Developing and characterizing a salmonid intestinal epithelial cell line for use in studies of inflammation in the fish gastrointestinal tract

by

Atsushi Kawano

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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

An intestinal cell line from rainbow trout, *Oncorhynchus mykiss*, was developed and challenged against several bioactive components. Primary cultures initiated from the distal segment produced the cell line, RTgutGC. RTgutGC showed optimal growth in L15 supplemented with 10-20% fetal bovine serum (FBS) at room temperature. RTgutGC has undergone over 100 passages and stained minimally for β-galactosidase, suggesting this to be an immortal cell line. Late passage cultures gave a consistent polygonal morphology with distinct borders. RTgutGC stained positive for alkaline phosphatase (AP) under certain culture conditions, hence may produce intestinal-specific alkaline phosphatase (IAP). Lipopolysaccharide (LPS) was used as a model microbial endotoxin for determining the sensitivity of the cells to a natural ligand in the gastrointestinal tract (GIT). Exposure of LPS was compared between RTgutGC and two mammalian intestinal cell lines (HT-29 and Caco-2). LPS induced cell death in RTgutGC, potentially through an alternative pathway seen in higher vertebrate response. Cytotoxicity of LPS against RTgutGC, seeded at normal density, was reduced in the presence of glutamine compared to L15 alone (t test, $p \leq 0.05$). RTgutGC seeded at a super density, where AP was strongly expressed, also showed less toxicity towards LPS. Two isoforms of tumor necrosis factor alpha (TNF-α) transcripts were up-regulated after LPS treatment in RTgutGC. Six rainbow trout cell lines, including RTgutGC, showed constitutive transcript expression of several immune-related genes: Major Histocompatibility (MH) class II α and ß. When MH activity was examined at the protein level, the cell lines showed constitutive expression of MH class I proteins, but not for MH class II molecules. RTS11, a rainbow trout spleen monocyte/ macrophage-like cell line, was the only line to express all MH transcripts and proteins. The utility of the anti-rainbow trout MH protein sera was demonstrated by exposing RTgutGC to poly IC. After a 3 day treatment, RTgutGC showed up-regulation of β2m protein expression. Thus, the cellular and immunological responses in fish intestinal cells can be modeled using the methods presented in this study.

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Dedication

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Chapter 1

General Introduction

GENERAL INTRODUCTION

The aim of this project is to develop and characterize a salmonid intestinal cell line. There are many pathological conditions associated with fish intestines; however, current research strategies rely heavily on whole-organism experiments. To date, there are no established intestinal salmonid cell lines. Benefits of establishing salmonid intestinal cell lines include improving aquaculture and other aspects related to fish intestinal functions.

1.1. BASICS OF THE GASTROINTESTINAL TRACT (GIT)

The mammalian gastrointestinal tract (GIT) plays a primary role in regulating processes necessary to obtain energy from ingested nutrients. In vertebrates, the entire length of the GIT contains four distinct layers: mucosa, sub-mucosa, muscularis, and adventitia or serosa. The mucosa regulates digestion, absorption, osmoregulation, and host defense. Intestinal epithelial cell types found in the mucosa include enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and M cells. Enterocytes represents the majority of epithelial cells found in the small intestine. Brush border enzymes are secreted by enterocytes to initiate digestive activities. Goblet cells are used to secrete mucous for lubrication to assist in the passing of feedstuffs. Enteroendocrine cells regulate hormonal responses in the intestinal region. Defensive actions are performed by Paneth and M cells. Paneth cells contain antimicrobial peptides known as defensins, as well as protective enzymes such as lysozyme and phospholipase A. M cells intake antigens from the lumen through phagocytosis and transport them to underlying Peyer's patches. The sub-mucosa, a loose connective tissue, provides structural support for the mucosa. Muscular contractions propel feedstuffs through the GIT. These movements are performed by the muscularis layers. The muscularis layers are supported by the adventitia in the esophagus or the serosa within the abdominal cavity.

1.1.1. GIT stem cells

Location of the GIT stem cell niche remains unidentified; however, mounting evidence provides encouraging hypotheses to explain its nature and function. Under normal conditions, epithelial cells of the GIT replenish every 2-7 days (Brittan and Wright, 2004a). The process of replacing damaged tissue may increase the rate of renewal by producing new cell progenitors (Brittan and Wright, 2004b).

Sequential compartmentalization along the crypt-villi axis characterizes the architecture of the mammalian small intestine. The crypt of Lieberkühn represents the proliferative region containing a rapidly renewing population of undifferentiated cells (Potten et al., 1997). Differentiated cells reside in the functional region, or villus. The intestinal mucosa comprises multiple cell lineages within the crypt-villus axis. Multipotent stem cells (MSCs) of the small intestine produce functional cell lineages from the proliferative region (Leedham et al., 2005). Intestinal stem cells appear to be anchored in the proliferative region and divides asymmetrically to sustain the cell population at the villus. Differentiated cells will migrate based on their functional role in the small intestine. Paneth cells are the only intestinal cell to remain in the crypt; the other intestinal cells migrate along the crypt-villus axis towards the lumen (Okamoto and Watanabe, 2004). In the colon, migration of differentiating stem cells displays characteristics from both the stomach and small intestine. MSCs of the large intestine are thought to be located in the mid-crypt of the ascending colon (Wright, 2000) and in the crypt base of the descending colon (Karam, 1999).

1.1.2. Properties of fish GIT

Fish share a similar GIT organization compared to higher vertebrates. However, fish posses a unique absorptive organ and specialized regions are absent in some fish. In some fish, appendages branch from the posterior portion of the pyloric sphincter called pyloric caeca (Buddington and Diamond, 1987). Pyloric caeca are structurally similar to the proximal intestine, hence contributes to food digestion and assimilation (Buddington and Diamond, 1987). Formation of caeca differs between species by the numbers formed and length (Buddington and Diamond, 1987). The predisposed feeding habits of fish influence its ability to secrete digestive enzymes and process certain nutrients. Digestive processes are initiated at the mucosal surface, particularly the epithelium. In the intestine, the tissue layers are arranged into folds to increase the surface area for digestion. The epithelial cells can also form villi or microvilli to further increase the surface area.

Structurally, fish fail to show a clear distinction between the small and large intestine. Different intestinal regions are designated by anatomical terms of location. The intestines of teleostean fish are composed of the proximal and distal segments. Some fish have distinct mid

intestinal segments. Distal intestinal regions have relatively larger diameters and higher densities of goblet cells in the epithelium (Reifel and Travill, 1979). The proximal intestine predominantly absorbs digested lipids (Garrido et al., 1993; Lie et al., 1987), proteins (Sire and Vernier, 1992) and carbohydrates (Moon, 2001). The distal intestine contains pinocytotic vacuoles involved in the absorption of whole proteins (Gautheir and Landis, 1972). There are four prominent cell types in the fish intestine that are derived from MSCs: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. The anatomical composition of fish intestines also depends on food availability and temperature. In times of food deprivation, salmonid fish experience a loss of intestinal mass through mucosal alterations, leading to decreased surface area for absorption. Prolonged exposure to colder temperature promotes an increase in intestinal mass. This unique response may result from the increased accumulation of RNA as a result of reduced RNA degradation (Foster et al., 1992).

1.2. PROTECTIVE MECHANISMS OF THE GIT

The GIT contains a substantial amount of resident microorganisms classified as either endogenous or transient. These microorganisms can manipulate nutrient absorption in fish by interacting with dietary intake. The intestinal segment maintains proper functioning by utilizing several host defense mechanisms including an intestinal epithelial barrier and complex immune system. The intestinal epithelial layer can regulate mucosal surface activities through specialized anatomical structures called tight junctions. Tight junctions control specific membrane pumps and channels to maintain proper flow of ingested content and prevent invasion of pathogenic microbes. Microorganisms possess conserved molecular patterns that are recognized by host organisms. All vertebrate hosts activate an innate immune response against microorganism when these conserved patterns are detected. Lipopolysaccharide (LPS), found on the outer membrane of gram negative bacteria, represents the most documented microbial component to stimulate the immune response. Activation of the innate immune response can trigger mechanisms associated with the adaptive immune response.

1.2.1. Physical barrier

Adjacent intestinal epithelial cells form specialized contacts to facilitate proper physiological processes. These contacts arise in the most apical pole of the junctional complex

and consist of an array of protein species, most notably, occludin and claudin (Yu and Yang, 2009). Tight junctions regulate paracellular transport by restricting passage to small, hydrophilic molecules, and ions (Baumgart and Dignass, 2002). Depending on the physiological situation, these structures can alter their degree of phosphorylation to accommodate the appropriate action (Baumgart and Dignass, 2002). A breach in these tight junctions could lead to pathogenic antigens penetrating the epithelial layer, thereby triggering an inflammatory response.

1.2.2. Innate immune response

In most vertebrate cells, microbes are initially detected through toll-like receptors (TLRs) that bind pathogen-associated molecular patterns (PAMPs). TLRs recognize broad patterns associated to commonly encountered microbes and not found on host cells. Due to the existence of a microbiota, the intestinal epithelial cells have developed intricate immunological processes to distinguish between commensal and pathogenic bacteria (Rescigno et al., 2008). For instance, differential location of TLRs may contribute to maintaining the symbiotic relationship. Certain TLRs that recognize bacterial PAMPs have been found at both the apical and baso-lateral epithelial membranes. Activation of the apical receptors tends to induce minimal pro-inflammatory responses. Interestingly, activation of baso-lateral TLRs can initiate an extensive production of inflammatory mediators (Lee et al., 2006). Other mechanisms seen in the innate immune response to eliminate foreign invader involves the use of different granulocytes, mast cells, and multiple glycoproteins associated with the complement system.

1.2.3. Adaptive immune response

In some situations, the pathogen might survive the innate immune response. These cases require the activation of the adaptive immune response to eliminate the pathogen. The adaptive immune response uses two major types of lymphocytes: B and T lymphocytes. Activated B lymphocytes bind to B-cell receptors (BCR), thereby developing antibodies with the same specificity for the antigen that triggered activation, generally called clonal expansion. Antibodies are utilized in the extra-cellular spaces of the body and bind to pathogens. T lymphocytes differ from B lymphocytes because they fail to bind antigen directly, and only recognize pathogen-derived antigens presented on the surface of host cells. Fragments of foreign peptides are presented on the surface from endogenous or exogenous protein sources by T-cell

receptors (TCR). These protein antigens are delivered to the surface bound to major histocompatibility complex (MHC) molecules. MHC molecules can be classified as either MHC classes I or II.

1.3. MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

In most vertebrates, major histocompatibility molecules are encoded from a cluster of genes and referred to as major histocompatibility complex (MHC). MHC class I molecules are found on surface of all nucleated cells. Structurally, the MHC class I molecule contains two sub-units: membrane bound heavy alpha chain non-covalently attached to a light chain termed beta-2-microglobulin (β2m). MHC class I present antigens derived from intracellular pathogens to immune cells. MHC class II molecules are less distributed, with the majority of expression being found on professional antigen presenting cells (APCs) (Glimcher and Kara, 1992). Similar to MHC class I, the heterodimeric MHC class II molecule contains two components: membrane bound alpha and beta units. MHC class II molecules present antigens derived from extra-cellular pathogens to immune cells.

1.3.1. MHC class I molecules

To eliminate intracellular altered polypeptides, MHC class I molecules are expressed to activate $CD8^+$ T-cells. Dysfunctional intracellular proteins are ubiquitylated and undergo proteasomal degradation. Peptides are brought into the endoplasmic reticulum (ER) for antigen presentation by the transporter associated with antigen processing (TAP) complex (Vyas et al., 2008). MHC class I molecules are transported into the ER by Sec 61 (Wiertz et al., 1996). Proper folding of the individual chains that comprise the MHC class I molecule are guided by two main chaperons: calnexin (CNX) and Erp57 (Cresswell et al., 1999). After heterodimer formation, calreticulin replaces CNX as the primary chaperone to facilitate final association with the TAP complex (Cresswell et al., 1999). The peptide-loaded MHC class I molecules are translocated to the cell surface through the Golgi complex.

There are reported cases of MHC class I molecules presenting exogenous peptides referred to as cross presentation (Bevan, 1976a, 1976b). Cross presentation requires exogenous peptides to be processed by proteosomes and actively transported into the ER (Guermonprez and Amigorena, 2005). The exact mechanism of this process remains unclear. Evidence has

suggested that dendritic cells (DC) have the necessary tools to cross-present exogenous peptides on MHC class I molecules (Guermonprez and Amigorena, 2005).

1.3.2. MHC class II molecules

MHC class II molecule assembly involves different molecules compared to MHC class I; however, the general steps of antigen presentation are similar. MHC class II molecules present foreign antigens from extracellular sources to activate $CD4^+$ T-cells. Antigens brought in from the extracellular environment are placed in phagosomes, a membrane delimited compartment (Bryant and Ploegh, 2004). This compartment eventually fuses with lysosomes to form phagolysosomes, an acidic environment that facilitates the degradation of proteins (Bryant and Ploegh, 2004). MHC class II molecules, produced in the ER, are delivered to the phagolysosome through vescular transport (Bryant and Ploegh, 2004). Peptide-loaded MHC class II molecules are eventually transported to the cell surface through a tubule protruding from the phagolysosome.

The processes involved in MHC class II antigen presentation appears to be regulated, in some part, by TLR activation. In studies evaluating the role of antigen binding coupled with TLR-4 bound to its ligand, showed this complex can activate T cells to a greater extent compared to the antigen alone (Blander and Medzhitov, 2006). Furthermore, the presence of LPS can also contribute to more effective peptide loading of MHC class II molecules (Blander and Medzhitov, 2006).

Along with conventional phagosomal processing of MHC class II peptides, other mechanisms are quickly emerging. Autophagy represents the most heavily studied alternate to the endocytic pathway. Lysosomal degradation associated with autophagy might contribute to processing antigens from pathogens in the cytosol or within phagosomes (Schmid and Munz, 2007). Thus, utilization of autophagy possibly expands the source of peptides presented on the MHC class II molecules to include some endogenous antigens.

1.4. INFLAMMATION

Inflammation represents one of the essential processes to control the spread of infection. The process involves recruiting macrophages, neutrophils, and other immune cells into the site of infection. The fundamental processes of inflammation include providing immunological effector

molecules to eliminate the pathogen by inducing blood clots, and repairing damaged tissue. Improper regulation of inflammation can be associated with severe conditions. Examples include sepsis, some forms of cancer, and inflammatory bowel disease (IBD).

1.4.1. Inflammatory Bowel Disease (IBD)

In North America, close to 10000-50000 new cases of inflammatory bowel disease are reported every year (Loftus, 2004). Hence, the detrimental effects of IBD have accelerated research concerning intestinal immune studies. Mucosal inflammations associated with IBD are represented by two major forms: ulcerative colitis (UC) and Crohn's disease (Xavier and Podolsky, 2007). Main features of UC include proximally extending mucosal inflammation from the rectum and development of superficial mucosal ulceration (Xavier and Podolsky, 2007). Crohn's disease involves aggregation of macrophages that form granulomas (Xavier and Podolsky, 2007). Unlike UC, Crohn's disease can potentially affect any site along the GIT (Xavier and Podolsky, 2007). Although the exact cause of IBD remains to be elucidated, promising studies have shed light on this problematic disease. Patients with IBD tend to have intestinal epithelial cells with modulated expression levels of TLRs. In both diseases, TLR-4 becomes significantly up-regulated (Shi et al., 2006). Recent studies indicate that that Crohn's disease patients may have a severely compromised innate immune response (Yamamoto-Furusho and Podolsky, 2007). When these patients were injected with killed bacteria, neutrophil accumulation and interleukin-8 (IL-8) production were decreased compared to healthy individuals (Marks et al., 2006). Thus, inheriting genes that result in a modified innate immune system, especially in TLR expression, may predispose patients to developing IBD. Other mediators of IBD include smoking, appendectomy, and environmental factors (Loftus, 2004).

1.5. AQUACULTURE AND FISH ENTERITIS

Aquaculture industries around the world produce over 30 million metric tons of fish and seafood products annually (Naylor et al., 2000). The potential to produce a constant supply of protein makes aquaculture one of the more popular solutions to reduce, and eventually eliminate, food shortages. In developing countries, mainly in Africa, establishing aquaculture practices represents an important step in alleviating the demand for affordable food. Countries in Africa are experiencing a decrease in per capita fish supplies due to falling wild stocks (von Bubnoff,

2005). Hence, reviving fisheries in Africa depends on making aquaculture cheap and practical. Currently, farming fish involves growing different species in enclosed conditions to facilitate optimal growth. Variations are seen country to country; however, all industries, in one way or another, depend on fish meal to raise fish. Fish-meal feeds are derived from small fishes and waste products from fisheries (Naylor et al., 2000). The small fish used to compose fish meal are acquired through aquaculture and/or wild stocks. Thus, reducing the amount of fish-derived inputs represents an essential component for aquaculture to thrive in the future.

Long-standing limitations associated with alternative fish feeds are preventing international large scale expansions of aquaculture. For decades, hundreds of studies have been dedicated to evaluating the potential of feeds formed from various sources, especially plants and cereals. Although plants provide adequate nutritional components, these sources contain anti-nutritional factors that limit their incorporation into feeds for aquaculture. Examples of these include soybean, wheat, rapeseed, and corn (Hardy, 1996). Although current economic trends are unpredictable, easy distribution of these commodities to multiple world sectors makes them a favored resource to exploit in aquaculture industries. Unfortunately, many studies have noted drawbacks to incorporating high percentages of plant-based meals in aquaculture feeds (Francis et al., 2001). Plants differ in their amino acid composition; some plants contain low concentrations of essential amino acids (EAA) (Csaky and Feteke, 2004). For example, soybeans, regarded as one of the more promising fish-feed alternatives, contain low levels three EAA: lysine, methionine, and threonine (Csaky and Feteke, 2004). Fish lack the necessary enzymes to digest complex carbohydrates found in soybeans and other legumes (Buddington et al., 1997). Most importantly, plants also contain anti-nutritional factors. Anti-nutritional factors are substances that interfere with food utilization, thereby negatively impacting the health of animals (Francis et al., 2001).

Unprocessed plant-based diets can lead to detrimental changes in the mucosa of the fish gastrointestinal tract (Buddington et al., 1997). The primary effects of fish fed plant-based diets are an increase in the number of goblet cells and substantial loss of absorptive function (Hardy, 1996). These abnormalities are attributed to the anti-nutritional factors found within plants. Studies using various animal models indicate lengthened exposure to anti-nutritional factors

reduces enterocytic activities by weakening structural integrity and shortening brush borders (Knudsen et al., 2007). The most bioactive anti-nutritional factors that are problematic to salmonid fish *in vivo* include lectins, protease inhibitors, phytoestrogens and saponins.

Among the anti-nutritional factors, saponins are likely the most detrimental to farming salmonid fish (Knudsen et al., 2007). Saponins are secondary plant metabolites composed of a water soluble sugar residues attached to a lipid soluble steroidal aglycone, or sapogenin (Lasztity et al., 1998). They are natural surfactants with several several biological actions. They can increase membrane permeability and stimulate the immune response (Knudsen et al., 2008). Saponins are found in virtually all terrestrial plants (Lasztity et al., 1998). At least five sapogenins are present in soybeans, called soyasaponins, and linked to varying sugar groups (Yoshiki et al., 1998). Salmonid species fail to digest soyasaponins and interact with the epithelial surface of the GIT (Moon, 2001). This exposure facilitates the onset of enteritis in Atlantic salmon fed full-fat soybean based fish feeds possibly by increasing permeability of epithelial cells in the distal segment (Knudsen et al., 2008).

Several processes have been employed to counteract these components during experimental feed developments. These methods include extrusion treatments, incorporation of dietary enzymes, and genetic modification of oilseeds and grains (Barrows et al., 2007; Infante and Cahu, 2007). Although significant progress has been made to introduce small portions of plant-based diets into aquaculture, the presence of anti-nutritional factors remains the leading obstacle for completely replacing fishmeal with plant products. Fortunately, the use of alternative feeds has steadily increased over the years due to increased knowledge about anti-nutritional factors and improvement of processing techniques.

1.6. GOALS OF THIS PROJECT

The goal of this project is to develop and characterize an adult rainbow trout GIT cell line to better understand the factors that influence intestinal properties. Firstly, this cell line will be evaluated to determine optimal growth conditions, ability to immortalize, and expression of selected immune response genes. Finally, this cell line will be exposed to pathogenic components to evaluate cellular and immune responses.

Chapter 2

Development of a fish cell line (RTgutGC) from an intestinal segment of rainbow trout to evaluate cytotoxic responses induced by Escherichia coli derived lipopolysaccharide (LPS)

OVERVIEW

An immortal cell line, RTgutGC, was developed from the distal intestinal segment of an adult rainbow trout (*Oncorhynchus mykiss*). RTgutGC, grown routinely in Leibovitz's L15, achieved optimal growth when supplemented with 20% fetal bovine serum (FBS). At room temperature, RTgutGC appeared epithelial-like in morphology. The cultures have been sub-cultivated over 100 times, consistently split with a ratio of 1:2 after growth container became confluent, and cryoperserved. Along with undergoing a relatively high number of passages, RTgutGC also failed to express significant amounts of β-galactosidase. Suggesting RTgutGC represents an immortal cell line. At high cell density, RTgutGC expressed a potential intestinal marker, alkaline phosphatase (AP). Cytotoxicity of lipopolysaccharide (LPS) from *Escherichia coli*, an endotoxic component of gram negative bacteria, was evaluated against RTgutGC. LPS treatment for 24 h caused cell viability to decrease in a dose-dependent manner. Cellular response to LPS was altered with cell density and L-glutamine supplementation. At low cell densities, RTgutGC were sensitive to 50 μg/mL LPS and induced death after 24 h. Supplementation with 2mM L-glutamine provided modest protection against LPS exposure when seeded at a normal density. RTgutGC seeded at densities capable of producing alkaline phosphatase, LPS induced minimal toxicity at high concentrations. Thus, glutamine and possibly AP protect against LPS cytotoxicity. Cell viability was evaluated with Alamar Blue (AB) for metabolic activity and carboxyfluoroscein diacetate acetoxymethyl ester (CFDA AM) for membrane integrity. This cell line represents a valuable tool in assessing intestinal epithelial properties of rainbow trout.

2.1. INTRODUCTION

The majority of mammalian intestinal *in vitro* studies are performed using cell lines. Mammalian cell lines have been established from most of the anatomical regions of the gastrointestinal tract (GIT): esophageous, stomach, small intestine, and colon/ rectum. The majority of these cell lines are from human GIT tumors. These include epithelial cell lines from esophageal adenocarcisquamous and squamous cell carcinoma (Boonstra et al., 2007), gastric adenocarcinoma (Li et al., 2002), and colorectal carcinoma (Oh et al., 1999). Conditionally immortalized cell lines have also been developed from the fetal small intestine (Quaroni and Beaulieu, 1997). Other examples include intestinal cell lines from chicken and rats that have spontaneously immortalized in culture (Quaroni et al., 1979; Velge et al., 2002). More complicated cases of deriving immortalized cell lines from the GIT involve transgenic mice carrying the SV40 ts-T-antigen (Obinata, 2007).

Due to the lack of normal intestinal epithelial cell lines, human colon carcinoma cell lines have been used as the representative model. Hundreds of human colon carcinoma cell lines are available through repository organizations. Several cell lines are able to produce differentiated properties representing the various cell types found in the intestinal epithelium. In 1964, Jorgen Fogh developed the first human colon carcinoma cell line, HT-29 (Simon-Assman et al., 2007). HT-29 exhibits properties of a multipotent intestinal cell line. The cells are undifferentiated when grown in normal media conditions. Interestingly, HT-29 can differentiate by altering media composition and supplementation with inducers (Simon-Assman et al., 2007). Various conditions have shown to facilitate differentiation of HT-29: removing glucose as an energy source, addition of alternate energy sources, and using alternate carbon sources (Simon-Assman et al., 2007). Differentiated HT-29 cells produce brush-border enzymes and/or goblet cells. The Caco-2 cell line represents the most extensively used intestinal model *in vitro* due to its ability to spontaneously differentiate into cells that exhibit properties of enterocytes (Engle et al., 1998). Non-confluent cultures are undifferentiated; however, well-differentiated cells are apparent after monolayer formation. After differentiating, Caco-2 cells can express brush border enzymes, form domes and tight junctions (Engle et al., 1998).

Cell models have contributed to understanding the complex processes associated with the immune response. In particular, mammalian cell lines are essential tools to explore the cellular mechanisms involved in the innate immune response. Through evolutionary encounters, vertebrate immune cells have developed receptors to recognize common molecular patterns unique to different pathogens. Among the many pathogenic components recognized by vertebrates, lipopolysaccharide (LPS) molecules are the most heavily researched due to the multiple symptoms it can induce through exogenous and endogenous sources. Endogenous microbiota contains gram negative bacteria that possess LPS on their outer membrane. Structurally, LPS is composed of three distinct segments: lipid A, inner core, outer core, and O-specific chain (Rietschel et al., 1994). The lipid portion, lipid A, represents the pathological component of LPS. Lipid A contains two phosphorylated N-acetyl glucosamine groups attached to a fatty acid chain and a variable acylation pattern (Rietschel et al., 1994). The core polysaccharides are divided into the inner core and outer core. The inner core includes heptose (Hep) and 2-keto3-deoxyoctonic acid (KDO) (Rietschel et al., 1994). Compared to the inner core, limited information is available for the diverse outer core structure.

Cellular decomposition of gram negative bacteria liberates LPS molecules in host organisms. Higher vertebrates possess defensive mechanisms capable of recognizing the structural features from pathogenic microbes, including LPS. Profuse exposure to pathogenic organisms can lead to undesirable physiological responses induced by excessive production of immunological cytokines. In mammalian models, LPS induce immunological responses with relatively low concentrations. Interestingly, fish (and other lower vertebrates) require high concentrations of LPS to generate similar results seen in mammals. Although fish contain similar pathogen recognition pathways as mammals, the components utilized in mammalian LPS activation may be lacking or involved in other processes (Iliev et al., 2005). Thus, much debate exists regarding the response of fish to LPS.

LPS activation of the mammalian toll-like receptor 4 (TLR-4) signaling pathway represents the most understood member of the TLR family. The process begins when LPS molecules in the circulatory system bind to LPS-binding protein (LBP) (Aderem and Ulevitch, 2000). This complex eventually binds to the CD-14 receptor located on the cell surface (Aderem

and Ulevitch, 2000). Monomeric segments of LPS, primarily composed of lipid A molecules, are translocated to LY96 (also known as MD2) attached to the TLR-4 receptor, thereby activating the TLR-4 pathway (Aderem and Ulevitch, 2000). TLR-4 mediates two pathways: MyD88-dependent (Burns et al., 1998) and MyD88-independent (Horng et al., 2001). Activation of the MyD88-dependent pathway stimulates the activation of p38, a class of mitogen activating protein kinases, that eventually leads to the translocation of nuclear factor kappa beta (NF-κβ) into the nucleus to produce tumor necrosis factor alpha (TNF-α) and other pro-inflammatory genes (Aderem and Ulevitch, 2000). Activation of the MyD88-independent pathway can also produce inflammatory mediators through NF-κβ. However, the MyD88-independent pathway activates another set of kinases to produce antiviral protein interferon, interferon beta (IFN-β) (Aderem and Ulevitch, 2000).

A substantial amount of resources are committed to protecting the GIT. The most prominent of these mechanisms involves close regulation of microbial penetration into intestinal cells and preferential expression of TLRs. Most recent intestinal epithelial studies have suggested protective effects provided by glutamine supplementation and expression of intestinal alkaline phosphatase (IAP). Glutamine has been found to have both nutritive and protective roles *in vivo* (Souba et al., 1990; Sukhotnik et al., 2007) and the cellular basis of these roles have been studied successfully with human intestinal cell lines (Lenaerts et al., 2006; Turowski et al., 1994). In zebrafish, IAP plays an important role in building mucosal tolerance to LPS by dephosphorylating the lipid A component (Bates et al., 2007). However, not all intestinal cell lines express alkaline phosphatase (Nollevaux et al., 2006; Velge et al., 2002).

Although fish show similar GIT features as higher vertebrates, surface interaction processes are poorly understood at the intestinal epithelial level. Mammal intestinal cell lines have been useful in studying some aspects of LPS tolerance, but at high concentrations significantly reduce viability of these cells (Abreu et al., 2001; Bocker et al., 2003; Lenoir et al., 2008). In order to study the complex interactions seen at the intestinal surface of fish, particularly to LPS, an intestinal cell line was developed from rainbow trout. Fish cell lines have been prepared from most tissues and organs, but not from the GIT segment (Bols and Lee, 1991). The first step in obtaining a cell line involves the preparation of primary cultures from

the cells, tissues or organs of fish. There are several successful cases of developing fish GIT cell cultures, including the initiation of primary cultures from spotted sand bass (Guzman-Murillo et al., 2000). This report discusses the successful development of a rainbow trout intestinal cell line, termed RTgutGC. Initial experiments characterized RTgutGC based on multiple parameters. Optimal growth conditions were determined using Leibovitz's L15 as the basal medium and supplementation with fetal bovine serum (FBS). To evaluate the cell line as a valid and sustainable *in vitro* model, the cells were screened for their ability to produce several expression markers. After characterizing RTgutGC, the cell line was used to determine the effects of LPS on cell viability by exposing cultures to varying concentrations of LPS under different conditions. End points for cell viability were determined by monitoring metabolic activity and cell membrane integrity after LPS treatments.

2.2. MATERIALS AND METHODS

2.2.1. Materials

The following products were purchased from Sigma-Aldrich (Oakville, ON): Leibovitz's L15 (L15), Fetal Bovine Serum (FBS), penicillin-streptomycin solution, gentamicin solution, trypsin from bovine pancreas, Dulbecco's phosphate buffered saline (D-PBS), tissue grade water, GenElute™ mammalian Total RNA miniprep kit, leukocyte alkaline phosphatase kit, senescence cell histochemical staining Kit, and lipopolysaccharide (LPS) from *Escherichia coli*.

2.2.2. Cell culture

Primary cultures of rainbow trout intestinal segments from a small female rainbow trout (*Oncorhynchus mykiss*) were prepared and developed into a cell line, RTgutGC, by Dr. Kristin Schirmer. The fish was not fed for two days prior to sampling and anesthetized in an aqueous solution of 1:10 000 tricaine methanesulphonate (MS222; Syndel, Vancouver, BC). The GIT was carefully removed and cleaned with tissue cell culture grade water. Segments were placed in D-PBS free of Ca^{2+} and Mg^{2+} supplemented with gentamicin. Thereafter, pieces of approximately 1 mm^3 were isolated from the distal intestinal portion and placed in 12.5cm^2 tissue culture flasks. Distal intestinal segments were grown in L15 supplemented with 30% FBS and 1% penicillin-streptomycin solution. The medium was adjusted to barely cover the tissue fragments. The flasks were placed in a 20°C incubator in ambient air and monitored regularly. The medium was exchanged every two weeks. After a period of eight weeks, one flask was sub-cultivated using versene and 0.1% (w/v) trypsin into another 12.5cm^2 tissue culture flask containing L15 supplemented with 20% FBS. Eventually, RTgutGC cultures were maintained at room temperature in 75cm^2 tissue culture treated flasks containing L15 supplemented with 10% FBS and 1% penicillin-streptomycin solution. Cultures were sub-cultivated into two flasks (1:2) every 4-7 days. The passage numbers used in this study are 75-112.

2.2.3. Proliferation assay

RTgutGC cells were plated into 12-tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NY) at a density of 5.0 x 10^4 cells per well in 2 mL of L15 supplemented with 10% FBS and allowed to attach overnight. After attachment, the cells are washed and replaced with media containing desired serum composition. Total number of cells counted every 3 days

over a 15 day time period using the Coulter Z2 particle count and size analyzer (Coulter, Burlington, ON).

2.2.4. β-galactosidase detection

RTgutGC cultures were tested for β-galactosidase using a cell senescence kit (Sigma). Approximately 1.0 x 10^6 cells were plated into a 35 mm^2 tissue culture Petri dishes (Falcon) and grown to confluency overnight. The cells were fixed with 1X fixation buffer from the kit. Cells were exposed to the staining mixture for 12 hours. Images were taken with a Nikon Eclipse TS100 inverted phase contrast microscope and Nikon Coolpix 8400 digital camera.

2.2.5. Alkaline phosphatase detection

RTgutGC cultures were tested for alkaline phosphatase (AP) using a histo-chemical leukocyte alkaline phosphatase kit (Sigma). Approximately 1.0 x 10^6 cells were plated into slide flasks (Nunc, Roskilde, Denmark). The cells were fixed using a citrate-buffered acetone solution and washed with deionized water. Cells were exposed to the substrate solution composed of AS-MX phosphate alkaline solution combined with diazonium salt for 2.5 hour. Cells were counterstained with Mayer's hematoxylin solution (Sigma) for 10 minutes. Images were taken with a Nikon Eclipse TS100 inverted phase contrast microscope and Nikon Coolpix 8400 digital camera.

2.2.6. Cytotoxicity assay

RTgutGC cells were plated into 96-microwell culture plates (Falcon, Becton Dickinson, Franklin Lakes, NY) at a density of 4.0 x 10^4 cells per well in 200 μL L15 supplemented with 10% FBS and grown overnight. RTgutGC cells were washed and replaced with L15 media without serum. Cells were exposed to various concentrations of LPS. All exposures were conducted at room temperature for 24 hours. Cell viability after exposure conducted using two fluorescent dyes: Alamar Blue and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester). These dyes were used concurrently and prepared in L15ex to give final concentrations of 5% (v/v) Alamar Blue and 4 μM CFDA-AM. Cells were exposed to indicator dyes for approximately 1 hour and quantified with the SPECTRAmax® GEMINI XS microplate reader (Molecular Devices, Sunnyvale, CA at respective excitation and emission wavelengths of 530 (±30) and 595 (±35) for Alamar Blue, and 485 (±22) and 530 (±30) for CFDA-AM.

2.2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cells collected at desired time points after LPS treatment. Pellet washed with D-PBS, RNA extracted using GenElute mammalian total RNA miniprep kit (Sigma). RNA quantified using NanoDrop 100 (Thermo Scientific, Wilmington, DE). 2 µg RNA incubated with DNase I for 30 minutes at 37°C and 5 minutes at 75°C to remove genomic DNA. cDNA from RNA obtained using the following steps. 1 µL of 0.5 µg/mL oligo-$(dT)_{23}$ anchored primer (Sigma) added to each DNase treated RNA sample and heated to 70°C for 10 minutes and put on ice. 4 µL 5X buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; Invitrogen, Burlington, ON), 1 µL 10 mM deoxynucleotide triphosphate mix (dNTP; Fermentas, Burlington, ON), 2 µL 0.1 M dithiothreitol (DTT; Invitrogen), and 80 U Superscript III reverse transcriptase (Invitrogen) added to each sample. Samples equilibrated at room temperature for 10 minutes. Samples incubated for 50 minutes at 42°C and 5 minutes at 95°C. Resulting cDNA stored at -80°C. All polymerase chain reaction (PCR) reactions contained: 0.5 µL 10 mM dNTP mix, 1.25 U Taq polymerase (Sigma), 1.5 mM $MgCl_2$ (Sigma), 2.5 µL 10X reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl; Sigma), 1.25 µL 10 µM forward and reverse primers (Sigma), 2.5 µL undiluted cDNA and nuclease free water to a total volume of 25 µL. The primer sequence, cycle number, and annealing temperature for each primer listed in Table 1. PCR reactions carried out using Mastercycler personal thermocycler (Eppendorf, Mississauga, ON). Cycle conditions for each reaction: 5 minutes at 95°C, a set number of cycles with 95°C for 30 seconds, 30 seconds at primer specific annealing temperature, a set number of cycles with 75°C for 1 minute, and a final extension at 72°C for minutes. PCR products visualized on 1.8% agarose gel with 15 minute post-stain in 0.5 µg/mL ethidium bromide (EtBr) and 15 minute de-stain in MiliQ water. UV trans-illumination performed using Fluorochem 8000 (Alpha Innotech, San Leandro, CA).

Table 2-1 Summary of primers used in study, including PCR product size and number of cycles.

Gene	Primers	Annealing temperature	Product size (bp)	Cycle Number	Primer Reference
β-actin (Gene Accession: AJ438158)	F 5' ATCGTGGGGCGCCCCAGGCACC 3' R 5' CTCCTTAATGTCACGCACGATTTC 3'	53°C	514	30	(Brubacher et al., 2000)
TNF-α1 (Gene Accession: NM_001124357)	F 5' TGGCTATGGAGGCTGTGTGGGGTC 3' R 5' GCCTTCGCCAATTTCGGACTCAGC 3'	68°C	512	30	Unpublished
TNF-α2	F 5' TGGAGAGGGGCCTTGAAAATAG 3' R 5' CGTCCTGCATCGTTGCCA 3'	68°C	206	30	(Komatsu et al., 2008)

2.2.8. Data analysis

Statistical analyses were done using GraphPad InStat (version 3.00 for Windows 95, GraphPad Software, San Diego, CA, www.graphpad.com). For graphical presentation and derivation of effective concentration (EC_{50}) values, the data was processed with GraphPad Prism 4 (GraphPad Software, San Diego, CA) using a variable slope dose-response sigmodial model. EC_{50}s values were compared using an unpaired t-test, with results indicated on Table 2-2. In all cases a *P*-level ≤ 0.05 was considered significantly different.

2.3. RESULTS

2.3.1. RTgutGC development and characterization

In order to create a fish intestinal cell line, adult rainbow trout (*Oncorhynchus mykiss*) were sacrificed and dissected. Enzymatic treatments with trypsin were used to release individual cells from intestinal tissue segments. A rainbow trout intestinal cell line, RTgutGC, developed by sub-cultivating primary cultures derived from the distal portion. Cell morphology was examined using a phase contrast microscopy. Early passage cultures (P1-10) appeared to have mixed cultures of fibroblast-like and epithelial-like cells (Figure 2-1). Long-term cultures eventually produced consistent cobblestone-shaped epithelial-like cells (passage cultures above 70; Figure 2-1). Staining of late passage RTgutGC cultures failed to stain significant amounts of β-galactosidase, a marker for cellular senescence in cell cultures (Figure 2-2). Hence, RTgutGC cultures might be an immortal cell line. Based on molecular genotyping methods, these cells were sequenced by the Canadian Barcode of Life Network (Guelph, ON) to verify the origin of species as rainbow trout (data not shown). At low densities, RTgutGC cells only proliferated in L15 basal media with FBS supplementation (Figure 2-3). RTgutGC did not proliferate in basal media without FBS or with 10% dialyzed FBS (dFBS). Optimal growth was seen with 20% FBS supplementation; however, sufficient grow was apparent at 10% FBS.

2.3.2. Alkaline phosphatase (AP) expression in RTgutGC

Production of alkaline phosphatase (AP) in RTgutGC was found to be density-dependent (Figure 2-4). RTgutGC produced AP when seeded at a density approximately double required to produce a monolayer (referred to as super density), but not at any density below. AP represents one of many embryonic cell markers; hence, an embryonic zebrafish cell line (ZEB2J) was used as the positive control (data not shown; Xing et al., 2008).

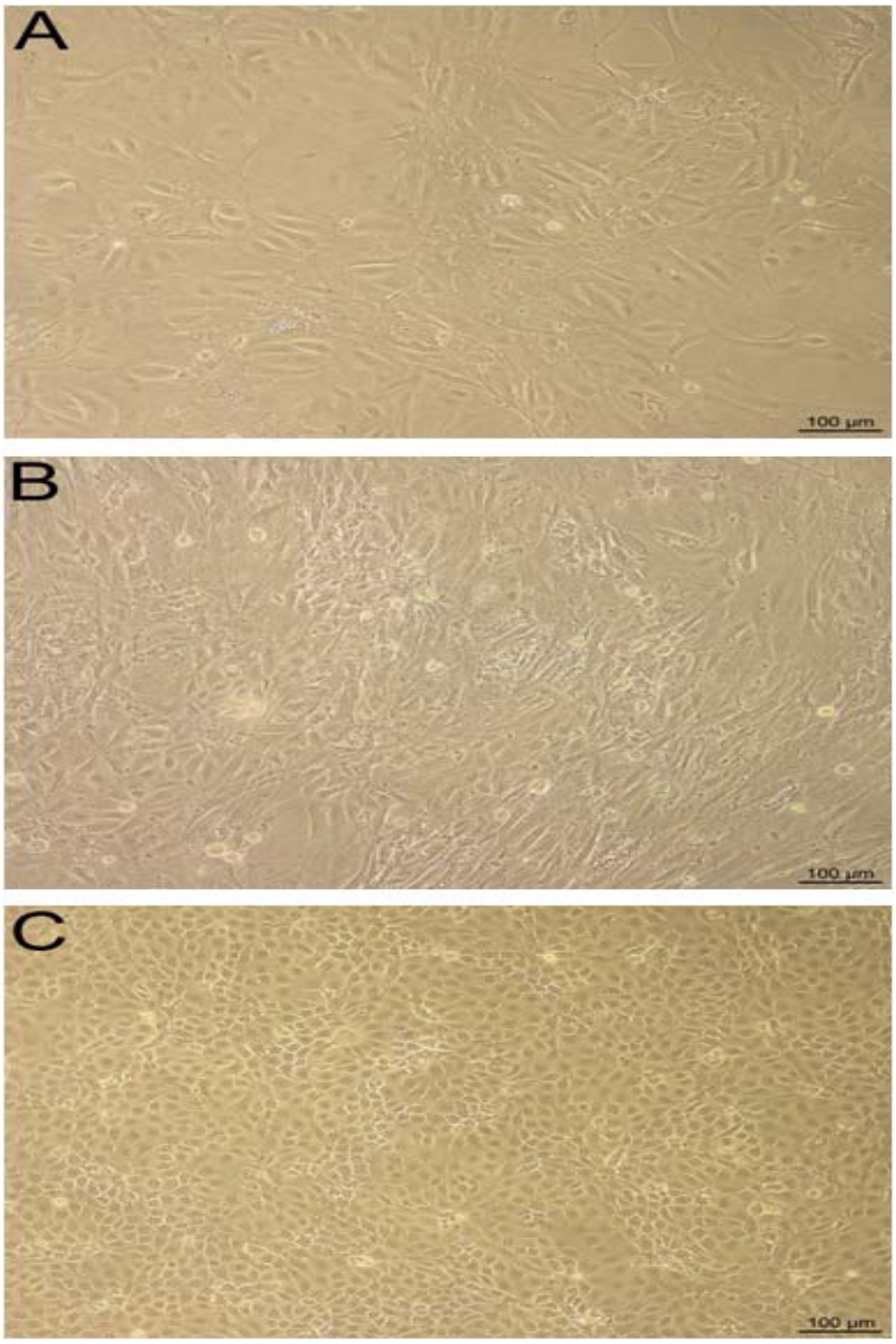

Figure 2-1. Appearance of RTgutGC cultures at different passage numbers.

Photomicrographs of passage 6 (*A*), 12 (*B*), and above 100 (*C*) were taken on an inverted phase contrast microscope. *Scale bar* indicates 100µm.

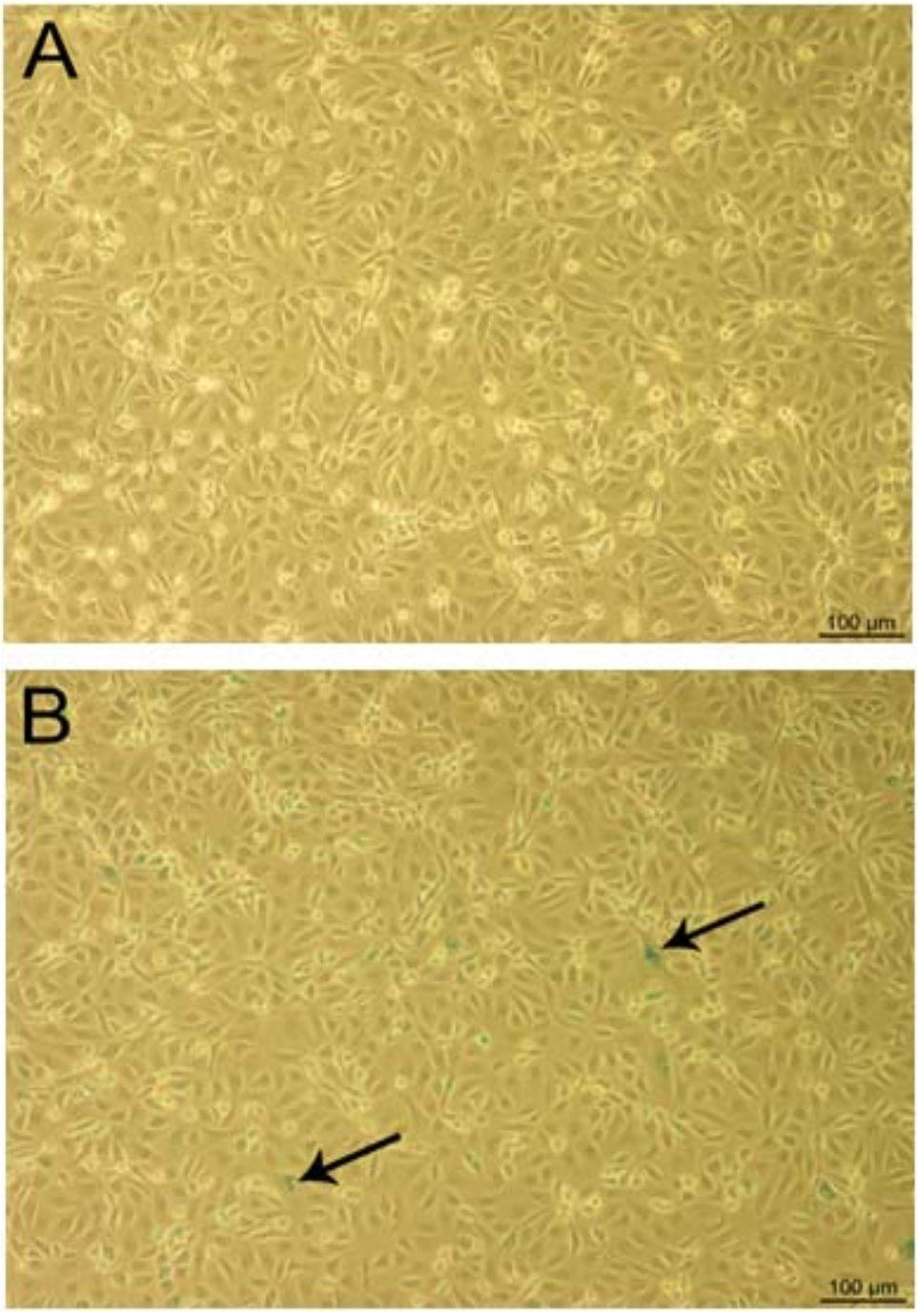

Figure 2-2. Examination of RTgutGC cultures for β-galactosidase activity.

A histochemical stain for β-galactosidase activity was applied overnight to cultures of RTgutGC. Photomicrographs are shown for fixed (*A*) and stained (*B*) cells. Cells showing blue staining were found in a small percentage of RTgutGC cultures, with some indicated by *arrows*. *Scale bar* indicates 100 µm

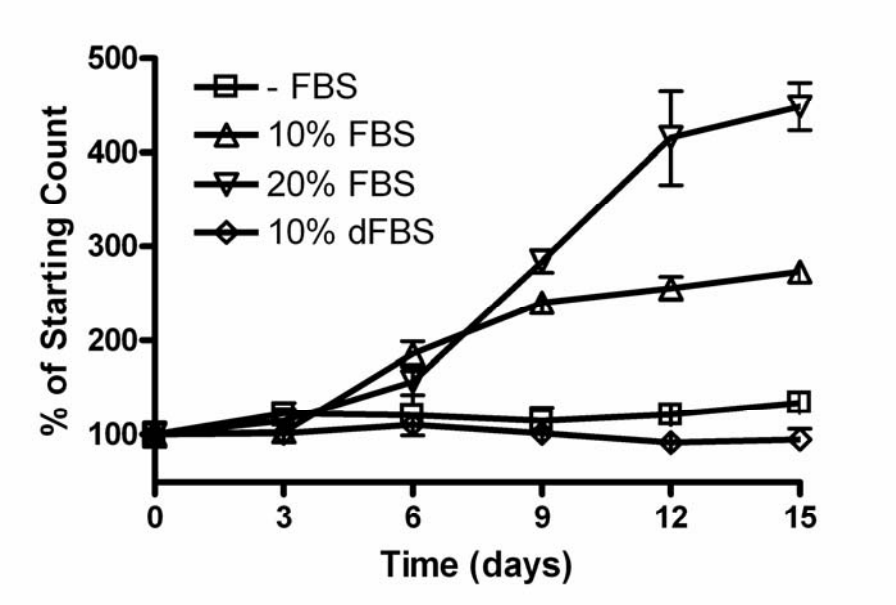

Figure 2-3. Growth curves for RTgutGC grown in different L15 media compositions.

Cultures were initiated at approximately 5.0 x 10^4 cells per well in 12-well tissue culture plates at room temperature. The next day, cell number was determined with a Coulter counter for three wells (starting count) and cells were grown in L15 alone, 10% fetal bovine serum (FBS), 20% FBS, or 10% dialyzed FBS (dFBS). Subsequent cell counts were made every 3 days over a 15 day period and expressed as a percentage of the starting count.

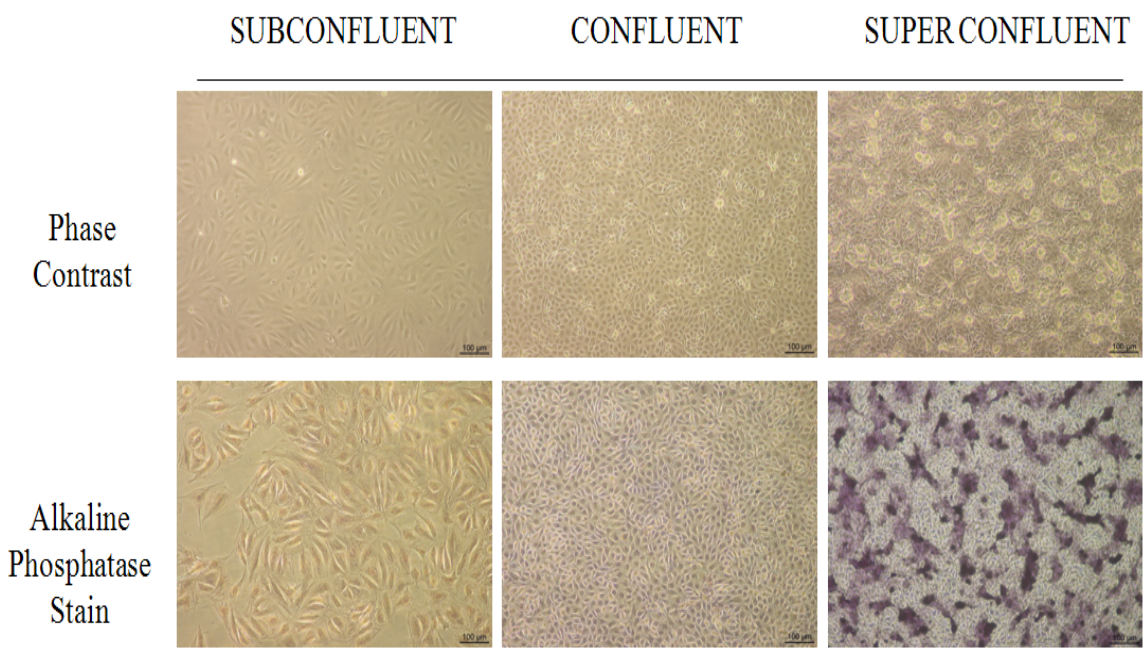

Figure 2-4. Examination of RTgutGC cultures for alkaline phosphatase activity at varying cell densities.

A histochemical stain for alkaline phosphatase activity was applied for 2.5 h to cultures for RTgutGC. The top row shows RTgutGC seeded at varying densities prior to detecting alkaline phosphatase activity. The bottom row shows RTgutGC seeded at varying densities after alkaline phosphatase staining. *Scale bar* indicates 100 µm.

2.3.3. Response of intestinal cell lines to lipopolysaccharide (LPS)

The viability of RTgutGC cultures to increasing concentrations of LPS was compared in L15ex and L15 alone, with and without 2 mM glutamine. L15ex consists of L15 salts supplemented with galactose and pyruvate. RTgutGC cultures were grown to confluency in 96-well plates and exposed to LPS for 24 h. RTgutGC cells without glutamine showed more pronounced changes in appearance, compared to cultures with glutamine (Figure 2-5). Exposure to above 15 μg/mL LPS brought about a dose-dependent response in RTgutGC, reducing relative fluorescent readings of Alamar Blue (AB) and carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Figure 2-6). In both cases, the dose response curves for both AB and CFDA-AM were shifted to the right in the presence of glutamine, producing higher effective concentration (EC_{50}) values (Table 2-1). For a comparison with mammalian intestinal epithelial cell lines, cultures of HT-29 and Caco-2 seeded at 4.0×10^4 cells per well in DMEM with glutamine, were exposed to increasing concentrations of LPS and evaluated for viability after 24 h with AB and CFDA-AM. Mammalian intestinal cell lines, HT-29 and Caco-2, required a higher concentration range (0-500 μg/mL) to produce dose-response curves (Figure 2-6). Comparison of EC_{50} calculations between RTgutGC and the mammalian intestinal cell lines were significantly different from one another (Table 2-1). These values with the human cell lines were higher than the EC_{50}s for RTgutGC at 4.0×10^4 cells in L15 with glutamine.

2.3.4. Effect of cell density on LPS cytotoxicity

Significant differences in cell viability measurements in response to LPS were observed with changes in cell density. RTgutGC cells seeded at varying densities (1.0×10^4 to 8.0×10^4 cells per well) in 96-well plates exposed to 50 μg/mL LPS for 24h. Endpoints were evaluated with AB and CFDA-AM (Figure 2-7). Both AB and CFDA-AM indicated a decline in cell viability; however, the magnitude of the decline depended on the cell density. RTgutGC cells seeded at 1.0×10^4 cells per well showed a 79.2% ± 6.6 (n=2) for AB and 68.9 ± 10.7 (n=2) for CFDA-AM. In contrast, when cells were seeded at 8.0×10^4 cells per well viability decreased 8.2% ± 7.6 (n=2) for AB and 5.0% ± 7.0 (n=2) for CFDA-AM.

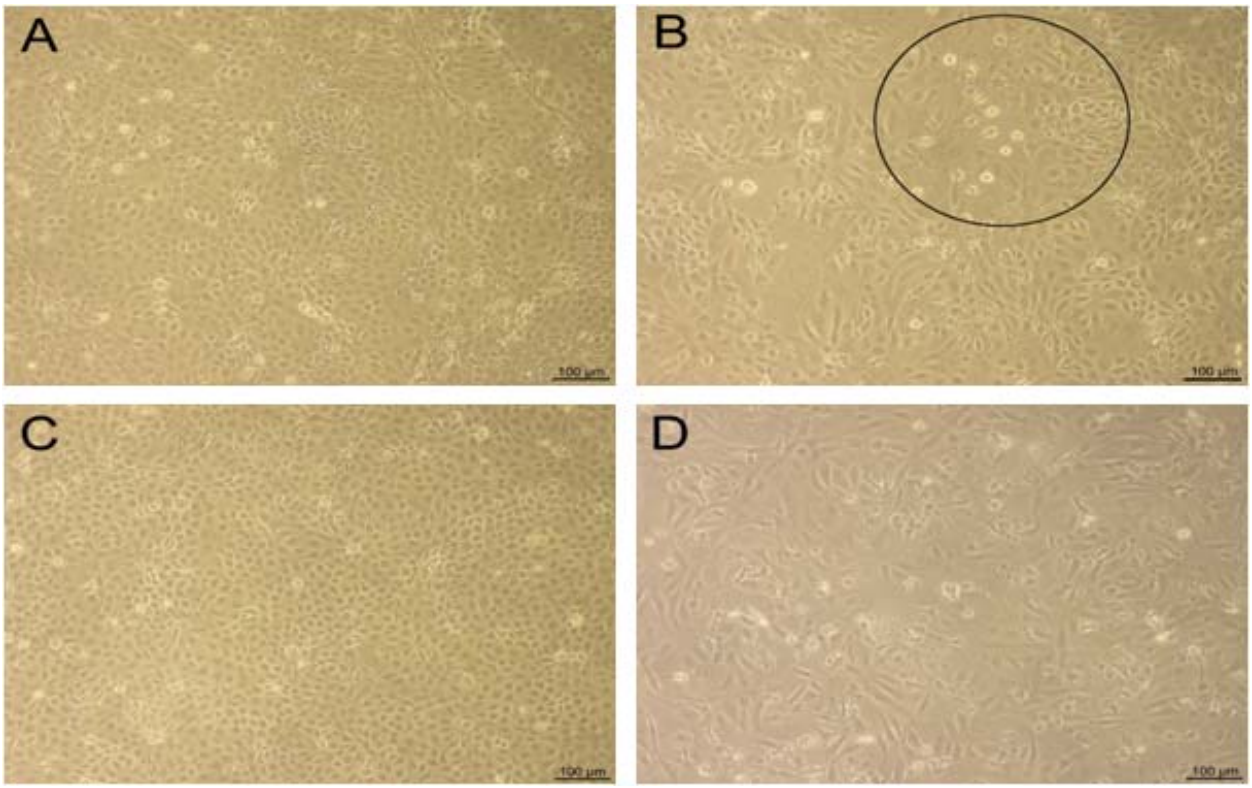

Figure 2-5. Appearance of RTgutGC cultures after lipopolysaccharide (LPS) treatment.

RTgutGC cells were seeded into 25cm^2 tissue culture flasks in L15 with and without glutamine. After 24 h exposure with 50 µg/mL LPS with glutamine, images were taken: control (*A*) and dosed (*B*). Similarly, images were taken of exposures without glutamine: control (*C*) and dosed (*D*). Some areas of cytotoxicity are indicated with a *circle*. *Scale bar* indicates 100µm.

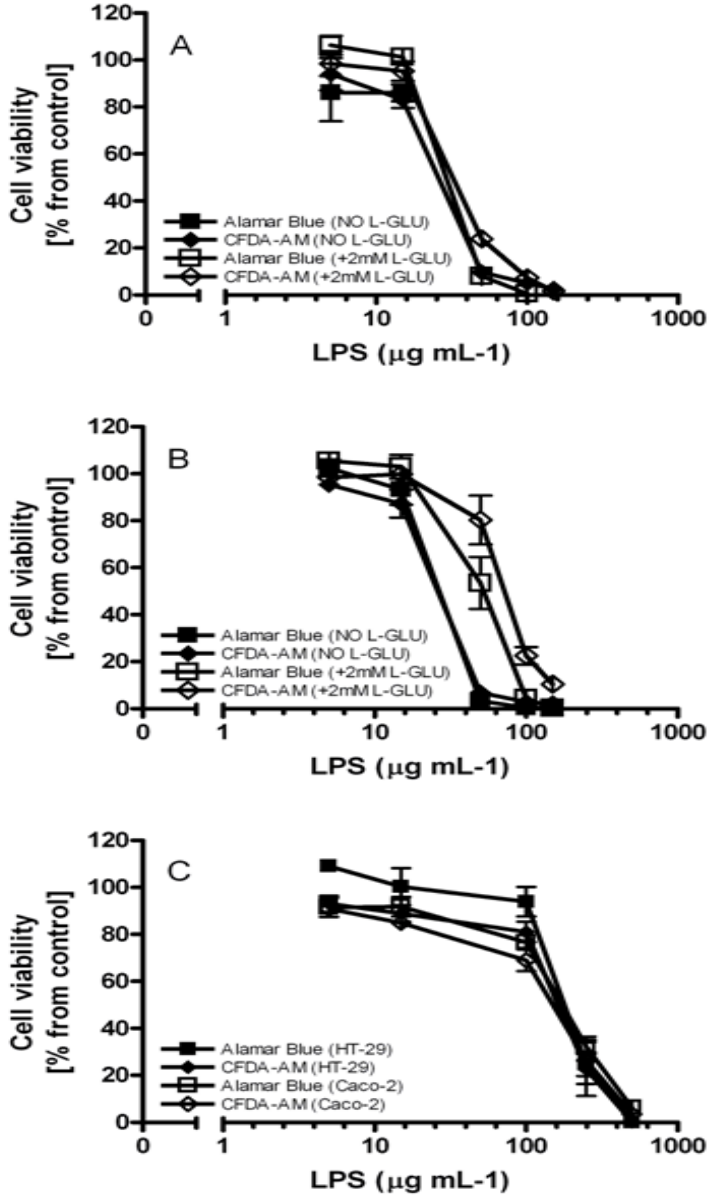

Figure 2-6. Cytotoxicity curves of three intestinal cell lines after lipopolysaccharide (LPS) treatment.

Cultures were initiated at approximately 4.0 x 10^4 cells per well in a 96-well culture plate. The next day, varying concentrations of LPS were directly dosed to six wells for each concentration. RTgutGC was exposed to LPS with and without glutamine in L15ex (*A*) or L15 (*B*). Two mammalian intestinal cell lines, HT-29 and Caco-2, were exposed to LPS in DMEM (*C*). After 24 h the viability of cultures was evaluated with Alamar Blue for energy metabolism and CFDA-AM for cell membrane integrity. Both assays used fluorescent dyes and read as relative fluorescent units (RFUs), which were expressed as percentages of the RFUs in control wells. The means with standard deviations for the percentage cell viability with the two assays are plotted against LPS concentration for one of two independent experiments.

Table 2-2 Cytotoxicity of LPS to intestinal cell lines

Cell line	Culture conditions [a]		Cell viability endpoints	
	Basal Media	Glutamine	Alamar Blue EC_{50} (n)	CFDA EC_{50} (n)
RTgutGC [a]	L15ex [b]	None	18.3 ± 1.0 (2)	25.7 ± 0.9 (2)
RTgutGC [a]	L15ex [b]	2mM	35.4 ±5.3 (2) [d]	36.2 ±1.1 (2) [d]
RTgutGC [a]	L15 [c]	None	27.0 ±2.3 (2)	30.1 ± 6.9 (2)
RTgutGC [a]	L15	2mM	49.7 ± 2.6 (2) [d]	70.2 ± 4.9 (2) [d]
HT-29 [a]	DMEM	2mM	174.9 ± 2.9 (2)	161.7 ± 2.9 (2)
Caco-2 [a]	DMEM	2mM	173.5 ± 26.7 (2)	156.7 ± 9.8 (2)

Mean LPS concentration (µg/mL) causing 50% decline in cell viability (EC_{50}).

[a] 4.0×10^4 cells per well

[b] L15 salts supplemented with sodium pyruvate and galactose

[c] Leibovitz's L15 without glutamine

[d] Statistically significant (t test; $p \leq 0.05$) compared to equivalent endpoint in basal media without glutamine

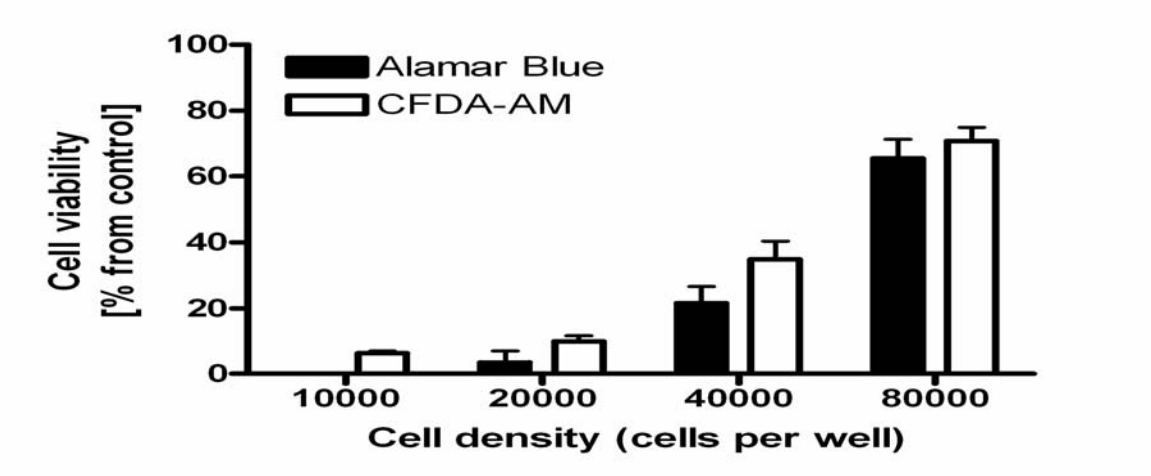

Figure 2-7. Bar graphs of RTgutGC exposed to LPS seeded at varying cell densities.

Cultures were seeded at approximately 1.0 x 10^4, 2.0 x 10^4, 4.0 x 10^4, or 8.0 x 10^4 cells per well in a 96-well culture plate with L15. The next day, a fixed LPS concentration (50 µg/mL) was directly dosed to six wells for each cell density. After 24 h the viability of cultures was evaluated with Alamar Blue for energy metabolism and CFDA-AM for cell membrane integrity. Both assays are fluorescent and read as relative fluorescent units (RFUs), which were expressed as percentages of the RFUs in control wells. The means with standard deviations for the percentage cell viability with the two assays are plotted against LPS concentration for one of two independent experiments.

2.3.5. TNF-α induction in RTgutGC by LPS

RTgutGC seeded at 7.0 x 10^6 cells per 75cm^2 tissue culture flask was exposed to 20 μg/mL LPS for 4 h without glutamine or 50 μg/mL for 24 h with glutamine. All cells were plated in L15 media without FBS supplementation. Two isoforms of tumor necrosis factor alpha (TNF-α) expression were monitored at the transcript level (Figure 2-8). Gene expression appeared stronger after the 24 h exposure relative to the 4 h exposure.

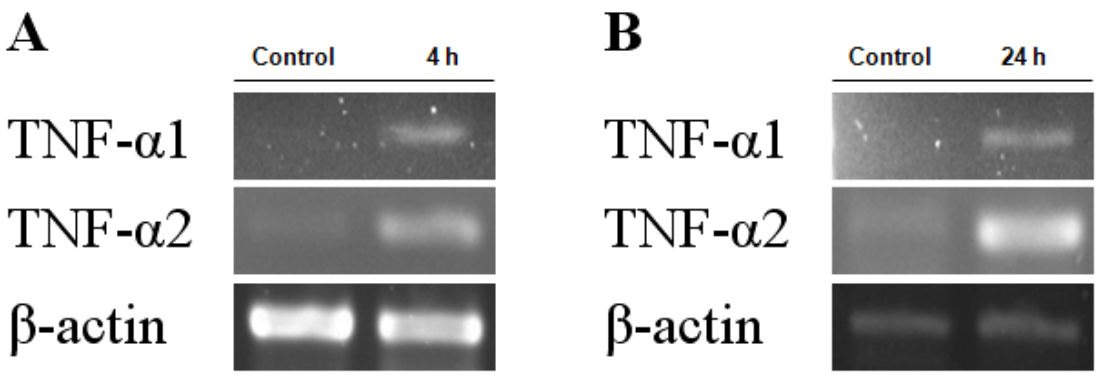

Figure 2-8. The effect of LPS exposure on TNF-α expression in RTgutGC.

RTgutGC cultures were exposed to 20 µg/mL LPS for 4 h (*A*) or 50 µg/mL LPS for 24 h (*B*). Cells were collected and RT-PCR performed to measure TNF-α expression. Individual bands represent expression of TNF-α1, TNF-α2, and β-actin at the transcript level as indicated in the labels on the figure.

2.4. DISCUSSION

A cell line, RTgutGC, is described for the first time from the gastrointestinal tract (GIT) of fish. RTgutGC was confirmed as derived from rainbow trout, *Oncorhynchus mykiss*, by amplifying and sequencing a 652 bp region of the mitochondrial cytochrome c oxidase I gene (COI), which has been used to barcode fish (Hubert et al., 2008). This sequence covers the 648-bp segment of the 5' region of the COI gene that had been used to form the library of primary barcodes for the animal kingdom (Hebert et al., 2003) and that had been used to successfully identify many other cell lines (Cooper et al., 2007). The development and basic characteristics of RTgutGC were similar to other cell lines from rainbow trout. The cell line seemed to be continuous as some cultures have been maintained continuously for 4 years, subjected to over 100 passages, and expressed minimal levels of β-galactosidase. The cell line was heteroploid. Nearly all rainbow trout cell lines developed to date appear to have immortalized spontaneously and are heteroploid (Bols et al., 1995). Like these cell lines, RTgutGC required serum for growth and could be cryopreserved. RTgutGC cells had an epithelial-like morphology. Several epithelial-like cell lines have been developed from a variety of adult rainbow trout tissues, including the gill, liver, pituitary, and spleen (Bols et al., 1995; Bols et al., 1994; Ganassin and Bols, 1999; Lee et al., 1993). However, in comparison to them, the cells in confluent cultures of RTgutGC are more uniformly cobblestone. RTgutGC should be a useful tool for investigating many topics on fish intestinal epithelial cells. This is illustrated below by investigating their requirement for glutamine, their expression of alkaline phosphatase and their cellular response to lipopolysaccharide (LPS).

The survival and growth of RTgutGC in medium without glutamine is similar to the behaviour of some mammalian intestinal epithelial cell lines and similar to non-intestinal fish cell lines. The rat intestinal cell line, RIE-1, underwent apoptosis upon glutamine starvation (Papaconstantinou et al., 1998). By contrast, RTgutGC survived in the complete absence of glutamine. Previously fish cell lines from embryos and from the liver, spleen, and skin of adults also were shown to survive in L15 in the absence of serum and glutamine (Bols et al., 1994). With a supplement of serum, L15 without glutamine supported the proliferation of these fish cell lines (Bols et al., 1994) and in this study RTgutGC. Thus, only the small amount of glutamine in

serum was sufficient to support the growth of these cell lines. Similar results have been obtained with the Caco-2 cell line. This human intestinal cell line grew in the absence of glutamine in basal medium that was supplemented with growth factors or FBS (Lenaerts et al., 2006; Turowski et al., 1994). At least one fish cell line grew in the complete absence of glutamine. CHSE-214, an embryonic cell line from Chinook salmon, proliferated in L15 with dialyzed FBS and no glutamine (Bols et al., 1994). However, RTgutGC did not. Rather than being a difference between the two cell lines, the results might reflect the use of dialyzed FBS with a molecular weight (MW) cutoff of 1,000 for CHSE-214 and 10,000 for RTgutGC. With the higher 10,000 MW cutoff, more small nutrients and growth factors would have been lost, possibly preventing this dialyzed serum from supporting growth in the absence of glutamine. Further research will be needed to determine whether the response of RTgutGC to glutamine deprivation is general to fish cells or to intestinal epithelial cells.

As with many mammalian intestinal epithelial cell lines, RTgutGC expressed alkaline phosphatase (AP) activity only under some culture conditions. Little or no AP activity was seen in normally grown human and rat intestinal epithelial cell lines, such HT-29, T84, LoVo, and IEC-6 (Herz and Halwer, 1990; Nollevaux et al., 2006). However, AP activity could be induced by a variety of treatments, such as sodium butyrate (Herz and Halwer, 1990; Fukushima et al., 1998). Another human cell line, Caco-2, expressed AP when the cells were grown to confluency (Matsumoto et al., 1990). The AP induced under these conditions was the intestinal form of AP (IAP) and considered a marker of differentiation, as *in vivo* only differentiated enterocytes express IAP. However, for some of these cell lines, other AP isoforms also were induced (Fukushima et al., 1998; Herz and Halwer, 1990). For RTgutGC, AP activity was not detected cytochemically in cultures under normal growth conditions. However, seen in cultures that had been initiated at very high cell densities, where clumps of cells attached on top of the monolayer. Determining the nature of the observed AP as induction of IAP, which has been identified in fish (Bates et al., 2007), and whether this represents enterocyte differentiation will be interesting questions to explore with the cell line in the future.

LPS at concentrations that were broadly similar to the lethal concentrations reported with mammalian intestinal epithelial cell lines reduced cellular viability of RTgutGC in sub-confluent

cultures. Literature values for the LPS concentrations cytotoxic to mammalian intestinal epithelial cells varied, likely due to differences in exposure conditions and endpoints, but ranged from approximately 40 μg/ml for SCBN (Chin et al., 2006) to 600 μg/ml for Caco-2 (Hirotani et al., 2008). When the same endpoints that were used with RTgutGC were applied to HT-29 and Caco-2, the EC_{50}s were between 150 to 175 μg/ml, which were within the published range but higher than with RTgutGC. The differences in the basal media and temperature used to culture cells for the two species may account for the differences between the rainbow trout and human intestinal epithelial cell lines. Fish cells are often considered less sensitive to LPS than mammalian cells (Iliev et al., 2005; Maier et al., 2008), but usually viability has not been the response measured and intestinal epithelial cells have not been compared.

Generally fish cells are thought to be less responsive to LPS because they appear to lack the CD14/LY96/TLR4 recognition and signaling system (Iliev et al., 2005). Mammalian intestinal epithelial cells are known to be hyporesponsive to LPS because they express no or low MD2 (Lenoir et al., 2008) and possibly TLR4 (Abreu et al., 2001; Bocker et al., 2003). Hence, for intestinal cell viability there might be little difference between mammals and fish in their response to LPS. The reduction in cellular viability caused by LPS might be initiated through a mechanism independent of the CD14/TLR4-signaling pathway. Impairment of mitochondrial function represents one action of LPS independent of CD14 (Glover et al., 1996). Changes in Alamar Blue reduction indicates changes in energy metabolism (O'Brien et al., 2001), including mitochondrial activity (Zhang et al., 2004). Thus, the decline in Alamar Blue reduction by RTgutGC in response to LPS might be a CD14/LY96/TLR4 independent response. The steps between the impairment of energy metabolism in RTgutGC and the loss of membrane integrity remain to be elucidated. Furthermore, the loss of cell viability could be associated with apoptosis or by some alternative mode of cell death.

The expression of at least one cytokine could be induced in RTgutGC. The inducer was lipopolysaccharide (LPS) and the cytokine was tumor necrosis factor alpha (TNF-α), which in mammals is a major pro-inflammatory cytokine and an immunoregulator (Cruse and Lewis, 2004). Transcripts for two TNF-α isoforms were induced in RTgutGC. Previous *in vivo* studies have shown the induction of TNF-α2 transcripts in the rainbow trout intestine (Mulder et al.,

2007). Recently, the addition of living *Aeromonas salmonicida*, but not by *Escherichia coli*, to primary cultures of rainbow trout epithelial cells was shown to up regulate TNF-α2 transcripts (Komatsu et al., 2009). Induction was also visualized *in vivo* at the protein level. Immunohistochemistry for TNF-α revealed staining in intestinal epithelial cells of rainbow trout in which *A. salmonicida* had been placed via the rectum into the posterior intestine for six hours (Komatsu et al., 2009). RTgutGC should be a convenient *in vitro* alternative for studying pro-inflammatory cytokine expression in the fish intestine.

The sensitivity of RTgutGC to the toxic actions of LPS was influenced by several culture conditions that hint at possible protective mechanisms. As the cell density of cultures was increased, the cells became less susceptible to LPS. In other culture systems, cell density has been found to modulate the induction of NO synthase by LPS (de Oliveira et al., 2002), but modulation of cell killing by LPS has not been previously reported. One possible reason for the cells being less susceptible to LPS-killing at high cell density was the elevated levels of alkaline phosphatase activity (AP). Recently, several studies have suggested that AP in the gut detoxifies LPS (Bates et al., 2007; Vaishnava and Hooper, 2007). Thus, in the future RTgutGC could be used to study the possible protective action of AP against LPS at the cellular level.

Another protective mechanism could involve glutamine. In the presence of 2 mM glutamine, the dose-response curve for the cytotoxicity of LPS to RTgutGC was shifted to the right, meaning that a higher concentration of LPS was needed to reduce cellular viability by 50%. In mammals, glutamine was found to be generally protective for intestinal epithelial cells both *in vitro* (Chow and Zhang, 1998; Evans et al., 2005) and *in vivo* (Sukhotnik et al., 2007) and appears to achieve this in multiple ways. Glutamine can protect by supporting the synthesis of glutathione and heat shock proteins and by suppressing the induction of cytokines and the activation of apoptosis (Roth, 2008; Evans et al., 2005). The mechanisms responsible for glutamine attenuating LPS remains to be investigated, but the results hint that glutamine could also have a protective role in the fish intestine. Finally, no cytotoxicity was observed when FBS was present. The serum presumably protects by binding with LPS, making it less available to cells, and by generally supporting the health of cells.

Chapter 3

Exploring Major Histocompatibility (MH) expression in fish cell lines

OVERVIEW

Similar to mammals, fish express major histocompatibility (MH) receptors to present peptide antigens to T lymphocytes. This chapter demonstrates MH expression in multiple rainbow trout cell lines using reverse-transcription polymerase chain reaction (RT-PCR) and western blotting. RT-PCR was completed using primers designed against published sequences to determine MH class II transcript expression. Western blotting with previously developed antibodies to rainbow trout MH homologues was used to compare MH protein expression. In total, six cell lines were directly compared: a rainbow trout spleen monocyte/ macrophage-like cell lines, termed RTS11, and five adherent cell lines. The five adherent cell lines originated from different tissues: gonad (RTG-2; fibroblast-like), gill (RTgill-W1; epithelial-like), intestine (RTgutGC; epithelial-like), liver (RTL-W1; epithelial-like), and hepatoma (RTH-149; epithelial-like). All cell lines expressed transcripts for MH class II alpha and MH class II beta genes. However, MH class II polypeptides were only expressed in RTS11. To demonstrate the utility of the anti-sera, RTgutGC was exposed to poly IC and monitored for changes in MH expression. A 3 day treatment with poly IC, RTgutGC showed up-regulation of β2m protein expression. This information makes these cell lines and anti-sera useful in fish MH regulation studies.

3.1. INTRODUCTION

Vertebrates are capable of responding to pathogens using evolutionarily developed host mechanisms. There are two broad immune responses that protect the host of foreign invaders: innate and adaptive. Although each mechanism shows unique features, they must work together to maintain homeostatic activity in the host. Elements of the innate immune response act as the initial barrier to host infection. The response in mammals begins within minutes or hours through activities of specialized cells. After initiating the innate response, several mechanisms are available to eliminate the pathogen: phagocytosis, respiratory burst, complement, and cytokine production. The adaptive immune response relies on the clonal expansion of antigen-specific lymphocytes and requires more time, compared to the innate response, to be fully effective.

In mammals, major histocompatibility genes are confined to a large genetic region with multiple loci in a single chromosome, hence referred to as a complex (MHC) (Cruse and Lewis, 2004). The MHC class I molecules are expressed on the surface of nearly all nucleated cells. Functionally, MHC class I serves to differentiate self/ non-self recognition to circulating $CD8^+$ T lymphocytes. Structurally, MHC class I consist of two polypeptide chains. The heavy chain (or α chain), approximately 44 kDa, contains three domains spanning the membrane (Cruse and Lewis, 2004). The MHC class Iα chain non-covalently binds to a 12 kDa light chain called β_2-microglobulin (β_2m) (Cruse and Lewis, 2004). Genes responsible for producing the light chain of the MHC class I molecule are encoded from a chromosome not associated with the MHC molecules. Although β_2m domains are derived from a different chromosome, its association represents an integral part of the MHC class I molecule. The antigens are presented by the MHC class I molecules are typically of endogenous origin. Proteasomal degradation generates peptides from endogenous pathogens for antigen presentation. Upon peptide loading to the MHC class I molecule in the endoplasmic reticulum, the entire complex eventually translocates to the cell surface.

The MHC class II molecules are expressed primarily in antigen presenting cells (APC): B lymphocytes, dendritic cells, monocytes, macrophages, and thymic cells (Cruse and Lewis, 2004). Expression of MHC class II can also be induced by non-APC in environments rich in

inflammatory cytokines. Examples of non-APC include fibroblasts, and epithelial cells. MHC class II molecules work to present exogenous peptides to $CD4^+$ T-lymphocytes. Two non-covalently associated trans-membrane glycoprotein chains comprise the MHC class II molecule, an alpha segment between 32-34 kDa and a beta segment between 29-32 kDa (Cruse and Lewis, 2004). The peptides presented by MHC class II molecule are processed through the lysosomal pathway.

In lower vertebrates, such as teleost fish, major histocompatibility molecules are encoded from different chromosomes and referred to simply as MH genes (Shand and Dixon et al., 2001; Stet et al., 2003). Although fish MH molecules arise from different linkage groups (Sato et al., 2000), functionality of the molecules appears to be conserved across vertebrate species (Shum et al., 2001; Vallejo et al., 1991). Studies indicate molecular weights of rainbow trout MH polypeptides are similarly sized compared to mammals. Variations might arise due to glycosylation (Nath et al., 2006). Teleost fish, shown to possess MH molecules, produce highest expression levels in immune tissues.

Tissue expression of MHC genes has been extensively studied in mammalian models, and to a lesser extent in fish. Generally, MHC class I proteins are found on nucleated cells with MHC II restricted to professional antigen-presenting cells (APCs) (Cruse and Lewis, 2004). Additionally, some human intestinal epithelial cells have shown to constitutively express MHC class II genes (Mayer et al., 1991). MHC class II proteins can be induced in some other cell types in response to IFN-γ (Radka et al., 1986). The pattern of tissue expression for MHII α and MHII β polypeptides in fish parallels mammalian results. They have been found in leucocytes and hematopoietic tissues (Moulana et al., 2008; Nath et al., 2006). Interestingly, MH II expression has been shown in other tissues, including intestine and gills. However, MH II expression seen in these tissues may have resulted from resident APCs or other exogenous cells.

Mammalian cell lines have contributed to the understanding of MHC expression and regulation. These studies reveal similar expression patterns seen in tissues. Although MH studies in fish are limited, a similar distribution appears to exist in teleosts. In order to determine fish MH activity *in vitro*, multiple cell lines were used to compare expression levels at the transcript and protein level, including RTgutGC. As some mammalian intestinal epithelial cell

lines can express MHC II genes (Van Niel et al., 2003). Further, transcripts for MHIIβ has been detected in rainbow trout intestine (Hansen et al., 1999; Nath et al., 2006), thus this chapter describes the comparison of MH gene expression in RTgutGC along with cell lines from several other fish tissues, including the monoycte/ macrophage cell line from the spleen, RTS11. Protein expression of MH Iα and β2m was shown in all rainbow trout cell lines. However, MH IIα and MH IIβ expression was only evident in RTS11. These anti-sera were also used to monitor MH expression after treatment with a double stranded (ds) RNA, polyinosinic: polycytidylic acid (poly IC), in RTgutGC. This information should make the cell lines and these anti-sera useful for studying the regulation of fish MH genes.

3.2. MATERIALS AND METHODS

3.2.1. Cell culture

In total, 10 cell lines were used in this study. Samples were collected from six rainbow trout cell lines: RTG-2, RTS11, RTL-W1, RTH-149, RTgill-W1, and RTgutGC. All fish cell lines were cultured with L15 supplemented with 10% FBS, unless otherwise stated. Rainbow trout cell lines, except RTS11, were maintained at room temperature. RTS11 cultures were maintained at 20°C with L15 supplemented with 15% FBS. Cross-species comparisons to rainbow trout anti-sera were performed with three fish cell lines (ZEB2J, CHSE-214, and EPC) and HeLa. ZEB2J, derived from embryonic zebrafish cells, was maintained at 26°C. CHSE-214, derived from embryonic Chinook salmon cells, was maintained at 18°C. Epithelioma papulosum cyprinid (EPC), an epithelial carp cell line, was maintained at room temperature. HeLa cultures were maintained at 37°C with 5% CO_2 and grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% FBS.

3.2.2. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Samples from rainbow trout cell lines were collected and centrifuged for 4 minutes at 400 g. Pellets were washed with D-PBS, and RNA extracted using GenElute mammalian total RNA miniprep kit (Sigma). RNA quantified using NanoDrop 100 (Thermo Scientific). 2 µg RNA incubated with DNase I for 30 minutes at 37°C and 5 minutes at 75°C to remove genomic DNA. cDNA from RNA obtained using the following steps. 1 µL of 0.5 µg/mL oligo-$(dT)_{23}$ anchored primer (Sigma) added to each RNA sample and heated to 70°C for 10 minutes and put on ice. 4 µL 5X buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; Invitrogen), 1 µL 10 mM dNTP mix, 2 µL 0.1 M DTT), and 80 U Superscript III reverse transcriptase (Invitrogen) added to each sample. Samples equilibrated at room temperature for 10 minutes. Samples incubated for 50 minutes at 42°C and 5 minutes at 95°C. Resulting cDNA diluted 1:20 in nuclease-free water and stored at -80°C. All polymerase chain reaction (PCR) reactions contained: 0.5 µL 10 mM dNTP mix (Sigma), 1.25 U Taq polymerase (Sigma), 1.5 mM $MgCl_2$ (Sigma), 2.5 µL 10X reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, Sigma), 1.25 µL 10 µM forward and reverse primers, 2.5 µL diluted cDNA and nuclease free water to a total volume of 25 µL. The primer sequence, cycle number, and annealing temperature for each primer listed in Table 1.

PCR reactions carried out using Mastercycler personal thermocycler (Eppendorf). Cycle conditions for each reaction: 5 minutes at 95°C, a set number of cycles with 95°C for 30 seconds, 30 seconds at primer specific annealing temperature, a set number of cycles with 75°C for 1 minute, and a final extension at 72°C for minutes. PCR products visualized on 1.5% agarose gel with 15 minute post-stain in 0.5 μg/mL EtBr and 15 minute de-stain in MiliQ water. UV trans-illumination performed using Fluorochem 8000 (Alpha Innotech).

Table 3-1 Summary of primers used in this study, including PCR product size and number of cycles

Gene	Primers	Annealing temperature	Product size (bp)	Cycle Number	Primer Reference
β-actin (Gene Accession: AJ438158)	F 5' ATCGTGGGGCGCCCCAGGCACC 3' R 5' CTCCTTAATGTCACGCACGATTTC 3'	53°C	514	30	(Brubacher et al., 2000)
MH-Iα (Gene Accession: AF318187)	F 5'ACTATGGGAAGAGCACTCTG 3' R 5' GTGGGAGCTTTTTTGGAAGG 3'	55°C	212	30	Unpublished
β2m (Gene Accession: L49056)	F 5' TGTCAATCGTTGTACTTGGG 3' R 5' CTTCAGGTGGCGGACTCTGC 3'	55°C	302	30	Unpublished
MH-IIα (Gene Accession: AJ251433)	F 5' TGTGGTACGCAGACTTCAAC 3' R 5' GCTCAGTAAGGGCCTTGTGC 3'	55°C	432	30	Unpublished
MH-IIβ (Gene Accession: U20946)	F 5' AGTGATGCTGGGATCCTGGG 3' R 5' GGGTGTGTACTCCAGGTGGG 3'	55°C	306	30	Unpublished

3.2.3. Western blot analysis

Cultures were seeded to 2.6 x 10^6 cells per 25 cm^2 tissue culture flask. Protein was extracted from collected cells using a modified RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0) supplemented with protease inhibitor cocktail (Sigma). Cells were kept in the cocktail for 30 minutes and centrifuged at 10 000 g for 15 minutes. Protein concentration determined using Bradford assay. Equal amounts of protein boiled in Laemmli buffer (135 mM Tris, 4% (w/v) SDS, 0.06% (w/v) bromophenol blue, 20% glycerol, 2% (w/v) 2-mercaptoethanol) and cooled on ice. Samples ran on SDS-polyacrylamide gel. Electrophoresis performed using a mini-PROTEAN® Tetra Cell (Bio-Rad, Mississauga, ON). Pre-stained protein standard was used to estimate protein size (Fermentas). A Bio-Rad

mini-Trans Blot module (20mA, 8 hours) used to transfer protein onto nitrocellulose membrane (Bio-Rad). Nitrocellulose membrane stained with Ponceau S (in 5% w/v acetic acid) for 5 minutes to confirm equal loading of protein samples. In Figure 3-3, actin expression was also used to verify equal loading protein. Membrane blocked in 5% (w/v) nonfat dried milk in TBS-T (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 hour. The primary antibody, polyclonal rabbit anti-rainbow trout MH proteins, was diluted in blocking solution 1:100. In Figure 3-3, a polyclonal anti-actin (Sigma) was used as a positive control. The polyclonal primary anti-actin antibody was diluted 1:100 with blocking solution. Membrane incubated with primary antibody for 1 hour. Membrane washed three times with TBS-T (5 minutes per wash) and incubated in alkaline phosphatase conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad) diluted 1:30000 for 1 hour. Membrane washed three times with TBS-T (5 minutes per wash). Visualized using NBT/BCIP (Roche, Mississauga, ON) and recorded digitally on flatbed scanner. Each blot performed at least twice with independent samples.

3.3. RESULTS

3.3.1. MH Iα protein expression

The polyclonal anti-serum to rainbow trout MH Iα detected a similar set of bands in western blots of extracts from six rainbow trout cell lines. A polypeptide of approximately 45 kDa was seen consistently in extracts of the epithelial-like cell lines from gill (RTgill-W1), intestine (RTgutGC), liver (RTL-W1), and hepatoma (RTH-149). Also, MH Iα was detected in the monocyte/macrophage-like cell line from the spleen (RTS11) and the fibroblast-like cell line from gonads (RTG-2). Additionally, most extracts had a band at 56 kDa and occasionally the 45 kDa band appeared as a doublet (Figure 3-1). A polypeptide at 25 kDa was seen when the gel had been loaded with more protein than the usual 50 μg of protein per lane. Other bands appeared inconsistently. No constant cell line difference was seen except for RTS11. With this cell line, the bands were much more intensely labeled and tended to smear into one another. The anti-serum also detected polypeptides in cell lines from other fish species: Chinook salmon (CHSE-214), carp (EPC) and zebrafish (ZEB2J) (Figure 3-2). For CHSE-214 a band was found at approximately 56 kDa; for EPC, a band at approximately 42 kDa. Bands at 40 and 34 kDa were seen with zebrafish. No bands were seen in extracts of a human cell line, HeLa.

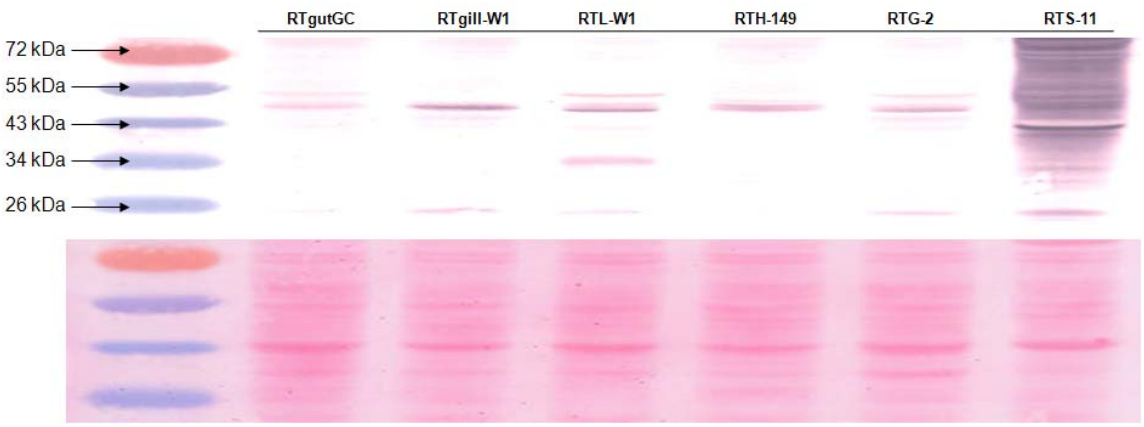

Figure 3-1 Western blot analysis of MH Iα protein in rainbow trout cell lines.

Western blot analysis of MH Iα expression in rainbow trout cell lines using purified anti-trout MH Iα anti-sera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

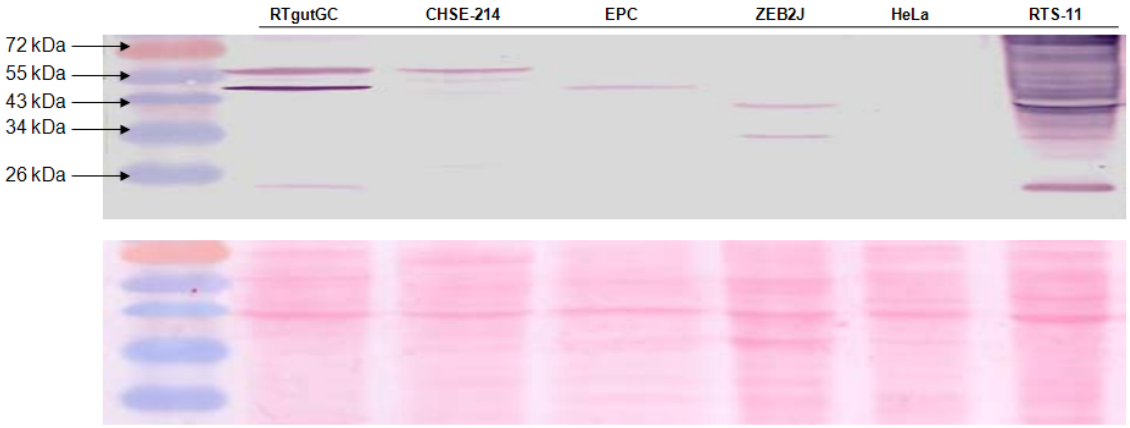

Figure 3-2 Western blot analysis of MH Iα protein in cell lines derived from different species.

Western blot analysis of MH Iα expression in cell lines from different species using purified anti-trout MH Iα anti-sera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

3.3.2. β_2-microglobulin (β2m) protein expression

Initially, western blots with polyclonal anti-sera to either a truncated or a mature sequence of recombinant rainbow trout β2m, a polypeptide of approximately 11 kDa was detected in extracts consistently for RTS11, but inconsistently for adherent rainbow trout cell lines. One hypothesis to account for this inconsistency was that scraping rather then trypsinizing cells off the flask surface better retained β2m in cell pellets. Another was that potentially the β2m in the medium supplement, fetal bovine serum (FBS) (Shields et al., 1998), could exchange with the β2m of the fish cells, and be the cause of the erratic results. When a comparison was made between extracts from adherent cells that had been prepared by either scraping or trypsinizing, the 11 kDa band was observed only in the scraped cells (data not shown). Likewise if cell pellets were washed multiple times with PBS prior to lysis, the 11 kDa band was not detected (data not shown). These results support the hypothesis that the method of removing cells from flasks represents a source of variability in the β2m western blots. As background information for testing the second hypothesis, the ability of the anti-sera to detect polypeptides in FBS and rainbow trout serum was investigated. No bands were seen for FBS, while a band at 11 kDa was seen in the fish serum (data not shown). When adherent cells were maintained in 50% FBS and extracts prepared by scraping the cells off the plastic surface, extracts from all cultures showed a 11 kDa polypeptide, although the intensity of the band might have been reduced slightly in 50% FBS (Figure 3-3). This suggests that any β2m exchange with FBS in the medium is minor and does not interfere with the detection of β2m in rainbow trout cell line extracts.

When collecting the cell pellet was standardized, a polypeptide at 11 kDa was detected consistently in the adherent rainbow trout cell lines as well as in the loosely adherent RTS11 (Figure 3-4). Although not shown, a 11 kDa band was detect in other adherent cell lines, RTee, which was from an early embryo, and an early passage spleen stromal cell line, Low 16. Although no attempt was made to quantify differences between cell lines, RTS11 often stood out as having a more intensely stained 11 kDa polypeptide. For all the cell lines, usually just this band was present. However, RTgutGC occasionally produced an additional band was found at

approximately 17 kDa and multiple bands were seen with RTS11 between approximately 10 and 17 kDa.

For extracts from other species, the anti-sera to rainbow trout β2m detected no polypeptides in ZEB2J, RAW 264.7, and HeLa, but did detect bands in CHSE-214 and EPC (Figure 3-5). A single band at 11-12 kDa was found in CHSE-214. In EPC, bands were not found with anti-serum to the mature β2m, but a polypeptide at approximately 17 kDa was seen with the anti-serum to the truncated β2m.

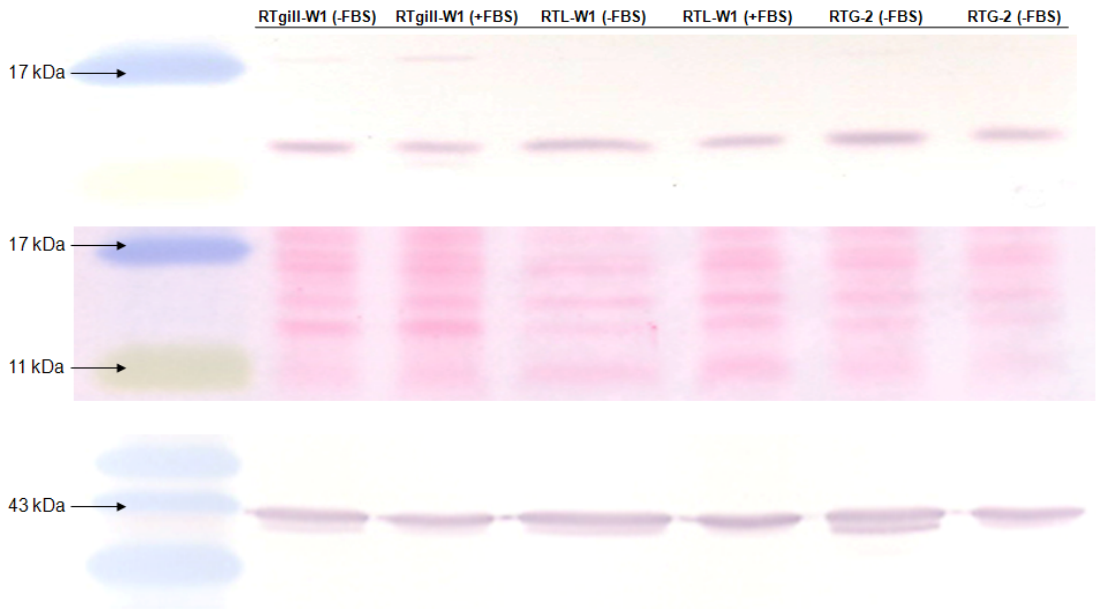

Figure 3-3 Western blot analysis of β2m protein in rainbow trout cell lines incubated with FBS.

Rainbow trout cell lines were incubated with and without 50% FBS for 24 h prior to lysate extraction. Western blot analysis of rainbow trout β2m expression between the different treatments using purified anti-trout β2m anti-sera. Ponceau staining and actin protein expression, below, indicates total protein transfer prior to membrane probing and equal loading.

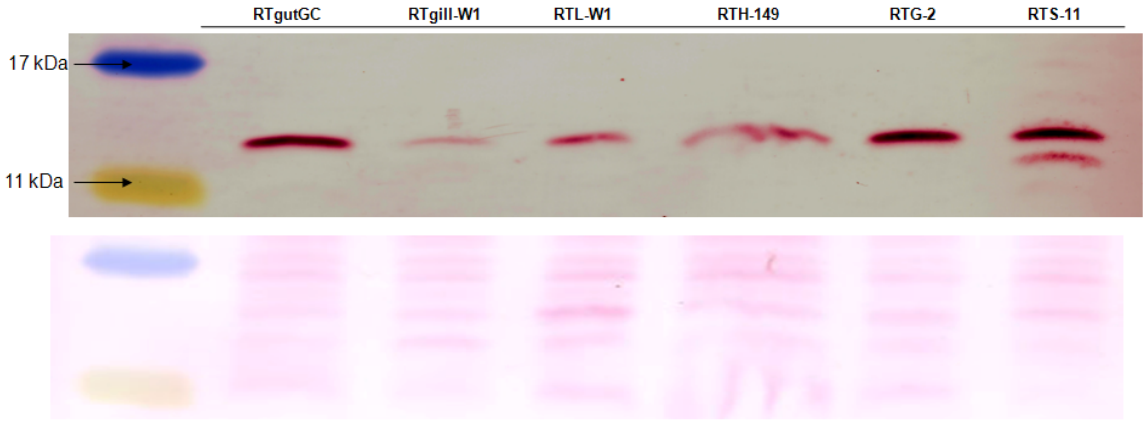

Figure 3-4 Western blot analysis of β2m protein in rainbow trout cell lines.

Western blot analysis of rainbow trout β2m expression in rainbow trout cell lines using purified anti-trout β2m anti-sera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

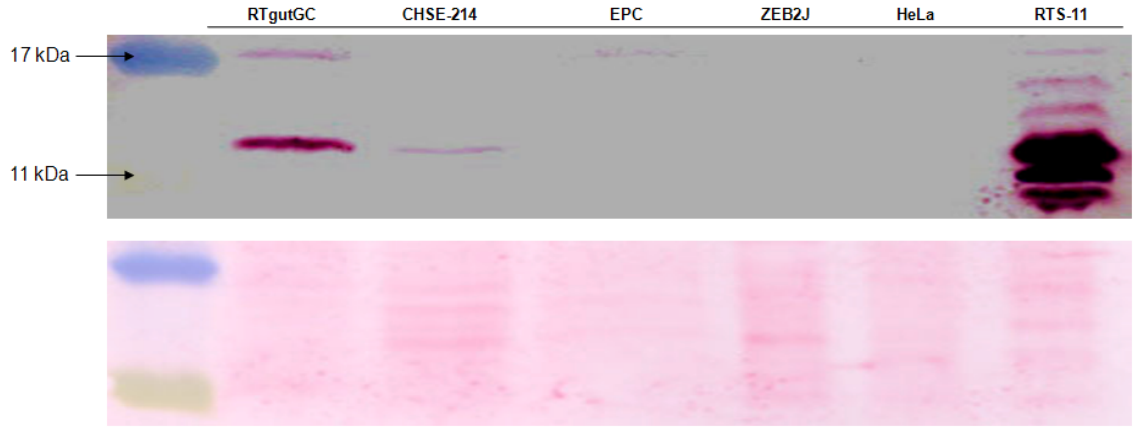

Figure 3-5 Western blot analysis of β2m protein in cell lines derived from different species.

Western blot analysis of rainbow trout β2m expression in cell lines from different species using purified anti-trout β2m anti-sera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

3.3.3. MH IIα/ β gene expression

Transcripts for MH IIα and MH IIβ were detected in all the cell lines by RT-PCR (Figure 3-6). Previously MH IIβ transcripts have been demonstrated in RTS11 (Brubacher et al., 2000) and RTG-2 (Dijkstra et al., 2003). In the current study, no attempt was made to quantify transcript levels, but the level of MHIIα transcripts seemed similar in all cell lines, whereas MH IIβ transcripts appeared consistently higher in RTS11.

In western blots with the polyclonal anti-serum to rainbow trout MHIIα, polypeptides were detected only in extracts of RTS 11 (Figure 3-7). A band was seen at approximately 30 kDa, and a second, less intensely stained one, was observed at approximately 35 kDa. The anti-serum failed to detect bands in cell extracts from CHSE-214, ZEB2J, and HeLa (data not shown).

In western blots with the polyclonal anti-serum to rainbow trout MH IIβ, only RTS11 had a reactive band (Figure 3-8). The RTS11 band was often diffusely stained and appeared at between 28 and 34 kDa.

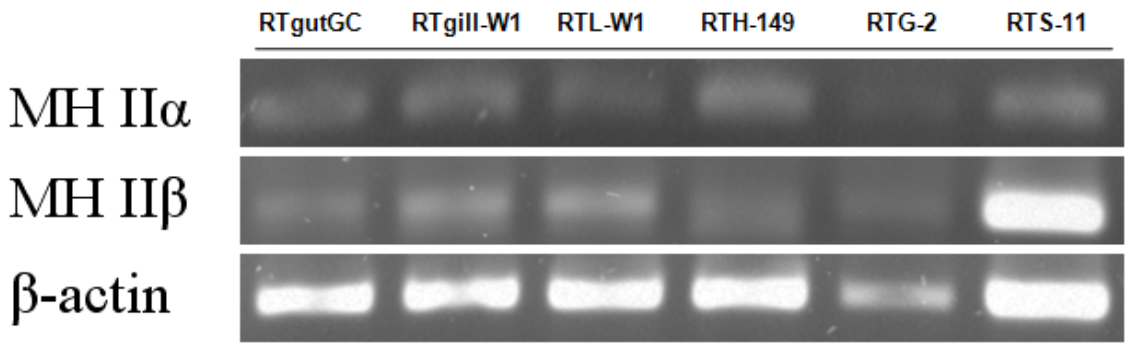

Figure 3-6 MH II transcript levels in rainbow trout cell lines.

RT-PCR analysis of MHII transcripts from different rainbow trout cell lines using primers designed from published sequences. Beta-actin (β-actin) served as an internal standard. Target cDNA and PCR cycle numbers are indicated in Table 3-1.

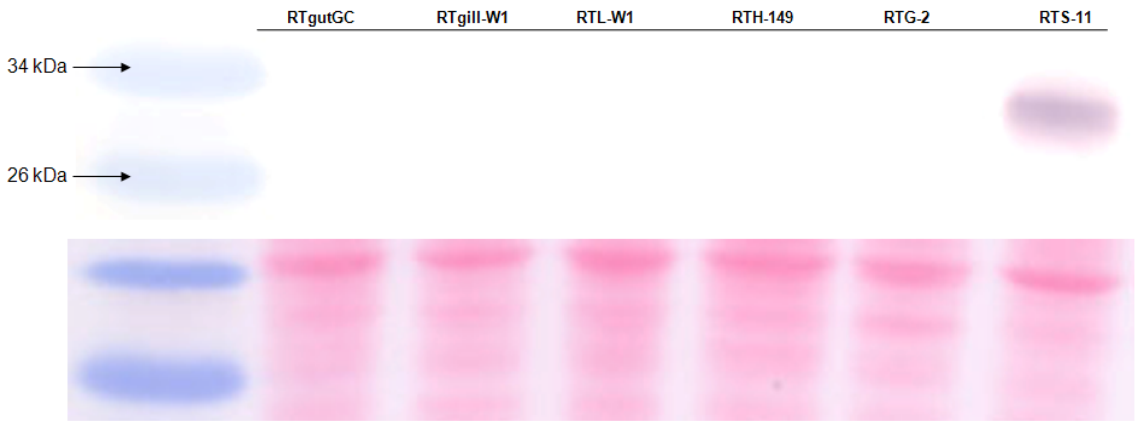

Figure 3-7 Western blot analysis of MH IIα protein in rainbow trout cell lines.

Western blot analysis of rainbow trout MH IIα expression in rainbow trout cell lines using purified anti-trout MH-IIα anti-sera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

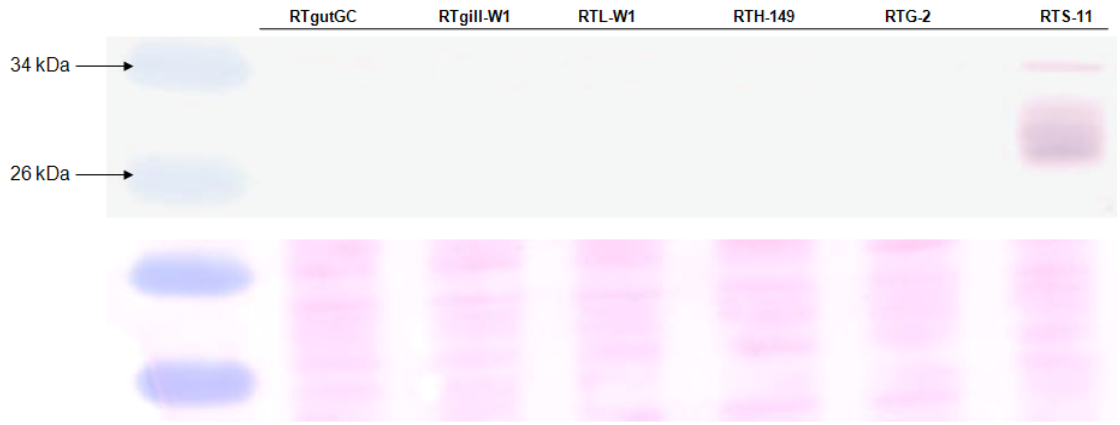

Figure 3-8 Western blot analysis of MH IIβ protein in rainbow trout cell lines.

Western blot analysis of rainbow trout MH IIβ expression in rainbow trout cell lines using purified anti-trout MH-IIβ anti-sera. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

3.3.4. Response to poly IC

Western blots revealed an increased accumulation of β2m protein in cultures of epithelial-like, fibroblast-like and monocyte/macrophage-like cell lines treated with 50 μg/ml of poly IC for 2 to 6 days. This was investigated with RTgutGC, RTG-2 and RTS11 and an increase was visible with each cell line as shown for RTgutGC in Figure 3-9. The magnitude of the protein increase was not determined. Modulations of MH Iα, MH IIα, and MH IIβ levels were not obvious and will require more thorough examination to definitely say whether they change in response to poly IC.

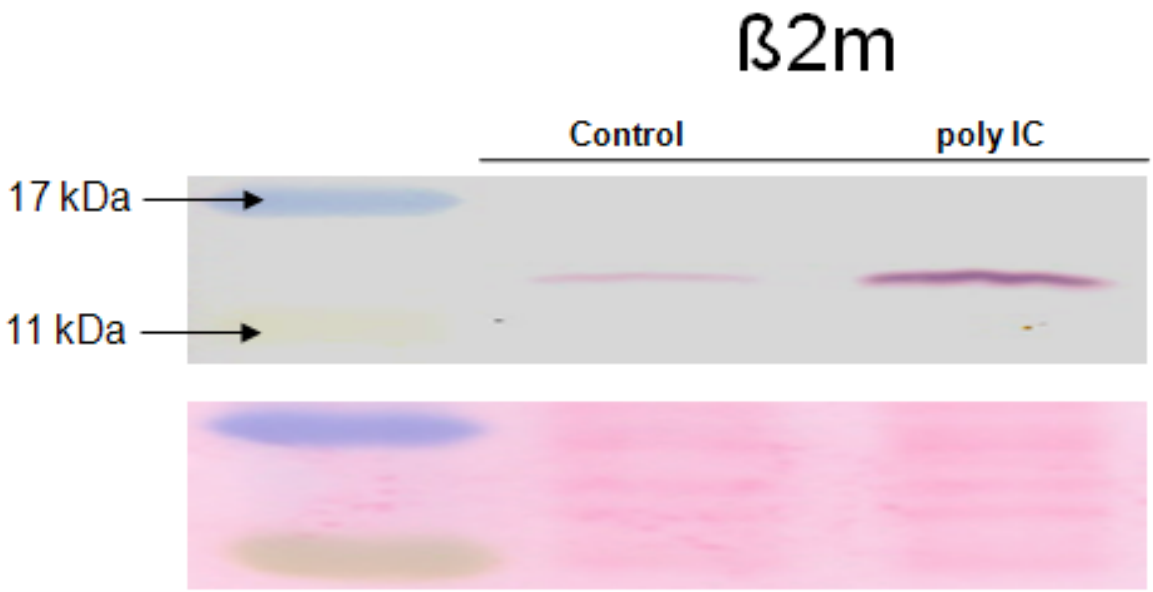

Figure 3-9 The effect of poly IC exposure on β2m protein expression in RTgutGC.

RTgutGC cultures were exposed to 50 µg/mL poly IC for 72 h. Cells were then collected and western blotting performed to measure β2m protein expression. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

3.4. DISCUSSION

Expression of major histocompatiblity (MH) genes was characterized in epithelial-, fibroblast- and macrophage-like rainbow trout cell lines with polyclonal anti-sera to recombinant rainbow trout MH polypeptides.

All rainbow trout cell lines expressed MH Iα, as judged by the detection in western blots with anti-serum to recombinant rainbow trout MH Iα of a polypeptide at approximately 45 kDa. Four of the cell lines were epithelial-like: RTgill-W1 from gill, RTgutGC from intestine, RTL-W1 from liver, and RTH-149 from hepatoma. Two others were the monocyte/macrophage-like cell line from the spleen, RTS11, and the fibroblast-like cell line from gonads, RTG-2. The same anti-serum consistently reacted with a polypeptide of approximately 45 kDa in extracts of gill, liver, spleen, and intestine (Kales, 2006). Similarly, a monoclonal antibody (H9) to the extracellular domain of a common MH Iα allomorph (Onmy-UBA*501) recognized a polypeptide at 44 kDa in extracts of RTG-2 (Dijkstra et al., 2003a). However, the polyclonal anti-serum to recombinant rainbow trout MH Iα used in the current study detected other bands in extracts of both the rainbow trout cell lines and tissues. These were seen less consistently and occurred at 56, 42, 36, and 25 kDa. As rainbow trout MH Iα is glycosylated (Kales, 2006; Dijkstra et al., 2003), some of these additional bands might be accounted for by glycosylation. Although these bands are incompletely understood, they are clearly found both *in vivo* and *in vitro*.

Like most fish tissues and mammalian cell lines, all the rainbow trout cell lines contained β2m. Nine cell lines from seven tissues had an 11 kDa polypeptide that reacted with polyclonal anti-serum raised to recombinant rainbow trout β2m. The same anti-serum detected an 11 kDa polypeptide in all examined trout tissues, including spleen, liver and intestine (Kales et al., 2006). Among the trout cell lines, less intense bands were seen on occasion at approximately 17 kDa with RTgutGC and between 10 and 17 kDa with RTS11. These minor bands also were occasionally seen with rainbow trout and Atlantic salmon tissue extracts (Kales et al., 2006), hence these additional bands are unlikely to be just a feature of the cell lines. Among mammalian cell lines, a few have been found not to express β2m. These have been cell lines from tumors and include Daudi from a Burkitt lymphoma (Evrin and Nilsson, 1974) and some

cell lines from embryonal carcinomas (Morello et al., 1982). However, no exceptions were found among the rainbow trout cell lines, even with a tumor cell line (RTH149) and an early embryo cell line (RTee) expressing β2m. Thus, expression of β2m appears to be a common characteristic of rainbow trout cell lines.

In western blots of extracts from cell lines of different species, anti-sera to fish β2ms have been found to have restricted species cross reactivity. Anti-serum to cod β2m detected a 15 kDa polypeptide in extracts of a haddock cell line (HEW), but not in RTS11 (Bryson et al., 2006). In the current study, anti-serum to rainbow trout β2m detected a polypeptide at 11 kDa for a Chinook salmon embryo cell line (CHSE-214), but failed to react with any polypeptides in the zebrafish cell line (ZEB2J). In past studies, the anti-serum failed to react with an embryonic haddock cell line (HEW). However, anti-serum to the truncated, but not the mature rainbow trout β2m, was reported to detect a band at approximately 11 kDa in gill extracts from the carp (Kales et al., 2006).

Western blotting with anti-serum to rainbow trout MH IIα detected polypeptides only in the monocyte/macrophage cell line, RTS11. The most intensely stained polypeptide was about the same size (approximately 30 kDa) as the polypeptide detected in western blots of extracts from rainbow trout tissues (Nath et al., 2006). The second band that clearly was evident in some of the cell lines might represent glycosylated forms because the rainbow trout MH IIα has been shown to be glycosylated (Nath et al., 2006). In addition, only RTS11 expressed MH IIβ as detected in western blots with anti-serum raised to rainbow trout MH IIβ. The band in RTS11 extracts was in the 28-34 kDa range, the size anticipated from several previous observations. The rainbow trout MH IIβ is expected to be 28 kDa based on amino acid sequence (van Lierop et al., 1998), has been shown to be glycosylated (Nath et al., 2006), and detected in western blots of tissue extracts at approximately 35 kDa (Nath et al., 2006). No bands were detected in extracts of the epithelial and fibroblast-like cell lines. A similar observation has recently been made with cell lines and monoclonal antibodies to MH IIβ from catfish (Moulana et al., 2008). Four catfish leukocyte cell lines had surface MH IIβ, but a fibroblast cell line (GF5) did not express the protein. MH IIβ transcripts were not detected in GF5, but have been detected in RTG-2 (current

report, Dijkstra et al., 2003). MH II transcripts also have been seen in the highly phagocytic cell line TO from Atlantic salmon (Pettersen et al., 20008).

Confined expression of the MH II polypeptides in RTS11 among the compared cell lines reinforces immunological principles derived with mammals (Cruse and Lewis, 2004) and fish (Koppang et al., 2003). APCs should constitutively express MH II molecules, including macrophages, but not in epithelial and fibroblast cells.

The expression and location of MH class II polypeptides in RTS11 compared with the expression of MHC class II antigens in mammalian myelomonocytic cell lines raises interest because these cell lines have been powerful models for studying the activation and differentiation of macrophages, including the possible differentiation into dendritic cells (Auwerx, 1991; Collins, 1987; Saxena et al., 2003). The constitutive expression of MHC class II antigens is usually low or absent in myelomonocytic lines, such as THP-1 (Lee et al., 2005; Feng et al., 2008), U937 (Barbaro et al., 2005), WEHI-3 (McNicholas et al., 1983), HL 60 (Yunis et al., 1989), and RAW 264.7 (Shen et al., 2008). Although hard to compare directly because western blotting has been used infrequently for studying the expression of MHC class II chains in these cell lines and when used the detection systems have been different (Lee et al., 2005), the results with RTS11 hint at stronger constitutive expression because the signals in western blots for α and β chains of RTS11 were strong. In mammalian cells, MHC class II antigens were inducible by γ interferon (Yunis et al., 1989) and the location changed from an intracytoplasmic compartment to the cell surface as dendritic cells matured (Winzler et al., 1997). However in U937, induction of MHC class II chains by γ interferon was not accompanied by an increase in cell surface expression, which was possibly due to defects in posttranscriptional processing (Yunis et al., 1989). Additionally, intracellular transport was influenced by culture conditions, such as the availability of exogenous fatty acids (Schweitzer et al., 2006). RTS11 should be convenient for investigating the factors that modulate the induction and location of MH II chains in fish macrophages.

This study appears to be the first documentation of β2m protein increasing in cell cultures treated with poly IC, but the result might represent an example of a general phenomenon in which viruses modulate the MHC class I presenting pathway. In mammals, HIV-1 down

regulates β2m transcripts (Ambagala et al., 2005). By contrast in fish, β2m transcripts have been found to be up regulated in rainbow trout infected with infectious hematopoietic necrosis virus (IHNV) (Hansen and La Patra, 2002), in Atlantic salmon cell cultures infected with infectious salmon anemia virus (ISAV) (Jorgensen et al., 2006; Schiotz et al., 2008) and in the croaker treated with poly IC (Liu et al., 2007). Whether the enhanced accumulation of β2m protein in poly IC-treated rainbow trout cell cultures is due to changes in transcription or in post translational events are interesting questions for the future. This work provides background information on the cell lines and the polyclonal anti-sera that should allow investigations into the regulation of rainbow trout MH gene expression.

Chapter 4

Conclusions and Future Aims

4. CONCLUSIONS AND FUTURE AIMS

Despite years of research, the onset of inflammation remains a mystery in most organisms, including fish. A dynamic environment occurs in the intestine, with cells of the animal being closely associated with resident microbiota. There exists a continual need for host intestinal cells to maintain homeostatic environment with microbiota and, at the same time, respond to exogenous invaders. The intestine utilizes many physiological and immunological mechanisms to mediate these actions. These cytokines have been intensely characterized in higher vertebrate models. However, understanding the full extent of piscine intestinal inflammation requires studies at different levels with different approaches.

Intestinal cell lines offer many advantages in studies of gastrointestinal dysfunction, especially inflammation. Unfortunately, initiation of intestinal cultures requires stringent surgical techniques and adequate treatment with antibiotics. *In vivo*, intestinal cells depend on complex interactions with the underlying connective tissue to remain viable and differentiate. Hence, development of normal intestinal cell lines has been generally unsuccessful. Due to its difficult nature, limited cell lines are available from the intestinal segment. To date, no established intestinal cell lines are published for any teleost fish. This study reports the development of a novel rainbow trout intestinal cell line, RTgutGC, to be used as a tool to assess functional responses to substances associated with cellular functioning and immune response.

One of the most fascinating and poorly understood aspects about the fish intestinal system concerns interactions with microbiota. Many unknown processes likely are involved in sustaining the long-standing homeostatic relationship that offers benefits to both. Hence, RTgutGC was challenged with lipopolysaccharide (LPS) from *Escherichia coli*. RTgutGC displayed similar cellular responses to LPS compared to higher vertebrates. The exact mechanism for toxicity was not examined in this study; however, the literature suggests fish most likely respond to LPS using an alternative pathway from higher vertebrates. Although LPS treatment up-regulated tumor-necrosis factor alpha (TNF-α), this may or may not be accomplished by pathways independent of toll-like receptor 4 (TLR-4).

Several factors protected RTgutGC against LPS cytotoxicity: glutamine supplementation and cell-density. Glutamine may have reinforced or stabilized the intestinal barrier functions in

RTgutGC, similar to those seen in sepsis studies using adult rats. The intestinal epithelial barrier, in fish and higher vertebrates, represent an important physiological barrier to protect the host organisms against pathogens. Increasing cell-density might facilitate production of intestinal alkaline phosphatase (IAP), which might deactivate LPS.

Major histocompatibility gene expression was characterized in multiple rainbow trout cell lines. The light chain non-covalently associated with the major histocompatibility class I heavy chain (MHC I) receptor, beta-2 microglobulin (β2m), was found in all tested rainbow trout cell lines. Relative expression of β2m was strongest in RTS11, a cell line of immunological origins. Multiple bands were apparent in some of the cell lines. The multiple bands could have resulted through cleaving multiple cysteine sites of the β2m protein. A major concern with investigating β2m *in vitro* arises with the notion of 'β2m exchange'. Studies with β2m from fetal calf serum in culture medium have similar or higher affinities for MHC I expressed by human cell lines. This phenomenon was examined with various rainbow trout cell lines. Results showed that cultures incubated with high levels of fetal bovine serum (FBS) did not alter β2m protein expression levels. The unique polymorphic nature of β2m raises many questions regarding its functional role in salmonid species. To gain better understanding of the role β2m plays in response to potentially pathogenic components, RTgutGC was challenged with plant derived lectins and double stranded RNA mimic. Both components increased accumulation of β2m after respective treatments. Thus, up-regulating β2m through its multiple genes might be an evolutionary conserved response to deal with foreign invaders.

MHC I receptors in rainbow trout are expressed as N-glycosylated polypeptides on all nucleated cells. Similar to β2m, MH I was expressed by all tested rainbow trout cell lines. Multiple bands were seen outside the known molecular weight of MH I ranging from 25- 55 kDa. These are most likely attributed truncated MH I products, proteolytic degradation and/or glycosylation. MHC class II (MHC II) receptors are known to be expressed by professional antigen presenting cells (APC). This was shown with the tested rainbow trout cell lines, as RTS11 was the only cell line that expressed MH II receptor proteins. Interestingly, MH II was expressed at the transcript level in all cell lines. Treatments with poly IC failed to induce conclusive changes in MH Iα or MH class II expression. These results suggest rainbow trout cell

lines reflect similar expression patterns seen with *in vivo* MHC studies in fish and other vertebrates.

RTgutGC represents an adult rainbow trout intestinal cell line that can aid in many areas of basic and applied research. Aquaculture represents Canada's fastest growing food sectors. For the projected exponential growth in aquaculture, several problems must be addressed for the industry to succeed in the future. Some of these problems relate to viral infections and diminishing food supplies.

Viral infections in aquaculture facilities are costly problems with limited solutions (Lang et al., 2009). Currently, the only affective solution appears to be complete destruction of infected stocks. Although vaccines are available to notable viruses problematic to aquaculture (Sommerset et al., 2005), gaining a better understanding of viral infections at the cellular level can improve treatment strategies. Like higher vertebrates, the fish intestinal system demonstrates complex immune processes important to sustaining healthy stocks. Some viruses associated with aquaculture include piscine nodavirus (Barker et al., 2002), viral hemorrhagic septicemia virus (VHSV) (Meyers and Winton, 1995), and infectious pancreatic necrosis virus (IPNV) (Essbauer and Ahne, 2001). The intestine represents a potential site for viral entry and research with RTgutGC might reveal new information in managing outbreaks.

The emerging concern in aquaculture involves the detrimental decrease in wild stocks. Although aquaculture works to sustain wild stocks, farming fish depends heavily on feeds composed of smaller fish. If this viscous cycle persists, future demands cannot be satisfied by current aquaculture practices. To alleviate this crisis, researchers have attempted to produce alternative plant-based feeds. Among the many evaluated candidates, soybean meal (SBM) has proven to be the leading candidate (Gatlin et al., 2007). In general, soybeans are considered to be the best alternative because they are economical, nutritious, and readily available. Unfortunately, high inclusion levels of SBM have consistently induced intestinal dysfunction in fish species raised through aquaculture (Knudsen et al., 2007; Olsen et al., 2007; Uran et al., 2008). More specifically, SBM has shown to induce distal intestinal enteritis in Atlantic salmon (Knudsen et al., 2007) and the common carp (Uran et al., 2008) if not properly treated or combined with fish meal. Research over the years has increased knowledge about this persistent

problem. However, the exact cause of inflammation remains unknown. Current research methodologies to investigate this problem depend on whole organism trials. To reduce the burden of whole organism studies, RTgutGC and other *in vitro* models could assist in nutritional studies. Although cases of SBM induced enteritis in rainbow trout are not common (Nordrum et al., 2000), RTgutGC could still be used to screen soybean anti-nutritional factors at the cellular and molecular level. Results from this study have demonstrated that RTgutGC respond to potentially pathogenic components by up-regulating immune-related cytokines transcripts and MH associated proteins.

As an example, preliminary experiments with several plant-based lectins induced increased accumulation of β2m, similar to results seen with poly IC (Figure 4-1). Generally, lectins avoid digestion and bind to surface intestinal epithelium. Some lectins have shown to compromise membrane integrity and initiate immunological responses (Gatlin et al., 2007). Soybean agglutinin (SBA) have shown to interact at the intestinal surface in both rainbow trout and Atlantic salmon (Buttle et al., 2001), leading to varied responses depending on the intestinal segment. Affinity for SBA seems to increase towards the distal intestinal segment, inducing many symptoms of intestinal dysfunction: wasteful protein synthesis, shortening of villi, and morphological changes (Buttle et al., 2001). Thus, RTgutGC can be used to screen to understand the cellular and immunological responses induced by commercially important bioactive compounds.

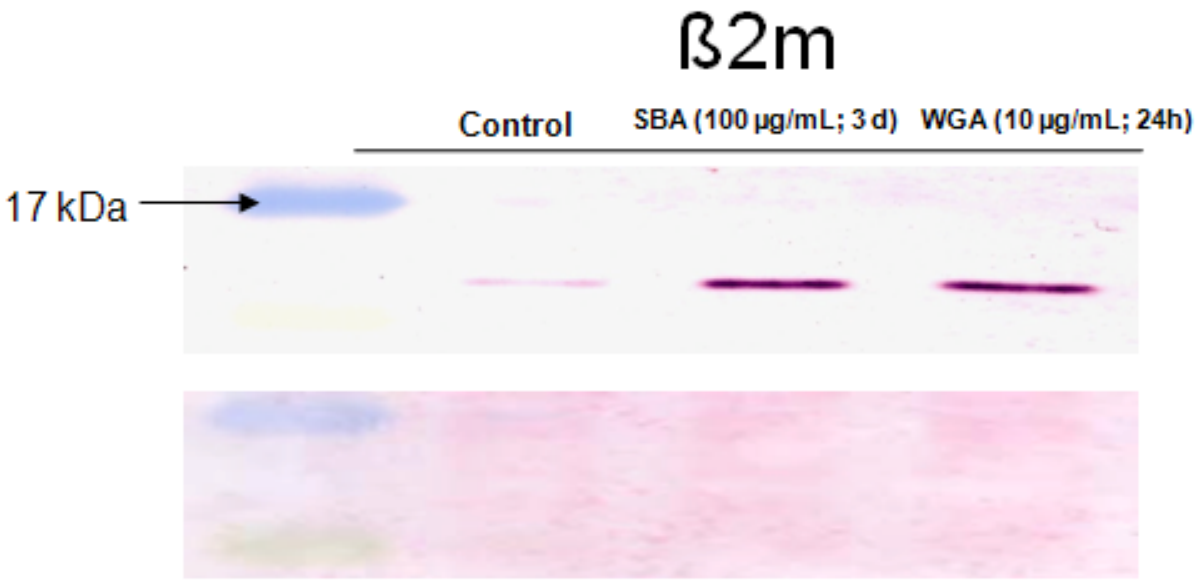

Figure 4-1. The effect of plant-based lectins on β2m protein expression in RTgutGC.

RTgutGC cultures were exposed to 100 µg/mL soybean agglutinin (SBA) for 3 d or 10 µg/mL wheat germ lectin (WGA) for 24 h. Cells were then collected and western blotting performed to measure β2m protein expression. Ponceau staining, below, indicates total protein transfer prior to membrane probing.

Taken together, the data in this study demonstrate that RTgutGC can be used to assess bioactive substances limiting the inclusion rates of plant-based fish feeds in aquaculture and contribute to fish GIT studies focused on sustaining current wild stocks. Although RTgutGC represents a promising tool to understand the multifaceted interactions associated with fish intestines, more experiments are required to evaluate the usefulness of RTgutGC as an intestinal model. To be fully accepted as a fish intestinal model, the differentiation potential should be examined in RTgutGC. Currently, only intestinal primary cultures and short-term cultures with fibroblastic support have shown to consistently produce different intestinal cell lineages. RTgutGC will become an indispensable fish intestinal model if bioactive compounds can be evaluated against differentiated fish intestinal cell types.

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General Introduction

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