plateau at 120 mins of treatment which was what was seen in RTS-11 dosed with rCK-2 (Agarwal et al., 1995). However, expression of IL-8 in the human study continued to increase until 240 mins whereas IL-8 transcript levels plateau in RTS-11 (Agarwal et al., 1995). In addition, the induction of IL-8 in RTS-11 by rCK-2 was much lower than its upregulation in human monocytes by LPS (Agarwal et al., 1995). A similar IL-8 mRNA expression pattern was seen in porcine macrophages induced with LPS, in which there was a significant increase in expression at 90 mins but did not reach its peak until 7hrs (Lin et al., 1994). All three cytokines began to downregulate expression after 240 mins in the human monocytes and except for IL-8, returned to basal levels at 24hrs which was also seen in the study with porcine macrophage (Agarwal et al., 1995; Lin et al., 1994). A study of IL-1β in rainbow

trout head kidney macrophages treated with LPS showed a dramatic increase in IL-1β transcript level after 240 mins of induction (Zou et al., 1999). However, this study did not determine when upregulation of IL-1β is initiated in rainbow trout macrophages. A 240 min treatment of RTS-11 with LPS was reported to significantly increase the mRNA expression of TNFα (Laing et al., 2001). Again, the time course of the induction of expression was not examined. The IL-8 transcript level in RTS-11 exposed to LPS was reported to increase significantly at 120 mins, peaking at 240 mins and lasting until 8hrs of treatment (Laing et al., 2002). The time points at which induction begins or ends were not determined in this study. Although it is possible that LPS induction of these pro-inflammatory cytokines begins earlier than the time points that were examined in these studies, it is also possible that chemokine-induction of pro-inflammatory gene expression begins earlier than LPS-driven stimulation. However similar to the studies of LPS-treated monocytes/magrophages, upregulation of IL-1β in human monocytes upon exposure to CCL5 was only examined and observed after 120 mins (Locati et al., 2002). RTS-11 dosed with the CC chemokine CK-6 were reported to upregulate IL-8 expression at the transcript level at 24hrs but the time course prior to this was not determined (Montero et al., 2008). In addition, IL-1β and TNF-α were not induced by CK-6 at the 24hr time point but it is possible that the increase in expression of these cytokines occurs earlier (Montero et al., 2008). Nevertheless, the induced expression of IL-1β, TNF-α and IL-8 in RTS-11 treated with rCK-2 was seen in this study to begin at 90 mins and lasted until the end of the time course examined.

The time courses of IL-1β, TNFα and IL-8 mRNA expression in RTS-11 in response to rCK-2 were similar to those seen in human, porcine and rainbow trout macrophages treated with LPS. However, the induction of IL-8 expression was not as high in cells exposed to rCK-2 as cells exposed to LPS. This difference could be due to the role of IL-8 in inflammation as a CXC chemokine. The reason for its secretion during an inflammatory response is primarily to attract leukocytes to the site of infection. CXC chemokines are responsible for directing the migration of neutrophils, lymphocytes and monocytes to the site of infection while CC chemkines mainly attract monocytes/macrophages. However, due to the presence of the ELR motif in IL-8, it is less effective in inducing the migration of neutrophils. It is possible that

macrophages in the infected tissue do not need to secrete as much IL-8 because monocytes are already being attracted to the site of infection by CK-2. IL-8 would still be needed to induce chemotaxis in lymphocytes. The overlap in target cells could be why rCK-2 did not elicit as large of a response in IL-8 expression that was reportedly caused by LPS. Apart from the lower level of IL-8 induction, rCK-2 stimulated a similar pattern of pro-inflammatory cytokine expression in RTS-11 as was seen by the treatment of LPS on human, porcine and rainbow trout macrophages. This may indicate that CK-2 is a sufficient signal for rainbow trout macrophages to initiate an inflammatory response.

The dose response of the expression of IL-1β, TNFα and IL-8 of RTS-11 to rCK-2 was also examined. The expression of IL-1β was the most augmented of the three pro-inflammatory cytokines and was induced at the concentration of 1000ng/mL. Likewise, the most significant induction of IL-1β in RTS-11 treated with rCK-6 was observed at a dose of 1500ng/mL (Montero et al., 2008). However, human monocytes were reported to only require 300ng/mL of CCL5 in order to generate a 19.5-fold upregulation of IL-1β compared to untreated cells (Locati et al., 2002). The statistically significant, albeit minor, increase in TNFα mRNA levels at 1000ng/mL of rCK-2 was not seen when RTS-11 was exposed to rCK-6 at any of doses that were tested in the study by Montero et al. (2008). rCK-2 elicited a decrease in IL-8 expression at lower levels of the chemokine but did not cause a response at the 1000ng/mL dose. Similarly, there was a slight decrease in IL-8 transcript in RTS-11 dosed with low levels of rCK-6 however there was a substantial induction of the gene at the 150 and 1500ng/mL concentrations. Thus, the dose response effect of rCK-2 on pro-inflammatory genes in RTS-11 reflects what was observed in studies of other chemokines on monocytes/macrophages.

When a sample of untreated RTS-11 from the same passage was examined by flow cytometry, it was seen that the majority of the population consisted of monocyte-like cells (Figure 3.17). It has been previously shown that human monocytes that have begun the process of differentiation into macrophages, increase their production of cytokines related to bacterial LPS-induction (Gessani et al., 1993). In light of this, it is possible that CK-2 is also

involved in monocyte differentiation. If this is the case, it would be expected that exposure to the chemokine would induce much higher levels of IL-1β and TNFα than seen in the time course experiment. One possibility is that much more expression of the cytokines may be occurring at the protein level rather than the mRNA level. Another possibility is that the dramatic increase expected in the transcript level of IL-1β and TNFα happens much later in the time course. However, it was seen in the study of these cytokines in human monocytes exposed to LPS that their expression at the mRNA level begins to return to basal levels after 240 mins, although it should be noted that the regulation of gene expression in fish may be vastly different than in mammals (Agarwal et al., 1995). The dose response data may corroborate with the hypothesis of further upregulation of the pro-inflammatory cytokines at a later time. It could be postulated that at low concentrations, when monocytes are still in the bloodstream and away from the site of infection, CK-2 has no effect on IL-1β and TNFα. However, once the monocytes have entered the site of infection where the concentration of CK-2 would be the highest, IL-1β and TNFα expression increases as CK-2 begins to induce their differentiation into macrophages.

It can be surmised that CK-2 influences several aspects of the innate immune response of macrophages. It may induce macrophages to initiate inflammation by increasing the production and secretion of IL-1β, TNFα and IL-8, which facilitate the migration of leukocytes across the blood epithelium. CK-2 can play a role here as well, since it was seen in the chemotaxis assay that it is capable of inducing chemotaxis in monocytes. Once the monocytes have migrated into the infected tissue, they can once again be influenced by CK-2 to differentiate into macrophages.

At the very least, the increases in IL-1β, TNFα and IL-8 mRNA indicate that the monocytes are being stimulated by CK-2 (Auffray et al., 2009). However, more studies at the protein level of these genes must be performed in order to confirm the hypothesis that CK-2 has multiple roles in the innate immune response in macrophages. It is possible that the increases that were observed in the pro-inflammatory transcripts are not translated into protein. In addition, if the proteins are not secreted into the environment, they can not affect the cells

around them. Increased IL-1β production may also mean that there is increased IL-6 expression downstream. Future studies should look at a rise in IL-6 transcript and protein levels later on in the time course, as this could indicate the involvement of CK-2 in the activation of the acute phase response. Similarly, iNOS expression levels and the induction of respiratory burst should be examined since TNFα is an inducer of NO production.

4.3 Implication of CK-2 exposure on the rainbow trout immune response to intracellular pathogens

Although the principal purpose of macrophages is traditionally thought to be the detection of extracellular pathogens, these cells also have a co-dependent relationship with proteins involved in the response against intracellular pathogens. As evidenced by the viral mimicry of chemokines in an attempt to evade the immune system, it is well-known that chemokines are extremely important in the antiviral response (Melchjorsen et al., 2003). In addition, the monocyte/macrophage cell lineage both produces and responds to the CC-subfamily of chemokines (Fantuzzi et al., 2003). In light of this, the effect of CK-2 on the expression of some of the antiviral response genes in RTS-11, including MH Class I α-chain, IFN1 and IFN2 was examined.

As nucleated cells, monocytes/macrophages have the inherent capability of expressing MH Class I and presenting antigens from intracellular pathogens. The increase that was seen in MHIα mRNA after 105 mins of exposure may be an indication that CK-2 is capable of priming monocytes/macrophages to defend against intracellular pathogens. Rainbow trout infected with infectious hematopoietic necrosis virus (IHNV) have been reported to show increased expression of MHIα beginning at 72hrs post-exposure in spleen and head kidney (Hansen and La Patra, 2002). A study using an Atlantic salmon kidney cell line (ASK) also showed an increase in MHIα at 72hrs of infectious salmon anaemia virus (ISAV) infection (Jorgensen et al., 2006). The MHIα upregulation observed at this time point may possibly corroborate with the timeline of viral induction of CC chemokine expression and chemokine induction of MHIα. Rainbow trout infected with viral haemorrhagic septicaemia virus (VHSV) have been seen to express CC chemokines at 72hrs of exposure (Montero et al.,

2009). Similarly, upregulation of CC chemokines in mice infected with influenza A virus were also reported to begin at 72hrs (Wareing et al., 2004). However, none of these studies examined the time course of expression of CC chemokines or MHIα between 24 and 72 hrs. It is therefore possible that CC chemokine expression is induced by viral infection prior to the upregulation of MHIα in the temporal vicinity that was seen in the present study. The time course of expression of CC chemokines and MHIα should be investigated in a finer detail. It has also yet to be seen whether the increase in transcript level of MHIα is also seen in the protein level. However, the upregulation of MHIα mRNA in response to rCK-2 could indicate that CK-2 is capable of triggering an immune response towards a viral infection.

The lack of a statistically significant dose response effect of rCK-2 on MHIα may indicate that prolonged exposure to the chemokine is required for upregulation rather than a specific dose. It is interesting that there is a substantial decrease in IFN1 expression in response to rCK-2 which returns to basal levels at the 1000ng/mL dose. However, dose response data of chemokines on interferon expression could not be found for a comparison.

The increase in interferon expression that was seen in the time course of RTS-11 treated with rCK-2 may provide further evidence that CK-2 could have a role in initiating an antiviral response. Type I interferons encompass α and β interferons, which are induced in many cell types, including macrophages, in response to viral infections. They operate in autocrine and paracrine fashions, secreted by the infected cell and act on it and surrounding infected cells to inhibit viral replication by inducing the expression of interferon stimulated genes (ISGs) (Melchjorsen et al., 2006; Kileng et al., 2007). For example, one of the genes that is capable of being induced by IFN1 encodes the antiviral protein Mx, which is known to interfere with viral replication (Kileng et al., 2007). The 32% increase in IFN1 after 105 of exposure to rCK-2 indicates that CK-2 could possibly act as a signal to cells that there is a viral invasion and to activate to an antiviral state. Similar to MHIα, Type 1 interferons have been seen to be upregulated at the transcript level in spleen and head kidney of rainbow trout infected with VHSV starting at 48 and 72hrs of exposure, respectively (Zou et al., 2007). Kidney tissue of Atlantic salmon challenged with infectious pancreatic necrosis virus (IPNV) also showed an

increase in IFN1 and IFN2 at 72hrs then decreased in expression before peaking at day 6 (McBeath et al., 2007). IPNV-infected cells of the rainbow trout gonad cell line (RTG-P1) were seen to significantly increase in IFN1 transcript level at 24hrs of treatment (Collet et al., 2006). Likewise, an Atlantic salmon head kidney leukocyte cell line, TO cells, that had been exposed to ISAV showed a 10-fold increase in IFN1 at 24hr post-infection (Kileng et al., 2007). The discrepancy in the time points at which upregulation of the interferons is seen in whole fish as opposed to cell lines is most likely due to the time it takes for the viral infection to reach the tissues capable of response. As discussed above, virus-induced chemokine expression has not been observed until 72hrs post-infection in whole animals. Again, the time course of CC chemokine expression between 24 and 72hrs of exposure to virus was not elucidated. Thus, CC chemokines could be expressed before the induction of the interferons within that window. It is also possible that viral infection could stimulate interferon production before the upregulation of CC chemokines which could further increase the expression of type 1 and 2 interferons as seen in the response to rCK-2. It would be interesting to see if there is an enhancement of interferon expression when RTS-11 is infected with virus in conjunction with exposure to rCK-2.

It has previously been reported by DeMaeyer and DeMaeyer-Guignard that of the Type I interferons, macrophages mostly express IFNβ, while Saksela et al., showed that viral induction of IFNα is more prevalent in monocytes (DeMaeyer and DeMaeyer-Guignard, 1988; Saksela et al., 1984). The primers that were used to amplify IFN1 were designed from the mRNA sequence of rainbow trout type I interferon 1 (NCBI accession number AJ580911.2). This sequence was compared to the National Center for Biotechnology Information (NCBI) nucleotide database using BLAST in order to determine whether it was the sequence for α or β interferon. The sequence analysis came back with 96% sequence identity to the Atlantic salmon IFNα1 gene, 98% sequence identity to the Atlantic salmon IFNα2 gene, and 100% sequence identity to an Atlantic salmon IFNα1 pseudogene. It can therefore be assumed that the transcript that the primers used in this study were designed against is that of rainbow trout IFNα. This indicates that the upregulation of Type I interferon in response to rCK-2 was most likely occurring in the dominating monocyte population.

Therefore, it is possible that CK-2 has a two-fold role in antiviral immune response. Upon exposure to the chemokine, monocytes are activated to help fight a viral infection that is taking place somewhere in the organism. They would then use the chemokine gradient to lead them to where the viral infection is occurring. By the time the cells reach the viral-plagued tissue, they are primed and ready to secrete type I interferons that will interfere with viral replication in infected cells. At the same time, the IFN1 that is being produced by these monocytes/macrophages can further upregulate the expression of MH Class I (Murphy et al., 2007).

In addition, the autocrine production of IFN1 may ensure that cells can more readily present viral antigens to $CD8^+$ killer T-cells by inducing the expression of proteins that are involved in processing foreign intracellular peptides to be loaded into the binding cleft of MHI molecules. These supplementary proteins include the TAP proteins, which transport cytosolic proteins to the endoplasmic reticulum, and the LMP proteins that are components of the immune-induced proteasome, or immunoproteasome which is found in interferon-induced cells (Murphy et al., 2007). Furthermore, the generation of peptides that are of compatible length for associating with the MHI binding cleft is ensured by the endoplasmic reticulum aminopeptidase ERAAP, which can be upregulated by IFN2 (Murphy et al., 2007). It is therefore plausible that CK-2 plays a role in stimulating monocytes/macrophages to prepare for an antiviral response.

IFN2 is also well established as a principal influence in skewing the immune response towards a Type I helper T-cell mediated response. It was traditionally thought that IFN2 expression and secretion was restricted to T-cells and natural killer cells, however a few studies have shown that macrophages are also capable of producing the cytokine. In 1993, Fultz et al., reported that murine macrophages could be induced to express IFN2 by lipopolysaccharide (LPS). A year later a research group in Italy published evidence that IFN2 is constitutively expressed in resting mouse peritoneal macrophages and could even be self-upregulated (DiMarzio et al., 1994). The same group also showed that a significant increase in IFN2 expression could be induced by the cytokine IL-12 and could confer antiviral

protection (Puddu et al., 1997). Both groups suggested that the ability of macrophages to both express and respond to IFN2 may indicate that autocrine activation by IFN2 is important for optimal immune preparedness (Fultz et al., 1993; Puddu et al., 1997). The increase that was seen in IFN2 expression in response to treatment with CK-2 may provide further evidence that the chemokine induces the cytokine to work in an autocrine fashion to activate an immune response against intracellular pathogens. Therefore, one of the functions of the chemokine may be to act as a molecular warning that an antiviral immune response needs to be initiated. It would be interesting to see whether the increase in IFN2 transcript gets translated to the protein level and whether IFN2 is secreted by the cell. It would also be interesting to see if CK-2 expression is substantially increased during an *in vivo* viral infection and whether anti-CK-2 antibodies can block its upregulation of interferon and activation of an antiviral response, however there are most likely other systems that are able to signal the presence of a viral threat.

IFN1 and IFN2 are both known to prime monocytes/macrophages for the immune response against viral infections and increase the expression of MH Class I. However, they are unlikely to be responsible for the upregulation of MHIα expression that was seen in CK-2 exposed RTS-11, since it seems to be occurring in parallel to the increase in interferon transcript levels. On the other hand, it may be possible that the autocrine interferons would further increase MHIα later on in the time course. Since MHIα is being induced relatively early in the time course, CK-2 may directly affect its upregulation.

4.4 Effects of rCK-2 on genes related to the immune response to extracellular pathogens

As antigen-presenting cells, it was not surprising to observe a CK-2-triggered monocyte response in MHIIα expression. The transcript level of MHII genes is known to correspond well to cell surface expression level, making it quite probable that the increase that was seen in response to CK-2 exposure is actually occurring (Jasinski et al., 1995). MH Class II is known to be upregulated in mouse macrophages by IFN2 as well as TNF-α however its expression is seen to be suppressed in the presence when cells are treated with LPS

(Glimcher and Kara, 1992). Contrary to this, the stimulation of MHIIβ expression has been observed in rainbow trout head kidney macrophages treated with LPS for 4hrs (Knight et al., 1998). A study with RTS-11 exposed to LPS for 4hrs reported no apparent increase in MHIIβ transcript level (Brubacher et al., 2000). However, when expression of MHIIβ in control and treated samples is compared to the level of expression of the internal control gene, β-actin, in those samples, it appears that upregulation may be occurring (Brubacher et al., 2000). Nevertheless, LPS was also seen to induce the expression of MHIIα in the Atlantic salmon macrophage-like cell line (SHK-1) at 72hrs of treatment (Koppang et al., 1999). The upregulation of MHIIα that was seen in the present study in RTS-11 dosed with rCK-2 starting at 90 mins of exposure may seem early compared to the previous studies. However, it should be noted that the expression of MH Class II genes in response to LPS was not examined at time points prior to 4hrs, which was the upper limit of the time course in this study. As well, the increased level of MHIIα was maintained until the 4hr time point in the rCK-2 study.

Interestingly, the rainbow trout invariant chain S25-7 does not get upregulated along with MHIIin response to CK-2. Invariant chain is essential for the proper presentation of non-self peptides by MHII. It is known to be inducible in the mammalian immune system however, previous work in this lab has shown that S25-7 does not get induced in the presence of other immunostimulatory factors indicating that it may be non-inducible and that it is constitutively expressed (Christie and Dixon, unpublished). The lack of a significant response in S25-7 expression to rCK-2 treatment of RTS-11 provides further evidence of its constitutive nature.

Although the co-receptor CD4 is most famously expressed on the surface of T-cells, it has also been found expressed in macrophages (Filion et al., 1990). In fact, human monocytes have also been shown to express CD4 intracellularly (Filion et al., 1990). It is interesting that an increase was seen in CD4 transcript level in response to rCK-2 as research groups studying human monocytes showed that CD4 is downregulated in response to various stimulants (Graziani-Bowering and Filion, 2000). Herbein et al., reported that treatment of human monocytes with LPS, TNFα and IL-1β all caused decreases in both cytosolic and cell

surface CD4 (Herbein et al., 1995). It was also seen in this study that the inhibition of CD4 was occurring at the RNA level, indicating transcriptional regulation of CD4 expression (Herbein et al., 1995). This conclusion makes the observed increase in CD4 transcript in response to rCK-2 even more curious. In addition, Herbein et al. found that the CC chemokines they tested had no effect on the expression of CD4 (Herbein et al., 1995). Since rCK-2 was seen to cause an increase in TNFα and IL-1β, it is possible that if these pro-inflammatory cytokines are secreted that they could act in an autocrine manner and cause a potential decrease in CD4 expression later on in the time course. Future studies should investigate whether rCK-2 is capable of inducing CD4 at the protein level and whether this increase as well as the increase in CD4 mRNA is reversed at a later time.

It was concluded by Graziani-Bowering and Filion that the downregulation of monocyte cell surface CD4 was mostly likely caused by their differentiation into macrophages (Graziani-Bowering and Filion, 2000). In light of this, it can be postulated that the monocyte-like cells in the samples were not undergoing differentiation. Nevertheless, the upregulation that was seen in TNFα and IL-1β could indicate that rCK-2 is initiating the differentiation of the monocyte-like cells into macrophage-like cells through the induction of these cytokines. In fact, it was concluded from the Herbein et al. study that the LPS-inhibition of CD4 was mediated by TNFα and IL-1β (Herbein et al., 1995). If there is a decrease in CD4 later in the time course, it would then be possible that rCK-2 is a trigger for CK-2 differentiation.

However if the increase in CD4 is mirrored at the protein level and is seen to be a prolonged induction, it is possible that the rCK-2-induced increase in cell surface CD4 acts as a negative feedback mechanism to modulate the monocyte response to chemokines. CD4 is known to be a co-receptor for human immunodeficiency virus (HIV) in monocytes and macrophages, as well as T-lymphocytes. CD4 stimulation by the HIV envelope glycoprotein gp120 has been seen to cause internalization of chemokine receptors from monocyte cell surfaces (Wang et al., 1998). The downregulation in chemokine receptor expression caused decreased sensitivity to chemokines in monocytes (Wang et al., 1998). The RTS-11 monocyte response to rCK-2 was to increase the expression of cytokines that trigger

inflammation and the T-cell mediated response. The continued secretion of molecules such as IL-1β, TNFα and interferons is not beneficial since they have many downstream effects in addition to inflammatory and T-cell responses. Consequently, it would make sense for the chemokine signal that triggers their production to be turned off by negative feedback. In addition, activated macrophages need to migrate to peripheral lymphoid tissues in order to trigger the adaptive immune response and does so by following a chemokine concentration gradient. However, if the activated macrophages are still responding to the chemokines that they had followed to the site of infection there would be two opposing signals pulling the cells in different directions. The activated macrophage must first turn off its response to the initial chemokines in order to follow the second chemokine gradient to the lymphoid tissues. Again, there must be some mechanism of downregulating the first signal. Along with causing increases in cytokine mediators of the immune response, rCK-2 may induce CD4 expression in order to decrease the number of chemokine receptors present on the cell surface so that it cannot continue responding to CK-2. This modulation of chemokine stimulation may represent a function of CD4 in monocytes/macrophages which is currently unknown.

4.5 Concluding Remarks

Based on the gene expression profile that was derived from the exposure of the monocyte/macrophage-like cell line RTS-11 to recombinantly produced CK-2, it can be postulated that CK-2 is a stimulator of the rainbow trout immune system. Not only does CK-2 promote chemotaxis of leukocytes, it is also appears to be capable of triggering inflammatory responses. However, it seems as though the chemokine is non-specific in its actions since it upregulates mediators of the immune response towards both intracellular and extracellular pathogens. It is still possible that the upregulation is not perpetrated to the protein level. Therefore, future studies should look at the protein levels of the expression in the cytosol as well as whether the protein products are secreted or translocated to the membrane as expected. A longer time course should also be carried out in order to get a complete picture of the effects of CK-2.

In addition, the immune gene-related expression profile of the RTS-11 response to rCK-2 exhibited an interesting upregulation of CD4, the function of which in monocytes/macrophages remains unknown. The chemokine-stimulated increase in expression of CD4 may suggest a negative feedback mechanism to control the response to CK-2. It will be exciting to further investigate this hypothesis in future studies.

Appendix A

Abbreviations

ASK	Atlantic salmon kidney
β2M	β-2-microglobulin
β2Mf	β-2-microglobulin fragment
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CK-2	Chemokine 2
CK-2.1	Chemokine 2.1
cDNA	Complementary DNA
DAG	Diacylglycerol
ERAAP	Endoplasmic reticulum aminopeptidase
FBS	Fetal bovine serum
GPCR	G-protein coupled receptor
HOS	Human osteosarcoma-derived
Hr	Hour
IFN	Interferon
IL	Interleukin
IP_3	Inositol triphosphate
IPNV	Infectious pancreatic necrosis virus
IPTG	Isopropyl β-D-1 thiogalactopyranoside
ISAV	Infectious salmon anaemia virus
LMP	Low molecular mass polypeptide
LPS	Lipopolysaccharide
MHIα	Major histocompatibility class I alpha chain
MHIIβ	Major histocompatibility class II beta chain
MHIIα	Major histocompatibility class II alpha chain
Min	Minute
MYA	Million years ago
NBT	4-nitro blue tetrazolium chloride
NTA	Nitrilotriacetic acid
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemaglutinin
PIP_2	Phosphoinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis Toxin
PVP	Polyvinylpyrrolidone

rβ2Mf	Recombinant β-2-microglobulin fragment
rCK-2	Recombinant CK-2
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
TAP	Tapasin
TBE	Tris/Borate/EDTA Buffer
TBO	Toluidine blue O
TBST	Tris-buffered saline-Tween-20
TEMED	Tetramethylethylenediamine
T_H1	Type 1 helper T-cell
T_H2	Type 2 helper T-cell
TNFα	Tumour necrosis factor-α
VHSV	Viral haemorrhagic septicaemia virus

Appendix B

Figures

Figure B.1: RT-PCR analysis of IL-1β transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. IL-1β and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

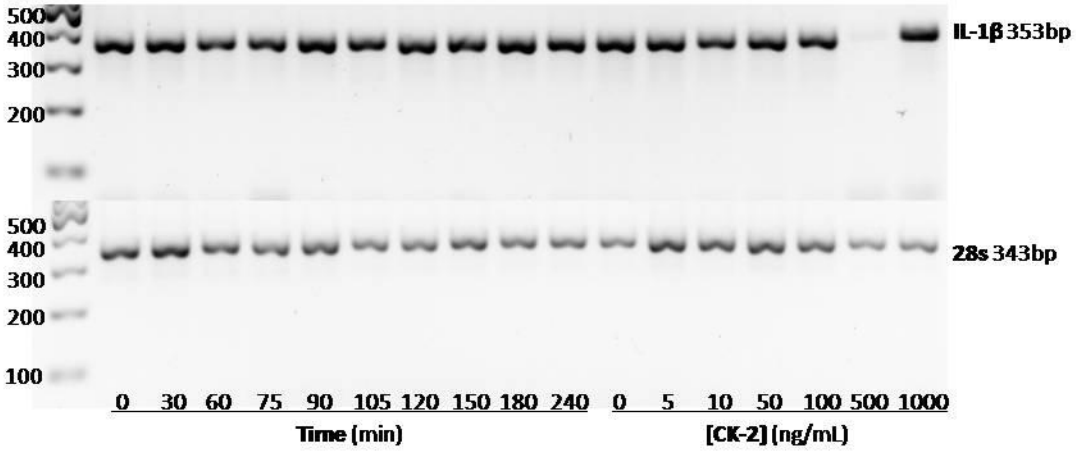


Figure B.2: RT-PCR analysis of TNFα transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. TNFα and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

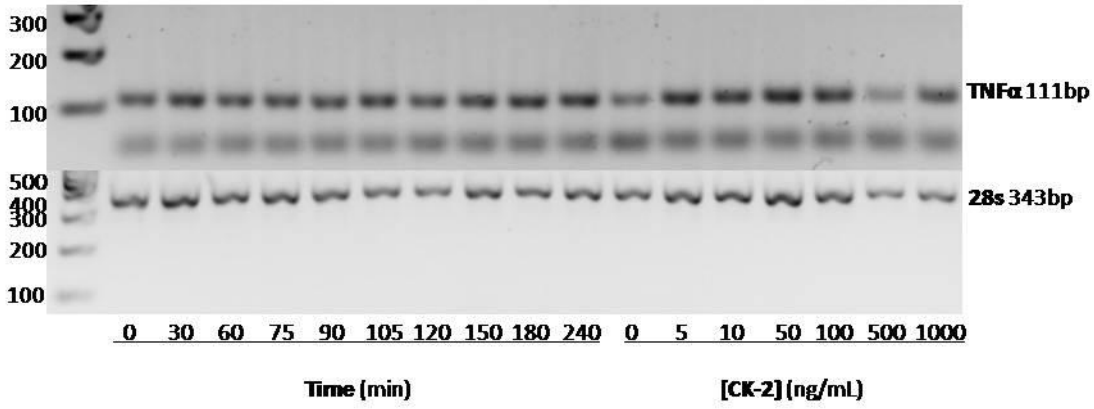


Figure B.3: RT-PCR analysis of IL-8 transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. IL-8 and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

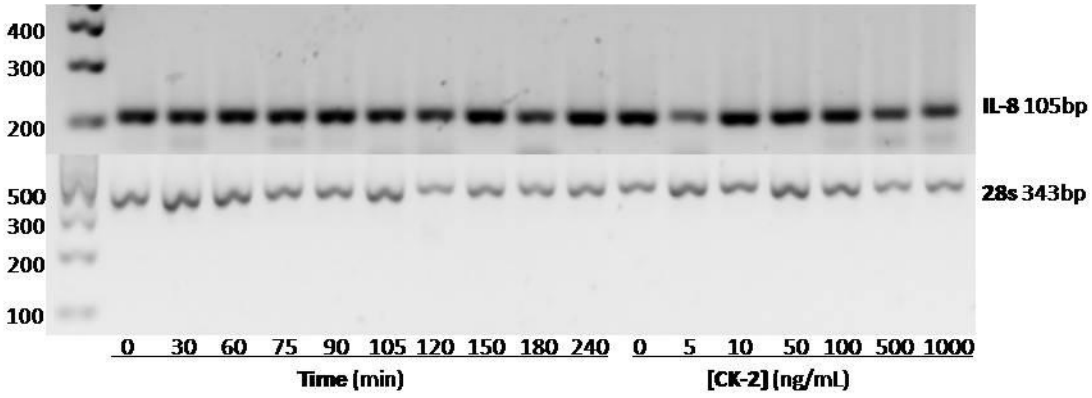


Figure B.4: RT-PCR analysis of MHIα transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. MHIα and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

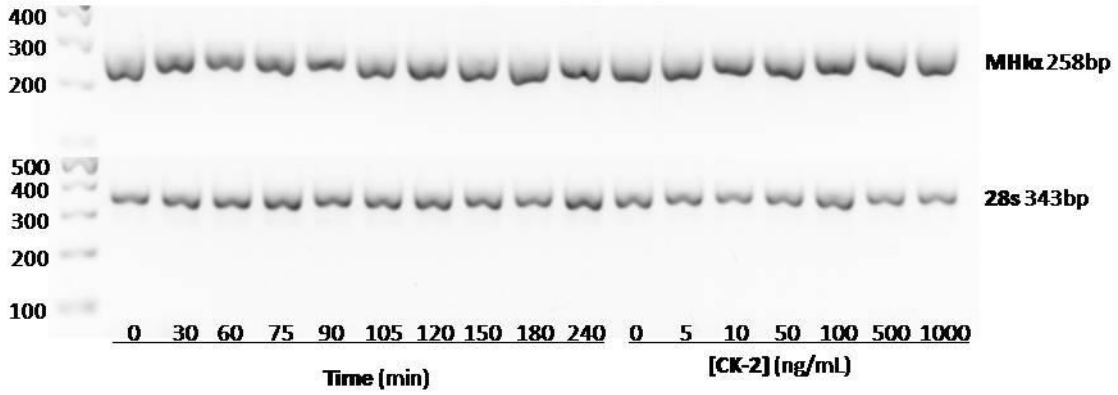


Figure B.5: RT-PCR analysis of IFN1 transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. IFN1 and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

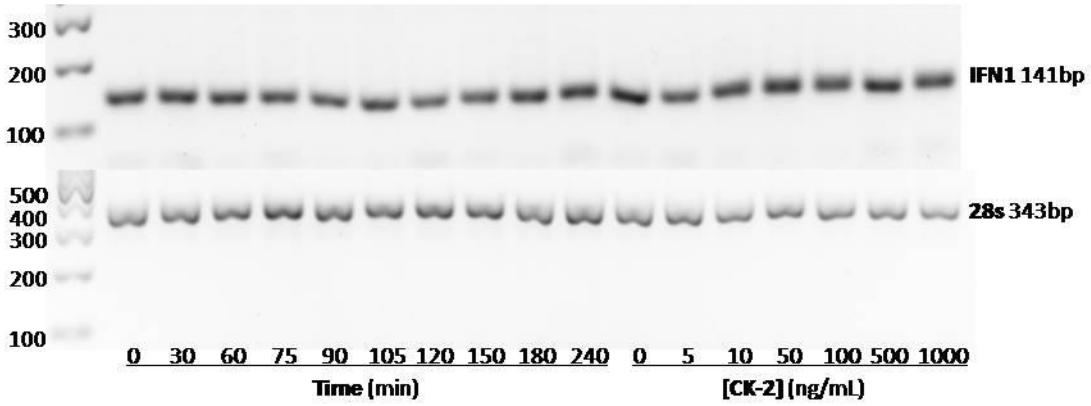


Figure B.6: RT-PCR analysis of IFN2 transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. IFN2 and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

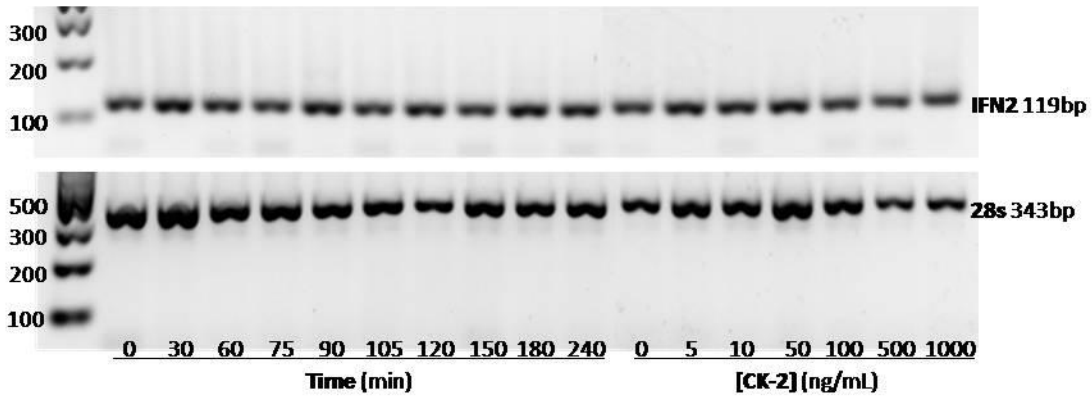


Figure B.7: RT-PCR analysis of MHIIα transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. MHIIα and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

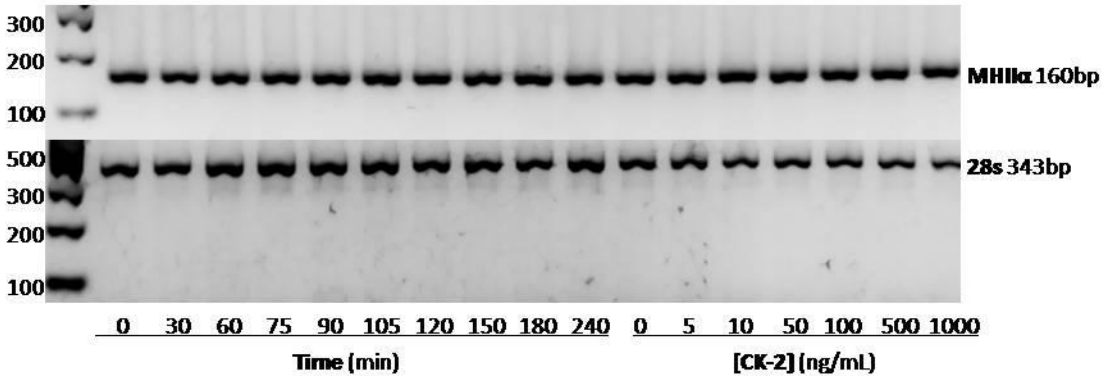


Figure B.8: RT-PCR analysis of S25-7 transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. S25-7 and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.

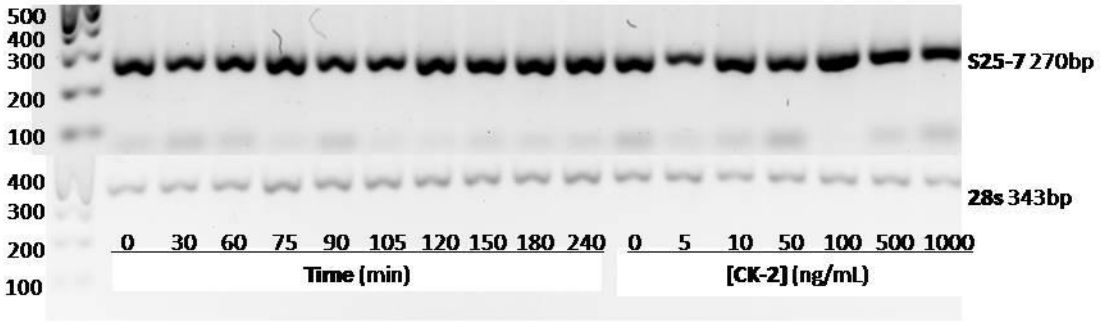
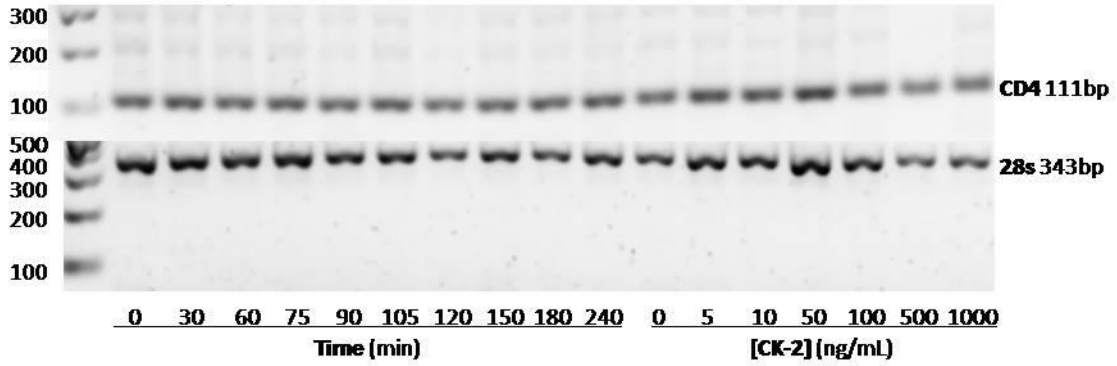


Figure B.9: RT-PCR analysis of CD4 transcript levels in rCK-2-treated RTS-11. Total RNA was extracted from two sets of RTS-11 and used to synthesize cDNA through RT-PCR. CD4 and 28s were amplified from this cDNA in two separate PCR reactions then run together on a GelRed-stained1.5% agarose gel in 0.5X TBE at 35V for 55 mins. Densitometry analysis was performed using ImageJ to determine band densities. The image of the gel from the experiment that was not shown in the Results section is presented here.



Appendix C

Tables

Table C.1: Cell counts of the migration towards each treatment in the chemotaxis assay. Three fields of view were counted in each well and are averaged here. Three wells were used for each treatment**.** The chemotactic index represents the ratio of the number of cells that migrated towards the substance of interest to the number of cells that migrated towards the negative control, PBS.

(A) Chemotaxis assay using RTS-11 cells to test the chemotactic ability of CK-2. The complement protein C5a was included as a positive control and β2Mf was a second negative control.

	Dose (ng/mL)	Well 1	Well 2	Well 3	Average	Std. Dev.	Chemotactic Index
PBS		0	0	82.67	27.56	41.5	1.00
β2Mf	1000	0	0	84.3	28.11	42.3	1.02
	500	0	0	0	0	0	0
	100	0	128.3	0	42.8	64.6	1.55
	50	127	134	101.3	120.78	27.2	4.38
CK-2	1000	173.3	185.7	178	179	22.2	6.49
	500	167	195	229	197	31.1	7.15
	100	228.2	236.2	191.7	215.4	20.6	7.82
	50	173	210	175.7	186.2	36.1	6.76
C5a		239	188	212.7	213.2	30	7.74

(B) Chemotaxis assay using PBLs to test the chemotactic ability of CK-2.

	Dose (ng/mL)	Well 1	Well 2	Well 3	Average	Std. Dev.	Chemotactic Index
PBS		61	0.7	19.7	27.1	30.9	1
β2Mf	1000	10.7	8.3	6.7	8.6	2	0.32
	500	30.7	59.3	51	47	14.8	1.73
	100	33.3	79	61.3	57.9	23	2.14
	50	67.3	27.3	33.7	42.8	21.5	1.58
CK-2	1000	53	24	52.3	43.11	16.6	1.59
	500	139	94.7	61.7	98.4	38.8	3.63
	100	94.7	139.7	151.7	128.7	30.1	4.75
	50	71.7	74	111.3	85.7	22.3	3.16
C5a		81.3	108.3	138	109.2	28.3	4.03

(C) Chemotaxis assay using RTS-11 blocked with PTX.

	Well 1	Well 2	Well 3	Average	Std. Dev.	Chemotactic Index
PBS	58.7	55.3	13.7	42.6	25.1	1
β2Mf	69	74.7	18.7	54.1	30.8	1.27
CK-2	96.7	98.3	114	103	9.6	2.42
PTX-CK-2	55	73.3	76	68.1	11.4	1.6
C5a	89.3	72	76.3	79.2	9	1.86

(D) Chemotaxis assay using PBLs blocked with PTX

	Well 1	Well 2	Well 3	Average	Std. Dev.	Chemotactic Index
PBS	6	23	24.7	17.9	10.3	1
β2Mf	143	122.7	87.3	117.7	28.2	6.62
CK-2	189.3	180.3	170	179.8	9.9	10.05
PTX-CK-2	78	92.3	122.3	97.6	22.6	5.45
C5a	166.7	149.7	177.3	164.2	13.9	9.24

Table C.2: Densitometry analysis of RT-PCR of IL-1β mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of IL-1β for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	IL-1β	28s	IL-1β/28s	IL-1β	28s	IL-1β/28s		
0	6.53	7.63	0.86	6.03	6.04	1.00	0.93	0.10
30	6.59	9.41	0.70	5.59	6.73	0.83	0.77	0.09
60	5.04	5.95	0.85	5.55	6.63	0.84	0.84	0.01
75	5.32	6.06	0.88	5.19	8.37	0.62	0.75	0.18
90	7.32	7.09	1.03	5.59	5.86	0.95	0.99	0.06
105	5.60	4.73	1.18	5.39	6.57	0.82	1.00	0.26
120	6.77	4.83	1.40	5.90	7.69	0.77	1.08	0.45
150	6.10	6.07	1.01	6.25	5.69	1.10	1.05	0.07
180	7.03	4.81	1.46	5.52	6.76	0.82	1.14	0.46
240	6.43	4.78	1.34	7.06	5.63	1.25	1.30	0.06
Dose (ng/mL)								
0	6.46	3.96	1.63	6.20	5.84	1.06	1.35	0.40
5	6.34	7.77	0.82	6.43	5.23	1.23	1.02	0.29
10	5.22	6.58	0.79	6.44	4.71	1.37	1.08	0.41
50	6.29	7.37	0.85	5.89	5.17	1.14	1.00	0.20
100	6.11	5.96	1.02	5.68	4.56	1.25	1.14	0.16
500	0.13	3.22	0.04	5.57	4.97	1.12	0.58	0.76
1000	6.74	3.80	1.77	5.72	3.56	1.61	1.69	0.12

Table C.3: Densitometry analysis of RT-PCR of TNFα mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of TNFα for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	TNFα	28s	TNFα/28s	TNFα	28s	TNFα/28s		
0	5.34	6.40	0.83	5.30	5.88	0.90	0.87	0.05
30	6.88	7.37	0.93	4.28	5.16	0.83	0.88	0.07
60	5.10	5.91	0.86	6.77	6.26	1.08	0.97	0.15
75	5.77	6.96	0.83	6.32	6.34	1.00	0.91	0.12
90	5.64	5.91	0.95	6.62	6.21	1.06	1.01	0.08
105	6.69	4.72	1.42	5.82	5.94	0.98	1.20	0.31
120	5.25	4.12	1.27	5.59	5.63	0.99	1.13	0.20
150	6.46	6.03	1.07	5.06	5.11	0.99	1.03	0.06
180	6.72	5.03	1.34	5.59	5.76	0.97	1.15	0.26
240	7.09	5.81	1.22	6.85	5.70	1.20	1.21	0.01
Dose (ng/mL)								
0	3.66	5.28	0.69	5.68	5.60	1.01	0.85	0.23
5	7.18	7.13	1.01	5.15	5.59	0.92	0.96	0.06
10	6.66	6.62	1.01	5.30	4.85	1.09	1.05	0.06
50	7.56	7.90	0.96	5.62	5.26	1.07	1.01	0.08
100	6.11	6.62	0.92	5.76	5.42	1.06	0.99	0.10
500	2.34	3.38	0.69	4.97	4.90	1.01	0.85	0.23
1000	5.59	4.80	1.16	4.19	4.24	0.99	1.07	0.13

Table C.4: Densitometry analysis of RT-PCR of IL-8 mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of IL-8 for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	IL-8	28s	IL-8/28s	IL-8	28s	IL-8/28s		
0	6.29	6.80	0.93	6.69	6.48	1.03	0.98	0.08
30	6.16	8.48	0.73	7.29	5.26	1.39	1.06	0.47
60	6.46	7.99	0.81	4.12	6.56	0.63	0.72	0.13
75	6.34	5.89	1.08	6.68	7.22	0.93	1.00	0.11
90	6.63	5.85	1.13	3.48	7.00	0.50	0.82	0.45
105	5.86	7.14	0.82	6.62	6.60	1.00	0.91	0.13
120	4.81	3.43	1.40	6.22	6.44	0.97	1.19	0.31
150	6.99	5.14	1.36	7.26	6.33	1.15	1.25	0.15
180	5.02	4.71	1.07	7.67	6.13	1.25	1.16	0.13
240	7.10	5.63	1.26	5.21	6.16	0.85	1.05	0.29
Dose (ng/mL)								
0	6.88	4.46	1.54	6.24	5.14	1.22	1.38	0.23
5	3.35	7.41	0.45	5.17	4.71	1.10	0.77	0.46
10	6.61	5.37	1.23	4.93	4.40	1.12	1.18	0.08
50	6.82	7.54	0.90	4.11	4.61	0.89	0.90	0.01
100	6.30	6.38	0.99	2.95	4.41	0.67	0.83	0.23
500	4.47	3.54	1.26	2.97	4.17	0.71	0.99	0.39
1000	3.92	4.25	0.92	6.29	3.54	1.78	1.35	0.60

Table C.5: Densitometry analysis of RT-PCR of MHIα mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of MHIα for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	MHIα	28s	MHIα/28s	MHIα	28s	MHIα/28s		
0	5.94	8.41	0.71	5.58	4.78	1.17	0.94	0.33
30	6.04	10.36	0.58	6.09	6.50	0.94	0.76	0.25
60	6.17	6.39	0.96	4.67	6.64	0.70	0.83	0.18
75	6.08	6.61	0.92	5.76	7.77	0.74	0.83	0.13
90	6.34	7.97	0.79	4.79	6.01	0.80	0.80	0.00
105	6.51	4.90	1.33	5.57	6.37	0.87	1.10	0.32
120	6.04	5.08	1.19	6.25	7.06	0.89	1.04	0.21
150	6.66	6.68	1.00	5.87	6.55	0.90	0.95	0.07
180	6.51	5.32	1.22	6.40	5.42	1.18	1.20	0.03
240	5.90	4.88	1.21	6.17	8.07	0.76	0.99	0.31
Dose (ng/mL)								
0	6.27	4.15	1.51	6.21	6.12	1.02	1.26	0.35
5	5.42	9.06	0.60	6.13	4.96	1.24	0.92	0.45
10	5.68	5.35	1.06	5.76	4.26	1.35	1.21	0.21
50	5.41	6.39	0.85	6.28	4.90	1.28	1.06	0.31
100	4.78	3.89	1.23	6.49	6.44	1.01	1.12	0.16
500	5.47	2.06	2.66	6.12	4.15	1.48	2.07	0.83
1000	4.80	2.48	1.93	5.85	4.02	1.46	1.70	0.34

Table C.6: Densitometry analysis of RT-PCR of IFN1 mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of IFN1 for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	IFN1	28s	IFN1/28s	IFN1	28s	IFN1/28s		
0	6.53	7.71	0.85	5.943	7.71	0.77	0.81	0.05
30	5.93	8.57	0.69	6.354	8.57	0.74	0.72	0.03
60	5.43	6.30	0.86	6.004	6.30	0.95	0.91	0.06
75	5.27	5.77	0.91	5.58	5.77	0.97	0.94	0.04
90	6.41	6.38	1.01	4.538	6.38	0.71	0.86	0.21
105	6.03	4.62	1.30	4.891	4.62	1.06	1.18	0.17
120	6.69	4.92	1.36	4.119	4.92	0.84	1.10	0.37
150	5.72	6.23	0.92	5.085	6.23	0.82	0.87	0.07
180	6.58	5.12	1.29	6.336	5.12	1.24	1.26	0.03
240	5.97	5.10	1.17	6.292	5.10	1.23	1.20	0.04
Dose (ng/mL)								
0	5.53	4.52	1.22	6.848	4.52	1.52	1.37	0.21
5	6.37	7.34	0.87	5.386	7.34	0.73	0.80	0.09
10	6.40	6.74	0.95	6.502	6.74	0.96	0.96	0.01
50	6.70	6.97	0.96	7.15	6.97	1.03	0.99	0.05
100	6.77	5.66	1.20	6.079	5.66	1.07	1.14	0.09
500	1.04	3.65	0.29	6.659	3.65	1.83	1.06	1.09
1000	6.63	4.41	1.50	6.234	4.41	1.41	1.46	0.06

Table C.7: Densitometry analysis of RT-PCR of IFN2 mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of IFN2 for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	IFN2	28s	IFN2/28s	IFN2	28s	IFN2/28s		
0	5.34	7.46	0.71	4.382	5.68	0.77	0.74	0.04
30	6.88	8.22	0.84	4.684	5.79	0.81	0.82	0.02
60	5.10	7.01	0.73	5.964	6.16	0.97	0.85	0.17
75	5.77	6.53	0.88	6.382	6.18	1.03	0.96	0.11
90	5.64	5.62	1.00	6.31	6.20	1.02	1.01	0.01
105	6.69	5.84	1.15	6.682	6.33	1.06	1.10	0.06
120	5.25	4.62	1.14	7.097	5.48	1.29	1.22	0.11
150	6.46	6.07	1.06	6.561	5.68	1.15	1.11	0.06
180	6.72	5.48	1.23	6.756	6.00	1.13	1.18	0.07
240	7.09	5.73	1.24	5.066	6.05	0.84	1.04	0.28
Dose (ng/mL)								
0	5.56	4.81	1.16	6.747	6.01	1.12	1.14	0.02
5	6.37	6.15	1.04	6.442	5.58	1.15	1.10	0.08
10	6.25	5.71	1.09	4.667	5.01	0.93	1.01	0.11
50	6.50	6.74	0.96	4.203	5.15	0.82	0.89	0.10
100	5.32	5.64	0.94	4.883	5.10	0.96	0.95	0.01
500	4.79	3.93	1.22	4.606	4.80	0.96	1.09	0.18
1000	4.74	4.44	1.07	3.407	3.63	0.94	1.00	0.09

Table C.8: Densitometry analysis of RT-PCR of MHIIα mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of MHIIα for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	MHIIα	28s	MHIIα/28s	MHIIα	28s	MHIIα/28s		
0	5.66	6.39	0.89	5.66	6.63	0.85	0.87	0.02
30	5.32	7.20	0.74	5.32	6.24	0.85	0.80	0.08
60	5.84	5.99	0.97	5.84	6.83	0.86	0.92	0.08
75	4.75	6.93	0.69	4.75	7.02	0.68	0.68	0.01
90	6.56	5.94	1.10	6.56	5.83	1.13	1.11	0.01
105	6.12	5.43	1.13	6.12	6.00	1.02	1.07	0.07
120	5.87	4.64	1.27	5.87	5.10	1.15	1.21	0.08
150	6.19	5.63	1.10	6.19	5.97	1.04	1.07	0.04
180	6.27	5.09	1.23	6.27	5.11	1.23	1.23	0.00
240	6.05	5.65	1.07	6.05	6.07	1.00	1.03	0.05
Dose (ng/mL)								
0	5.80	5.27	1.10	5.61	5.10	1.10	1.10	0.00
5	6.06	5.99	1.01	5.97	5.45	1.10	1.05	0.06
10	5.77	6.17	0.94	5.94	4.67	1.27	1.10	0.24
50	6.23	7.75	0.80	5.83	5.17	1.13	0.97	0.23
100	6.26	6.37	0.98	5.29	4.60	1.15	1.07	0.12
500	5.39	4.22	1.28	5.36	4.45	1.20	1.24	0.05
1000	5.86	5.34	1.10	4.79	4.15	1.15	1.13	0.04

Table C.9: Densitometry analysis of RT-PCR of S25-7 mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of S25-7 for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	S25-7	28s	S25-7/28s	S25-7	28s	S25-7/28s		
0	5.53	5.17	1.07	5.64	3.80	1.48	1.28	0.29
30	6.41	8.23	0.78	4.74	5.00	0.95	0.86	0.12
60	5.75	6.73	0.85	5.73	5.64	1.02	0.94	0.11
75	6.08	8.42	0.72	6.32	7.86	0.80	0.76	0.06
90	6.79	7.50	0.90	5.11	6.48	0.79	0.85	0.08
105	5.66	5.34	1.06	4.75	6.68	0.71	0.89	0.25
120	5.39	5.07	1.06	6.08	6.39	0.95	1.01	0.08
150	4.73	5.85	0.81	5.70	6.17	0.92	0.87	0.08
180	6.67	5.01	1.33	6.18	5.78	1.07	1.20	0.18
240	7.01	5.96	1.17	6.59	6.61	1.00	1.09	0.13
Dose (ng/mL)								
0	6.59	4.83	1.36	5.70	5.20	1.10	1.23	0.19
5	6.29	5.37	1.17	3.68	5.30	0.69	0.93	0.34
10	6.60	6.08	1.09	6.26	3.56	1.76	1.42	0.48
50	6.66	8.09	0.82	5.60	4.87	1.15	0.99	0.23
100	6.51	4.54	1.43	7.04	5.29	1.33	1.38	0.07
500	0.87	3.17	0.27	5.44	5.04	1.08	0.68	0.57
1000	6.47	4.64	1.39	5.01	3.94	1.27	1.33	0.09

Table C.10: Densitometry analysis of RT-PCR of CD4 mRNA in RTS-11 treated with rCK-2. Values are corrected for the expression of the internal control gene, 28s. The average relative expression levels of CD4 for each sample are presented with standard deviations.

Time (min)	Trial 1			Trial 2			Average	St. Dev.
	CD4	28s	CD4/28s	CD4	28s	CD4/28s		
0	5.84	7.72	0.76	4.40	5.73	0.77	0.76	0.01
30	6.33	7.27	0.87	5.07	5.07	1.00	0.93	0.09
60	5.59	6.82	0.82	5.66	7.33	0.77	0.80	0.03
75	5.60	6.92	0.81	5.95	7.03	0.85	0.83	0.03
90	5.27	5.82	0.90	6.84	6.97	0.98	0.94	0.05
105	5.49	5.76	0.95	7.20	6.89	1.05	1.00	0.07
120	4.66	3.67	1.27	6.60	6.38	1.03	1.15	0.17
150	5.09	5.53	0.92	5.85	6.42	0.91	0.92	0.01
180	6.03	4.39	1.37	6.54	6.88	0.95	1.16	0.30
240	6.61	5.57	1.19	6.58	6.58	1.00	1.09	0.13
Dose (ng/mL)								
0	5.63	4.64	1.21	6.07	5.62	1.08	1.15	0.09
5	7.90	7.10	1.11	5.41	5.03	1.08	1.09	0.03
10	6.89	6.14	1.12	5.14	4.18	1.23	1.18	0.08
50	8.05	8.43	0.96	4.38	4.43	0.99	0.97	0.02
100	5.79	6.34	0.91	4.52	4.89	0.93	0.92	0.01
500	4.56	3.57	1.28	4.98	3.76	1.33	1.30	0.03
1000	4.69	4.32	1.09	3.48	2.72	1.28	1.18	0.14

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