Effects of Dietary Bovine Lactoferrin on Intestinal Lymphocytes of Mice After Dextran Sulfate Sodium or Acute Exercise Challenge

by

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Paul A. Spagnuolo

Abstract

Background: Inflammation, if uncontrolled, can promote the formation of colon cancer. Intestinal lymphocytes (IL) are immune cells that can participate in inflammation through the generation of cytokines and by causing direct cellular injury. Apoptosis, or programmed cell death, regulates the population of lymphocytes and dysregulation of this process results in the prolonged activation of IL that occurs during inflammation. Therefore, inducing apoptosis of IL is a viable mechanism by which inflammation and possibly colon carcinogenesis could be prevented. Experimentally, inflammation may be induced in mice by: 1) the addition of the chemical irritant dextran sulfate sodium (DSS) to drinking water or 2) exposure to acute exercise (AE; intense treadmill running).

Bovine lactoferrin (bLf) is a dietary whey protein with demonstrated ability to promote anti-inflammatory responses by reducing pro-inflammatory or increasing anti-inflammatory cytokine concentrations within the intestine. There are also reports that bLf can alter apoptotic proteins in the intestinal epithelium to favour cell death. Moreover, athletes have been reported to supplement their diets with whey protein; it is also known clinically that after heavy competition some athletes may experience gastrointestinal distress. The role, however, of bLf in reducing inflammation by initiation of apoptosis or alteration of cytokine levels in IL has not been examined.

Objectives: The primary objective of this research was to determine whether dietary bLf affects mouse IL apoptosis and cytokine concentrations in: 1) a normal, non-inflamed state, 2) following AE challenge and 3) following DSS treatment. A second objective was to directly examine the potential protective effects of dietary bLf from

inflammatory damage caused by DSS and AE challenge when administered alone or in combination with a carcinogen.

Methods: A total of 252 female C57BL/6 mice were used in the experiments. Apoptotic proteins (Bcl-2, caspase 3, Bax, cytochrome c), inflammatory cytokine proteins (TNF-α and IL-10) and a transcription factor for pro-inflammatory cytokines (NFκB) were determined in isolated mouse IL by Western blot analysis. Flow cytometry was used to determine the extent of apoptosis in mouse IL subsets by measuring phosphatidylserine surface expression using Annexin V^+ (ANN^+) and propidium iodide staining. Tissue inflammation was determined by histology (H&E staining) on segments of mouse small and large intestine. Diets were prepared and pelletted to contain 20% total protein and contained either bLf or were the control formulation (no bLf). Mice were exposed to bLf containing diets for 4 d or 12 d prior to sacrifice. DSS was provided at 5% in the drinking water for 4 consecutive days prior to sacrifice. Animals were subjected to three repeated bouts of AE (each separated by 24 h rest) involving treadmill running and sacrificed either immediately or 24 h after the final exercise bout. In the experiment involving carcinogenesis, mice were given two subcutaneous injections of azoxymethane (AOM), followed by a two week incubation period, and subsequently exposed to bLf or the control diet.

Results: Results from the first experiment determined that 2.0% bLf was effective at reducing mouse IL levels of TNF-α ($p<0.05$) (pro-inflammatory) and increasing the percentage of apoptotic $CD4^+$ IL ($CD4^+/ANN^+$, $p<0.05$) in healthy mice. Thus, 2.0% bLf was used for the subsequent experiments. Dietary bLf administration in mice exposed to AE was associated with lower levels in mouse IL of the anti-apoptotic protein Bcl-2

($p<0.01$) and of TNF-α ($p<0.05$) and NFκB ($p<0.05$), both pro-inflammatory proteins. Further, the exercise protocol resulted in oxidative stress, as measured by 8-iso prostaglandin $F_{2\alpha}$ levels in plasma, but did not induce intestinal inflammation, evident by the absence of both tissue damage and infiltration of immune cells. Following DSS treatment, mice supplemented with bLf enriched diets had lower levels of both TNF-α (non-significant, 34% reduction) and NFκB ($p<0.05$) and increased concentrations ($p<0.01$) of cytochrome c, a mitochondrial protein associated with cell death. DSS exposure in mice resulted in gross morphological alterations and infiltration of immune cells in the small and large intestine; these changes in tissue histology were not affected by the addition of bLf. Mice injected with AOM and then subjected to DSS, but not AE, had increased numbers ($p<0.001$) of aberrant crypts, preneoplastic colonic lesions, compared to animals only receiving AOM injection. Dietary bLf did not affect any of these carcinogenic processes.

Conclusions: Collectively, these results suggest that dietary bLf administration reduces pro-inflammatory cytokine levels and has limited effects on apoptosis of mouse IL. Moreover, these modifying effects of bLf did not result in mucosal protection, as evident in the inability of this protein to reduce DSS-induced tissue damage or formation of aberrant crypts.

Physiological and Clinical Implications: Although the long term physiological consequences of bLf supplementation in the regulation of intestinal immune homeostasis require further study, the following clinical implications are tentatively suggested by the findings from this thesis research. First, dietary bLf supplementation does not provide direct protection of the intestine during inflammation either with or without exposure to

the carcinogen (i.e., AOM); hence, bLf (at least in the dietary concentration and exposure used in these experiments) may not be useful in reducing the formation of aberrant crypts and carcinogenesis. Second, dietary bLf should not be recommended as a supplement at this time for athletes experiencing intestinal distress since it had no impact on tissue indicators of disease in a model (DSS) shown to produce extensive inflammation and tissue pathology. Nonetheless, the findings raise the possibility that bLf can modify both cytokines and apoptotic protein expression in IL and may influence some aspects of inflammatory processes in the gut.

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SPECIAL DEDICATION

For Franky: His humour was contagious, his wit unparalleled and his charisma inspiring. Our final conversation has forever changed my life.

Frank Spagnuolo
(1963 - 2001)

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INTRODUCTION

Intestinal inflammation is characterized by tissue damage caused by the recruitment and excessive activation of intestinal lymphocytes. Under normal conditions lymphocytes contribute to mucosal protection. During prolonged inflammation, however; these cells could contribute to the creation of an environment that increases the risk of colon carcinogenesis. Apoptosis, or programmed cell death, is essential in regulating the immune response and dysregulation of this vital process perpetuates lymphocyte activation. Therefore, inducing apoptosis of activated intestinal lymphocytes could result in decreased inflammation and, by extension, reducing the risk of colon carcinogenesis.

Bovine lactoferrin (bLf) is a dietary milk protein with demonstrated ability to reduce inflammation mainly through modifying cytokine production. Addition of bLf reduces colonic tumour numbers in rats and has been shown to affect apoptotic proteins within the colonic epithelium. However, the ability of this important dietary constituent to influence apoptotic parameters in intestinal lymphocytes has not been examined.

Inflammation may be induced experimentally by several different protocols. The gold standard for induction of experimental intestinal inflammation is by the administration of dextran sulfate sodium (DSS) to the drinking water of rodents. Another possible strategy is by subjecting mice to strenuous exhaustive acute exercise (AE). Both protocols result in an excessive production of pro-inflammatory cytokines, a common characteristic of inflammation. The objectives of this research were: 1) to characterize the effect of dietary bLf on intestinal lymphocyte apoptosis and cytokine levels during a normal, non-inflammatory state and following DSS or AE challenge and 2) to directly

examine the ability of bLf to protect the intestinal mucosa from alterations induced by DSS or AE and when these protocols were provided following tumour initiation events.

Chapter 1 is a literature review aimed at providing a general overview of the important concepts and topics germane to this thesis. Chapter 2 provides an explanation of the study rationale and the working hypotheses. Chapters 3-7 are the results from experiments conducted from this thesis research. These chapters were written as scientific papers for publication and include a detailed design and outlined hypotheses for each study. Chapter 8 is an integrated discussion of the research findings and includes study limitations and offers recommendations for future research.

Chapter 1: Literature Review

The purpose of this literature review is to provide a background into the current state of knowledge regarding lactoferrin, apoptosis, inflammation and the intestinal immune system. This is not an exhaustive review but a summary focused on providing context into the potential influence of lactoferrin on apoptosis and intestinal inflammation. A list of abbreviations is found in Appendix I and may aid in the reading of this thesis.

1.1. Intestinal Immune System

The intestinal immune system is an integral component of overall host protection as it governs the immunological response in the most hostile environment in the human body. Intestinal lymphocytes are central in the maintenance of intestinal homeostasis as they participate in both regulator and effector functions. In this section a brief overview of the intestinal immune system, specifically focusing on intestinal lymphocytes, is presented.

The gastrointestinal mucosa is in constant contact with bacterial and dietary antigens. Given this anatomical location it is not surprising that the gut associated lymphoid tissue (GALT) is the largest and most complex lymphoid population in mammals (Abreu-Martin and Targan, 1996). GALT consists of organized tissues [e.g., Peyer's patches (PP) and mesenteric lymph nodes (MLN)] responsible for the induction phase of the immune response and effector sites which contain immune cells (i.e., leukocytes) within the epithelium and lamina propria (Mowat, 2003). A single layer of epithelial cells covered by mucus and secretory IgA acts as an interface between the

intestinal tissue and the luminal contents. This epithelium provides the first active line of defense against infection by segregating the luminal contents from the underlying complex immune network (Baumgart and Carding, 2007). The intestinal epithelium, however; is not impervious to luminal antigens and there are several methods by which the underlying lymphoid follicles (e.g., PP) sample luminal antigen: 1) dendritic cells (DC) within the lamina propria extend dendrites into the lumen, 2) antigens transverse the epithelia via microfold cells and encounter DC, and 3) intestinal epithelial cells (IEC) uptake antigen and present antigen to the DC together with proteins of the major histocompatibility complex (MHC) (Kunisawa and Kiyono, 2005). Once the antigen is in contact with the DC it can either be processed within PP or transported to MLN where lymphocytes are encountered and the fate of the immune response (i.e., tolerance or immunity) is decided (Mowat, 2003).

1.1.1. Intestinal Lymphocytes

There are two classes of intestinal lymphocytes (IL) that are differentiated based on immunological properties and location. In the lamina propria (LP) are lamina propria lymphocytes (LPL). Within the intestinal epithelium, above the basement membrane, resides a highly specialized population of intraepithelial lymphocytes (IEL). Together IEL and LPL perform critical effector and inducer functions and are integral components in maintaining immunological intestinal homoeostasis (Hershberg and Blumberg, 2003).

IEL account for about half of the peripheral T cell pool in mammals, and the small intestine of mice has an estimated IEL population of 50-500 x 10^6 cells depending on the strain, age and other factors (Beagley and Husband, 1998). Phenotypically, these

cells are 80-90% $CD3^+$T cells, and of these, 80% are $CD8^+$ IEL. The T-cell receptor (TCR) is essential for T-cell function and is classified as either αβ or γδ. All γδCD8 IEL express the CD8αα homodimer co-receptor whereas αβCD8 IEL expresses either the CD8αα homodimer or CD8αβ heterodimer (Beagley and Husband, 1998). Indeed two distinctive features of IEL from other peripheral blood lymphocytes (PBL) are that they exclusively express the CD8αα homodimer co-receptor and contain large numbers of γδ T cells (e.g., 53% vs. 3%, in mouse IEL vs. PBL, respectively) (Abreu-Martin and Targan, 1996; Ishikawa et al., 2007). Along the intestinal tract, populations of IEL share identical cellular phenotypes, which led to their characterization as oligoclonal (derived from only a few clones) (Gross et al., 1994). This feature of IEL may account for their restricted ability to respond and proliferate to previously unseen antigen (Fiocchi, 2001).

A unique feature of the IEL population is the expression of the memory phenotype (85% express $CD45RO^+$) and markers of activation (e.g., CD69, CT antigen and CD11c) which together indicate prior antigen exposure (Abreu-Martin and Targan, 1996). IEL are rapidly activated in the presence of anti-$CD3^+$ stimuli (Wang et al., 2002) and collectively these observations have led to the description of IEL as 'partially activated' T cells. Although the mechanism of activation is not fully understood, it has been suggested that IEL "wait" for the appropriate signals (e.g., antigenic stimuli, co-stimulatory molecules) to push them into full activation (Montufar-Solis et al., 2007). Once activated IEL are capable of lysing target cells (e.g., virally infected cells or foreign bacteria) and contain cytoplasmic granules with perforin and granzyme (Beagley and Husband, 1998). Apart from cytotoxcity, IEL secrete cytokines which are important cellular messengers (see section 1.2.1.). Although the cytokine profile is largely

dependent on the type of activation signal, in the active state IEL tend to favour a T_H1, cell mediated cytotoxic responses (Beagley and Husband, 1998). Thus, IEL are a very distinctive and critical lymphocyte population for the maintenance of immune homoeostasis through both cytotoxic and regulatory cytokine functions.

In comparison to IEL, LPL are more similar to conventional PBL. As in PBL, LPL are predominantly (95%) αβ TCR with small numbers of cells expressing the γδ receptor. Approximately 40-90% of LPL are T cells ($CD3^+$) with the greater majority (65-85%) expressing the $CD4^+$ co-receptor. Similar to PBL, LPL ratios of $CD4^+/CD8^+$ are approximately 65%:35% (Abreu-Martin and Targan, 1996). Within the LPL there is also a large percentage (70-90%) of IgA secreting plasma cells that aid in anti-inflammatory responses (Beagley and Elson, 1992). LPL are thought to be a population of highly activated lymphocytes, as they express a large percentage (66-96%) of the $CD45RO^+$ memory phenotype and share similar surface markers found on activated cells (Fiocchi, 2001).

LPL participate in both effector and regulator functions (Rothkotter et al., 1999). These lymphocytes express Fas ligand, and contain granules with perforin and granzyme, which can induce apoptosis in target cells (Melgar et al., 2002). In response to antigen, the proliferation and cytotoxicity of LPL is lower than in PBL, which may be a default mechanism attributed to the surrounding environment (i.e., constant presence of luminal antigen) (Fiocchi, 2001). Given the large population of $CD4^+$ cells in the LP compartment, it is not surprising that LPL function largely as regulatory cells. That LPL are critical to regulation is supported by their restricted capacity to clonally expand (i.e., proliferate in response to antigen) and their propensity and efficiency at secreting anti-

inflammatory and tolerogenic T_H2 cytokines (Abreu-Martin and Targan, 1996). In further support of this argument is the finding that LPL produce large concentrations of T_H2 cytokines that support anti-inflammatory responses (Fiocchi, 2001; Beagley and Elson, 1992). Cytokine balance is important in the regulation of inflammatory events (see section 1.2.1) and $CD4^+$ LPL in inflamed tissues show a predominance to produce T_H1 cytokines [e.g., increased IL-1β, IL-6 and tumour necrosis factor-α (TNF-α)] (Reinecker et al., 1993). In summary, LPL are more similar to PBL than IEL, represent an activated lymphocyte population with limited proliferative capacity, and have regulatory and cytotoxic functions.

1.2. Intestinal Inflammation

Inflammation is the complex physiologic response to tissue damage caused by pathogens, wounding, or irritation (Philip et al., 2004). It is a protective mechanism by which the individual attempts to remove or limit the harmful stimuli and initiate the healing process (Kelsall, 2008). The inflammatory reaction is initiated by a cascade of chemical mediators (e.g., cytokines) secreted by local macrophages, epithelial and mast cells that coordinate a response by which altered blood flow, increased vascular permeability and up-regulation of adhesion molecules lead to the recruitment of immune cells (Linden, 2001). The movement of leukocytes from the circulation to the interstitial space is explained by a multi-step paradigm of cellular events: 1) circulating leukocytes are slowed as they align and interact with the endothelium wall at sites of injury, 2) leukocyte interaction with adhesion molecules increases and leads to improved contact between the immune cell and the endothelium, and 3) adhered leukocytes transmigrate

into tissues (Bianchi et al., 1997; Springer, 1994). Inflamed tissues are dominated by neutrophils, macrophages and lymphocytes, which generate large amounts of growth factors, cytokines and reactive oxygen and nitrogen species that contribute to tissue breakdown and strengthen the defense against potential infection (Philip et al., 2004; Coussens and Werb, 2002; Lu et al., 2006). Under normal conditions, inflammation is self-limiting so that once the harmful stimulus is cleared and the damaged tissue repaired, the same cells that initiated inflammation are responsible for its down-regulation (i.e., negative feedback). The production of pro-inflammatory mediators is shifted towards production of anti-inflammatory mediators, which inhibit further activation and recruitment of immune cells (Philip et al., 2004). The healing process is initiated and there is rapid clearance of inflammatory cells (Lu et al., 2006).

The intestine is resident to over 500 different species of bacteria. Coupled with the constant presence of dietary constituents, the intestine is believed to be in a constant state of controlled inflammation termed 'physiological inflammation' (Fiocchi, 2001). This has been suggested because of the relatively peaceful co-existence between intestinal bacteria and the large numbers of mature and partially activated IL. The intestine has control mechanisms that limit GALT exposure to luminal antigen. These include: 1) physical control mechanisms such as an active epithelial lining and the organized structure of GALT that limit antigen exposure and 2) physiological control mechanisms such as increased IgA secretion, the tendency of LPL and IEL to have low proliferative capacity, and the propensity of LPL to act as helper cells through active immune suppression (i.e., secretion of IL-10 and TGF-β) (Beagley and Elson, 1992). Additional physiological control mechanisms include those that confer oral tolerance.

Oral tolerance is defined as the state of immune unresponsiveness to orally administered antigen such that an immune response (i.e., proliferation and activation of immune cells) does not occur (MacDonald and Monteleone, 2005; Chehade and Mayer, 2005). Immune unresponsiveness following antigen recognition is achieved through several cellular mechanisms including clonal anergy (lack of T-cell proliferation and activation), clonal deletion (death of T-cells) and active suppression (secretion of anti-inflammatory cytokines) (Mowat, 2003).

The balance between activation and regulation of lymphocytes is essential to the delicate balance of intestinal immunological homeostasis. Alterations to this balance, which could include excessive inflammatory stimuli (e.g., incorrect recognition of a harmless luminal antigen as foreign, alteration of intestinal barrier permeability) or failure of regulatory mechanisms (e.g., failed apoptosis of activated lymphocytes, breakdown in oral tolerance) could result in inappropriate immune activation and potentially detrimental inflammation (MacDermott, 1996). This could lead to pathological inflammation resulting in host tissue damage and, possibly, disease. For example, individuals with inflammatory bowel diseases (IBD) have recurring bouts of intestinal inflammation; although the etiology is unknown, it is believed to involve multiple factors (such as diet, genetics and environmental) that participate in altering this balance to favour disease pathogenesis (Neuman, 2007).

1.2.1. Cytokines

Cytokines are low molecular weight glycoproteins secreted by various immune and epithelial cells. They exert their function through receptor mediated pathways and

have important roles in biological processes. Cytokines involved in inflammation can be divided into two generic groups: pro-inflammatory cytokines, which include interferon-γ (IFN-γ), TNF-α, and interleukin (IL-) 1β and anti-inflammatory cytokines, which include IL-4, IL-5, and IL-10 (Williams, 2001). $CD4^+$ T cells are a main source of cytokines and they can be further classified into T_H1 or T_H2 cells based on the cytokines they secrete: T_H1 cells secrete pro-inflammatory cytokines and T_H2 cells secrete anti-inflammatory cytokines (Strober et al., 2002).

The release of pro-inflammatory cytokines results in the activation of immune cells (e.g., neutrophils and lymphocytes), enhancement of T-cell proliferation, increases in vascular permeability and up-regulation of adhesion molecules on the endothelial surface to facilitate leukocyte transmigration (Goldsby et al., 2003). An important T_H1 cytokine in intestinal inflammation is TNF-α, which is synthesized as a 26kDa membrane bound protein (mbTNF-α). Cleavage by TNF-α converting enzyme results in soluble TNF-α (17kDa) that acts on downstream targets expressing cognate receptors (Bradley, 2008). There are several functions of TNF-α, dependent on whether it is the soluble or receptor form. When available as a membrane bound receptor, mbTNF-α, through direct contact may confer cell survival. The more common soluble form (sTNF-α) participates in a wide variety of biological processes that include apoptosis, proliferation and differentiation, activation of proteases, promotion of angiogenesis and activation of immune cells (Aggarwal et al., 2006; Goetz et al., 2004). TNF-α is a potent T_H1 cytokine that is important in normal host defense against infection. When inappropriately or excessively produced, however, TNF-α is harmful and has been experimentally and clinically linked to IBD and colon cancer (Szlosarek et al., 2006; Bradley, 2008). TNF-α

was found elevated in the serum and stools of patients with IBD (Murch et al., 1991; Braegger et al., 1992) and in mice is overexpressed in LP macrophages (Neurath et al., 2001) and inflamed colonic tissues (Tomoyose et al., 1998). Moreover, several investigators have shown that treatment with neutralizing anti-TNF-α antibodies results in decreased disease indicators. For example, Van Dullemen and colleagues (1995) observed a decrease in Crohn's disease activity index scores and healing of mucosal ulcerations in patients following a single infusion of an anti-TNF-α human/mouse chimeric monoclonal antibody (cA2). Improvements in tissue histology and reduced numbers of infiltrating neutrophils and lymphocytes were observed following a single dose of infliximab (anti-TNF-α antibody) in patients with Crohn's disease compared to placebo controls (Baert et al., 1999). Thus, it appears that TNF-α may have an important role in inflammation induced tissue damage and the ability to reduce its expression has been correlated with decreased intestinal inflammation.

T_H2 cytokines are involved in regulating the inflammatory response. IL-10, secreted by $CD4^+$ cells, macrophages, dendritic cells and B cells, terminates inflammation by inhibiting activation and effector functions of T cells and macrophages (Moore et al., 2001). In addition to these activities, IL-10 regulates the growth and/or differentiation of natural killer (NK) cells, cytotoxic and $CD4^+$ cells, B cells, granulocytes, mast cells and dendritic cells (Moore et al., 2001). It is a potent inhibitor of TNF-α and IL-1β (Rosatto et al., 2007) and Crepaldi et al., (2002) demonstrated that in vitro stimulation of human neutrophils with IL-10 can further enhance an anti-inflammatory environment by inducing the production of an IL-1 antagonist. The importance of IL-10 in the regulation of inflammation is further emphasized in IL-10

deficient mice. These animals develop severe intestinal colitis characterized by massive infiltration of lymphocytes, macrophages and neutrophils as well as show increased concentrations of pro-inflammatory cytokines; amelioration of colitis occurs upon addition of IL-10 (Wirtz and Neurath, 2000). Moreover, mice treated with recombinant IL-10 had reduced extensiveness of tissue damage and less of an increase in TNF-α and IL-1 following the addition of a chemical irritant known to cause intestinal inflammation (Tomoyose et al., 1998). Thus, the relationship between the pro- (T_H1) and anti- (T_H2) inflammatory cytokines is such that a shift towards a T_H1 response can result in a state of inflammation whereas a shift towards a T_H2 response confers an anti-inflammatory environment (Elson et al., 2005; Wirtz and Neurath, 2000).

1.2.2. Nuclear Factor κ B

Transcription factors (TF) are proteins responsible for regulating the transfer of information from DNA to messenger RNA (mRNA) (Lewin, 2002). TF bind to DNA and assist in transcription which may eventually result in the production of proteins that perform cellular functions. One TF central to the inflammatory process is nuclear factor κ B (NFκB). NFκB is actually the general name for a family of transcription factors that influence biological processes such as inflammation, apoptosis and tissue repair (Nakanishi and Toi, 2005). Its relative importance as a therapeutic target is highlighted in several diseases, including IBD and colon cancer in which patients exhibit elevated levels of the activated TF (Karin, 2006; Tak and Firestein, 2001). NFκB is composed of various homo or heterodimer combinations of protein subunits and several target genes include those that produce cytokines, chemokines, cell adhesion molecules, growth

factors, immunoreceptors, anti-apoptotic proteins and transcription factors (Lee and Burckart, 1998; Barnes and Karin, 1999).

NFκB is found in the cytoplasm in an inactive state and it is associated with the inhibitory protein IκB. Upon activation from an external signal (e.g., TNF-α, IL-1, lipopolysaccharide (LPS), UV radiation or free radicals), the IκB protein is phosphorylated by the I kappa β kinase (IKKβ) which liberates NFκB from its inhibitor (Barkett and Gilmore, 1999). IκB is degraded and NFκB translocates to the nucleus where it exerts its role in transcriptional regulation. The link between inflammation, cancer and increased NFκB is well established. In knockout mice devoid of IKKβ in enterocytes there is a dramatic 75% decrease in tumour number compared to wild-type mice; reduced inflammation (e.g., reduced COX-2, IL-6 mRNA and IL-1β mRNA) and a 50% reduction in tumour number also occurred in mice that were lacking IKKβ in myeloid cells (Greten et al., 2004). NFκB was found elevated in hepatic tumours and this increase was attenuated by treatment with ibuprofen, a non-steroidal anti-inflammatory drug (NSAID). Complete absence of NFκB did not influence atypical proliferation but decreased tumour progression indicating a role of NFκB in tumour promotion rather than initiation (Pikarsky et al., 2004). Therefore, targeting and down-regulating NFκB appears to be a potentially valuable therapeutic target against inflammation and carcinogenesis.

1.2.3. Inflammation as a Promoter of Colon Carcinogenesis

Colon carcinogenesis is a complex multistep process that can be divided into three general stages: initiation, promotion and progression. The end result of this process is the

accumulation of the so called 'hallmarks of cancer' that convert a normal cell to an autonomous cell capable of uncontrolled growth and invasion of neighbouring tissues (Trosko, 2006). The hallmark features of cancer, as described by Hanahan and Weinberg (2000) are: 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) evasion of apoptosis, 4) limitless proliferation, 5) sustained angiogenesis, and 6) tissue invasion and metastasis. The multi-step progression of carcinogenesis is a framework to outline and define possible molecular events associated with the transformation of a normal, 'mortal' cell to a cell capable of limitless growth (i.e., immortal cell). Initiation involves the irreversible primary damage to, or alteration of, DNA in normal cells caused either by spontaneous mutation or from carcinogen exposure (Oliveira et al., 2007). The consequence of initiation is that the cell begins to accumulate some of the characteristics of transformed cells, which lead to the possibility that the cell could escape the typical limits of normal growth (Trosko, 2006). Experimentally, initiation events can be induced by injection with a known colon specific carcinogen such as 1, 2-dimethylhydrazine (DMH) or azoxymethane (AOM).

The initiated cell can persist in otherwise normal tissue for extended periods of time (Farber and Rubin, 1991). However, the conversion of this atypical cell into an immortal cell can be accelerated by events that create a favourable growth environment. These so called "promotional events", which include inflammation, cause the clonal expansion of the atypical cells (Farber and Ruben, 1991). The end result may be the eventual accumulation of all the hallmarks of cancer and, therefore, promotion is the process that allows the initiated cell to escape the suppressing and regulatory effects of normal cell growth (Pitot and Dragan, 1991). Finally, progression refers to the event that

confers the ability of the immortal cell to sustain its own growth, which may result in tumour formation, and to invade neighbouring tissue (Oliveira et al., 2007).

Colon cancer prevention strategies can be targeted against any of the stages of carcinogenesis. However, as Trosko (2006) points out, whereas initiation and progression may be irreversible and occur within a short duration, the promotion phase occurs over decades and may be reversible. Thus, appropriate therapeutic interventions would be directed against promotional events. Focusing on inflammation, there is ample evidence that implicates it is a promoter of colon carcinogenesis. Experimentally, inflammation can be induced by several methods such as rectal injection with the hapten trinitrobenzenesulfonic acid (TNBS), feeding of carrageenan (CAR), or administration of dextran sulfate sodium (DSS) in the drinking water (Strober et al., 2002). Okayasu et al. (1996) using DSS and D'Argernio et al. (1996) using TNBS, as promotional events following AOM injection observed increased numbers of colonic tumours when compared to animals receiving AOM alone. Similarly, AOM injected mice treated with DSS had a greater appearance of several inflammatory indicators (i.e., increased TNF-α, increased immune cell infiltration, enhanced COX-2 expression, body weight loss, bloody stools) and increased tumour numbers (Popivanova et al., 2008).

Perhaps the most striking evidence to support inflammation as a promoter of carcinogenesis are animal and human studies that show a reduction in the number of colon tumours after treatment with anti-inflammatory agents. For example, Seril et al. (2002) demonstrated a 20% reduction in the number of colon tumours in DSS-treated mice supplemented with N-acetylcysteine, an antioxidant with known free radical quenching capacity. Administration of aspirin, a NSAID, significantly reduced both

tumour incidence and the number of invasive carcinomas in the colon of AOM injected rats (Reddy et al., 1993). Studies also support the notion that anti-inflammatory agents protect against colon cancer development in humans. Flossmann and Rothwell (2007) [British Doctors Aspirin Trial, N=5139 and UK-TIA Aspirin Trial, N=2449] and Logan et al. (2008) [N=945, in patients with colorectal adenomas] reported that individuals ingesting 300 mg/day of aspirin was sufficient to reduce colon cancer risk and risk of recurrence of colorectal adenomas, respectively. Taken together, there is evidence from human and animal studies suggestive that inflammation promotes increased tumour number and that the addition of anti-inflammatory agents may reduce tumour number.

The mechanism by which inflammation promotes carcinogenesis is unknown, but in recent reviews by Philip et al. (2004), Lu et al. (2006) and Coussens and Werb (2004), inflammation is suggested to create an environment that favours cells to accumulate DNA and cellular damage through the persistent generation of reactive oxygen species (ROS). Moreover, this environment also favours proliferation since a consequence of tissue damage is a drive toward tissue repair. Therefore, the increased likelihood of cellular and DNA damage coupled with the tendency for increased cellular division results in the potential for atypical cell development and the accumulation of the hallmarks of cancer that may eventually lead to carcinogenesis.

1.2.3.1. Aberrant Crypts

Aberrant crypts (AC) are established pre-cancerous lesions in the colonic mucosa that begin as a single enlarged crypt but expand into larger clusters involving many crypts

(aberrant crypt foci - ACF). AC are distinguished from their normal counterparts in many ways that include changes in width, height and thickness of the epithelial lining, depletion of goblet cells, and irregular luminal openings (Bird, 1987; 1995; Bird and Good, 2000; Augenlicht et al., 2002). AC are the first known observable mucosal lesions that may develop into adenomas (Bird, 1987; Mori et al., 2005) and are a commonly used biomarker for colon carcinogenesis (Takayama et al., 2005). Several investigators demonstrated that inflammation enhances AC formation. Flynn et al. (2007) found that deoxycholic acid, a secondary bile acid and suspected tumour promoter, enhanced ACF formation when provided in the diet of mice following AOM injection. Addition of 2.5 % carrageenan (CAR) to the diet of rats injected with AOM resulted in increased numbers of ACF compared to rats on CAR-free diets (Tache et al., 2000). Sutherland and Bird (1994) reported increased ACF in AOM treated rats fed diets containing chenodeoxycholic acid (CDC), a primary bile acid and known tumour promoter; increased proliferation was also evident in CDC groups indicating accelerated cellular growth. AC are thus reliable and important markers of early tumourigenesis that increase during inflammation.

1.2.4. Methods of Inducing Intestinal Inflammation

1.2.4.1. Dextran Sulfate Sodium

As noted earlier in this literature review, animals given dextran sulfate sodium (DSS) develop intestinal inflammation. DSS is a sulfated polysaccharide that directly damages the intestinal mucosa and is currently the most common method used to induce intestinal

inflammation (Strober et al., 2002). Animals receiving DSS develop several pathological changes such as intestinal bleeding, loss of body weight, shortening of the intestine, mucosal ulcers and other epithelial changes including fibrosis and crypt loss (Hibi et al., 2002; Elson et al., 2005). The mechanism of DSS induced intestinal inflammation is not known but likely involves multiple pathways. DSS may damage the intestinal epithelial barrier allowing luminal bacteria to penetrate within the lamina propria, thus activating an immune response (Strober et al., 2002). DSS has also been shown to alter intestinal microflora (Okayasu et al., 1990), stimulate B cell expansion (Minchin et al., 1990), accumulate in macrophages (Kitajima et al., 1999) and alter adhesion molecules on intestinal epithelial cells that favour IL attachment (Ni et al., 1996). Whatever the mechanism, DSS induced colitis is characterized by an excessive T_H1 response attributed to activated neutrophils, macrophages and lymphocytes that accumulate within the damaged segments (Boismenu and Chen, 2000; Dieleman et al., 1998; Pizarro et al., 2003).

The conventional means of administering DSS to animals is through the drinking water. The concentration and duration of administration determines the extent of intestinal mucosal damage (Egger et al., 2000). Acute inflammation is usually triggered by exposure to DSS (1-5%) for a few days (usually between 4 and 7) and this is followed by euthanasia (Kullmann et al., 2001; Melgar et al., 2005). Chronic inflammation develops when periods (e.g., 1-2 weeks) of regular (DSS-free) drinking water is administered following DSS treatment (Strober et al., 2002; Kullmann et al., 2001).

1.2.4.2. Acute Exercise

Gastrointestinal disturbances such as abdominal pain, diarrhea and intestinal bleeding are common adverse effects of strenuous exercise in athletes (Peters et al., 2001b; Bi and Triadafilopoulos, 2003). These effects may be related to alterations of intestinal homeostasis caused by exercise. Indeed, oxidative stress and ischemia-reperfusion (IR) injury are two consequences of exercise that are implicated in the induction of intestinal inflammation (Shek and Shephard, 1998). These two processes may cause death of intestinal epithelial cells leading to a loss of intestinal barrier function. Failure of the epithelial layer to segregate the immune network from the luminal bacteria allows bacteria to penetrate into the lamina propria and interact with the intestinal immune network. The result would be the activation of immune cells and the induction of intestinal inflammation, which may contribute to the gastrointestinal problems associated with strenuous exercise in athletes (Marshall, 1998; Shek and Shephard, 1998).

During exercise intestinal ischemia occurs as blood is diverted away from the intestine to metabolically active skeletal muscle. Peters et al. (2001a) reported that blood flow in the superior mesenteric artery (SMA), which supplies blood to the intestine, was reduced by 49% compared to pre-exercise values in volunteers subjected to a cycling test (70% VO_2 max). A damaging effect of intestinal ischemia is death of epithelial cells. Following surgical occlusion of the rat SMA, Noda et al. (1998) and Giakoustidis et al. (2008) reported increased DNA fragmentation and apoptosis of intestinal epithelial cells (IEC); infiltration of inflammatory cells into the lamina propria was also evident. Increased intestinal permeability was observed following SMA occlusion in rats which

was also associated with elevated serum and intestinal mucosal levels of pro-inflammatory cytokines (TNF-α and IL-6) (Grotz et al., 1995). Therefore, exercise associated intestinal ischemia may lead to death of the IEC and loss of intestinal barrier function.

During aerobic exercise there is an increase in the production of reactive oxygen species (ROS). When this production alters the balance in favour of ROS over the endogenous cellular antioxidant defense enzymes which are capable of neutralizing ROS, a state of oxidative stress occurs (Opara, 2006). Sources of ROS during exercise include the electron transport chain, which also creates ROS during normal cellular respiration, the xanthine and NADPH oxidase enzyme systems, phagocytes and the iron catalyzed Fenton reaction (Niess et al., 1999; Jackson, 2008). ROS, such as the superoxide anion, the hydroxyl radical, and hydrogen peroxide can induce cellular damage and thus there are several defense mechanisms by which ROS are removed. For example, superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide which is subsequently converted to water via catalase enzymes (e.g., peroxidase) or is broken down by glutathione peroxidase (Hensley et al., 2000). These important cellular defenses (i.e., SOD, catalase and glutathione) are critical in cellular protection and if these defenses are overwhelmed, which can occur during exercise, the resultant state of oxidative stress can impart tissue damage.

Another important cellular defense mechanism is heat shock protein (e.g., HSP 70). It is rapidly induced in response to both oxidative stress and ischemia reperfusion injury and offers cellular protection against intestinal mucosal damage (Fehrenbach and Northoff, 2001; Otaka et al., 2006). The protective roles of HSP 70 include preventing

protein misfolding and aggregation and also reducing DNA fragmentation and apoptosis (Yenari et al., 2005). During exercise HSP 70 is elevated in human blood leukocytes (Fehrenbach et al., 2000) and in mouse intestinal lymphocytes (Hoffman-Goetz and Spagnuolo, 2007a); increased HSP 70 has been associated with reduced intestinal mucosal damage and neutrophil infiltration following ischemia (Sakamoto et al., 2005).

The consequences of ROS production include DNA damage, loss of both cellular integrity (via lipid peroxidation) and enzyme function, and cell death (Phaneuf and Leeuwenburgh, 2001; Hensley et al., 2000). Thus, similar to intestinal ischemia, oxidative stress can induce death of the IEC leading to loss of intestinal barrier function. Indeed, oxidative stress as a result of exercise is associated with physiological alterations suggestive of inflammation. For example, phagocyte infiltration, damage to plasma membrane lipids, proteins and DNA, and activity of NFκB are all increased as a result of exercise induced oxidative stress (Aoi et al., 2004). Mastaloudis and colleagues (2004) showed that antioxidant supplementation (e.g., vitamin C and RRR-alpha-tocopheryl acetate) attenuated the increase in plasma 8-iso prostaglandin $F_{2\alpha}$, a reliable marker of membrane lipid peroxidation caused by oxidative stress, in ultramarathon (50km) runners; the expression of pro-inflammatory markers (e.g., IL-6, TNF-α and C-reactive protein) were also observed post-exercise.

The suggestion that intense exercise induces intestinal inflammation may thus rely on the ability of the exercise regimen to induce damage to the intestinal epithelium. Such damage would result in the inability of the epithelium to segregate the bacteria from the intestinal immune network. As bacteria penetrate into the lamina propria the immune

cells would be activated, and provide the stimulus necessary to induce the inflammatory response.

1.3. Cell Death

Two terms frequently used to describe cell death are apoptosis and necrosis. Apoptosis is a rapid, genetically controlled process characterized by distinct cellular morphology such as: cell shrinkage, chromatin condensation, budding, little or no swelling of organelles and DNA degradation (Kerr et al., 1972). Necrosis is characterized by cellular swelling and eventual cellular and nuclear lysis that is accompanied by inflammation (Savill et al., 2002; Majno and Joris, 1995; Van Cruchten and Van Den Broeck, 2002). The following section is a brief review of apoptosis and necrosis with a focus on the molecular apoptotic pathways.

1.3.1. Apoptosis

Apoptosis, or programmed cell death, is an essential biological process involved in embryonic development, regulation of the immune response and tissue homeostasis (Hoffman-Goetz et al., 2005). It is orchestrated by a cascade of cellular events primarily carried out by a family of cysteine proteinase enzymes called caspases. These enzymes specifically cleave substrates at residues that follow aspartic acid and are classified as either initiator or executioner caspases. Initiator caspases (such as caspase 8 and caspase 9) function in upstream regulatory events and are responsible for the activation of the executioner caspases (caspase 3, caspase 7, and caspase 12). These in turn then

coordinate the apoptotic response (Danial and Korsmeyer, 2004; Baumann et al., 2002; Pinkoski and Green, 2005). Executioner caspases also activate DNA fragmentation factor (DFF) which is responsible for DNA degradation (Huppertz et al., 1999).

The complex apoptotic process can be broken down into three sequential phases: initiation, execution and death (Hoffman-Goetz et al., 2005). In the initiation phase cellular signaling results in activation of initiator caspase enzymes. The execution stage begins with the activation of executioner caspases; during this stage the cell reaches a point of no return and becomes committed to death. Finally, during the death stage, the nucleus collapses and apoptotic bodies are formed (Huppertz et al., 1999). One key event in this process is the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the surface (Vermes et al., 1995). This occurs early in the apoptotic cascade (during the initiation stage) and acts a signal to phagocytes that death is imminent (Huppertz et al., 1999; Krysko et al., 2006). This signaling is important as the recognition of dying cells facilitates their removal and prevents the onset of inflammation (Savill and Fadok, 2000).

Under certain conditions, failure to remove apoptotic cells results in a specific type of death known as secondary or apoptotic necrosis (Majno and Joris, 1995). The late apoptotic cells lose membrane integrity and leak intracellular components that may result in inflammation (Cohen et al., 2002). The conflicting reports about whether secondary necrosis can cause inflammation may be partly explained by the properties of the intracellular fragments. Krysko et al. (2006) argues that the intracellular contents of secondary apoptotic cells have undergone degradation by the apoptotic cellular machinery and thus the protein fragments may or may not be immunogenic. In addition,

upon phagocytosis apoptotic cells release IL-10 (Gao et al., 1998) and TGF-β (Chen et al., 2001), thereby, promoting an anti-inflammatory environment. The loss of the suppressing activity of the phagocytes engulfing the apoptotic bodies could also skew the response towards inflammation.

The two main pathways of apoptosis are the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. The intrinsic pathway of apoptosis is induced by various stimuli that include chemotherapeutic agents, UV radiation, oxidant stress molecules, glucocorticoids, and growth factor withdrawal (Gupta, 2005; Hoffman-Goetz et al., 2005). The permeability of the outer mitochondrial membrane, which is controlled by the ratio of the Bcl-2 family of proteins, mediates apoptosis by the intrinsic pathway (Hengartner, 2000; Youle and Strasser, 2008). Proteins of the Bcl-2 family include both pro- (e.g., Bax) and anti- (e.g., Bcl-2) apoptotic proteins. Pro-apoptotic proteins reside within the mitochondrial outer membrane and upon activation these proteins undergo conformational changes that contribute to pore formation (van Loo et al., 2002). This pore formation increases mitochondrial outer membrane permeability (MOMP) and allows the contents of the intermembrane space to be released into the cytoplasm. The anti-apoptotic effect of Bcl-2 is attributed to its ability to bind and prevent the conformational changes associated with pro-apoptotic protein activation (Youle and Strasser, 2008). Therefore, anti-apoptotic proteins negatively regulate MOMP, inhibiting pore formation, and preventing the escape of important down-stream proteins responsible for carrying out apoptosis.

Intermembrane space contents include cytochrome c and pro-caspases. Once in the cytoplasm, cytochrome c interacts with APAF-1 (apoptotic protease activating factor-

1), ATP (adenosine triphosphate), and pro-caspase 9; together they form a complex known as the apoptosome (Adams and Cory, 2002). The apoptosome activates caspase 9 and initiates a series of enzymatic reactions that results in the eventual conversion of pro-caspase 3 to the active executioner caspase 3 (Hoffman-Goetz et al., 2005).

In contrast, the extrinsic pathway of apoptosis is induced by death signals which are provided by interaction between a ligand and its cellular receptor. Cellular death receptors include Fas (CD95/APO-1) and tumour necrosis factor receptor (TNFR), and their ligands: Fas ligand (Fas L) and TNF- α (Huerta et al., 2006). Upon ligation, death signals initiate a cascade involving recruitment of adaptor proteins and the eventual activation of initiator (e.g., caspase 8) and executioner (e.g., caspase 3) caspases.

Cross-talk may occur between the intrinsic and extrinsic pathways. When pro-caspase 8 levels are low in the cytoplasm, the cell amplifies the caspase cascade via the mitochondrial pathway using the protein Bid (Hoffman-Goetz et al., 2005). In this pathway Bid is cleaved producing a truncated protein (tBid) that translocates from the cytoplasm to the mitochondria. Once in the mitochondria tBid exerts a pro-apoptotic effect by inducing conformational changes in Bax (pro-apoptotic protein) and inhibiting the activity of Bcl-2 (anti-apoptotic protein). This results in the release of cytochrome c and the eventual activation of caspase 3 (Budd, 2002; Gupta, 2005).

1.3.2. Alternative Apoptotic Pathways

The above section highlights the sequential and step-wise apoptotic process that results in the eventual activation of caspase 3. Although caspase activation is the principle mechanism of apoptotic death, caspase-independent pathways also exist. For

example, apoptosis inducing factor (AIF) and endonuclease G (endoG) are proteins located in the mitochondrial intermembrane space and are released following increased MOMP. These enzymes are capable of directly inducing apoptosis via nuclear translocation and DNA degradation (Kim et al., 2005). Other organelles such as lysosomes and endoplasmic reticulum are both capable of inducing apoptosis (Ferri and Kroemer, 2001). Lysosomes are "suicide" vacuoles which contain a large number of hydrolase enzymes that upon release damage integral cellular components. Alterations in the integrity of the lysosomal membrane facilitate the release of enzymes, such as cathepsins, that are directly involved in apoptosis (Guicciardi et al., 2004). Cathepsins are also capable of participating in up-stream apoptotic events that eventually result in an increased MOMP (Chwieralski et al., 2006).

The endoplasmic reticulum (ER) is capable of sensing cellular stresses through two mechanisms: accumulation of misfolded proteins and perturbation of intracellular calcium levels. Together these signals activate ER associated caspase 12, an executioner caspase capable of directly inducing apoptosis (Broker et al., 2005). Increases in intracellular calcium levels can activate calpains, which are cysteine proteases with similar activity to caspases (Wang, 2000). Calpains induce apoptosis by either activation of caspase 12 or by activating pro-apoptotic proteins (e.g., Bax) that increase MOMP (Ferri and Kroemer, 2001). In addition, calpains may alter lysosomal permeability leading to cathepsin release and apoptosis (Yamashima, 2004).

To summarize, at any point multiple apoptotic pathways may occur simultaneously and work together to determine the eventual fate of the cell. Cross-talk

between pathways may occur and the sensing of cellular events by various organelles allows for a coordinated and organized controlled form of cell death.

1.3.3. Necrosis

Necrosis does not involve DNA or protein degradation and is characterized by cytoplasmic and mitochondrial swelling prior to rupture of the plasma membrane (Kroemer et al., 1998). It occurs following cellular injury (e.g., ischemia-reperfusion, drug induced) and is described as a passive process, mainly because of its independence from ATP. Necrosis affects groups of cells rather than occurring on an individual cellular level as in apoptosis (Jaeschke and Lemasters, 2003). Necrosis may also occur if ATP is rapidly depleted at the onset of apoptotic stimuli (Nicotera and Melino, 2004). Thus, apoptosis and necrosis may occur following the same treatment such as ischemia/reperfusion induced hepatocyte death (Malhi et al., 2006) and DSS-induced colonic epithelial death (Renes et al., 2002). In necrosis, plasma membrane disruption and the resultant release of cellular contents cause inflammation and is a distinctive feature between necrosis and apoptosis (Gibson, 2004).

1.4. Intestinal Lymphocyte Apoptosis and Inflammation

Apoptosis of lymphocytes is an important step in removing the vast number of potent antigen specific T cells generated during an inflammatory response. Failure to execute apoptosis of activated IL results in prolonged inflammation leading to increased tissue damage and potentially disease (Levine and Fiocchi, 2001). Following an immune

response, apoptosis of lymphocytes can be induced by active or passive mechanisms. Active methods of apoptosis include activated induced cell death, whereby Fas L binds to Fas located on the surface of the T cells. Passive methods involve withdrawal of cytokines required for lymphocyte survival (Neurath et al., 2001).

LPL are susceptible to Fas mediated apoptosis and exhibit higher degrees of spontaneous apoptosis compared to PBL (Neurath et al., 2001). Apoptotic resistance of LPL is a common observation in individuals with IBD. For example, colonic T cells of patients suffering from Crohn's Disease (CD) had increased Bcl-2/Bax ratios and increased numbers of $CD3^+$ T cells; these cells were also resistant to Fas and TNF-α mediated death (Ina et al., 1999). Similarly, in both CD and ulcerative colitis (UC) patients, Fas-induced apoptosis was defective upon stimulation with CD2 (Boirivant et al., 1999) and apoptotic resistant T cells in inflamed tissues had increased levels of the anti-apoptotic protein Bcl-2 (Atreya et al., 2000).

Given the resistance to apoptosis of LPL in IBD, a potential therapeutic approach may be by inducing apoptosis of activated IL. Currently, the primary approach is the use of anti-cytokine treatments to indirectly induce apoptosis (Van Den Brande et al., 2002). However, any molecule capable of receptor binding, internalization and activation of the apoptotic machinery should theoretically be able to initiate IL death. Successful anti-cytokine treatments include those directed at TNF-α. Lugering et al. (2001) reported that following anti-TNF-α antibody treatment in patients with active CD, there was elevated expression of cytochrome c, Bax and Bak and the induction of caspase 9 and caspase 3 in blood monocytes. Similar observations were reported by Di Sabatino and colleagues (2004) whereby anti-TNF-α treatments resulted in the healing of mucosal ulcerations and

improved disease activity index scores in CD patients. Myers et al. (2003) demonstrated that antisense oligonucleotides specific for TNF-α mRNA effectively reduced TNF-α expression and resulted in a 44% reduction in the disease activity scores and prevented colonic shortening in mice following DSS treatment. Collectively, these results imply that induction of lymphocyte apoptosis during periods of inflammation may provide some degree of protection in the bowel from these potentially tissue damaging cells (or their products).

1.4.1. Acute Exercise and Intestinal Lymphocyte Apoptosis

In response to strenuous and prolonged exercise there is a well documented increase in plasma levels of the adrenal glucocorticoid (GC) hormone corticosterone (CORT) (see for example, Murosaki et al., 1997; Quadrilatero and Hoffman-Goetz, 2005; Mastorakos et al., 2005). The role of GC is to recruit important energy reserves required for exercise through the stimulation of gluconeogenesis and the mobilization of amino and fatty acids (Tharp, 1975). GC hormones are also potent anti-inflammatory and apoptosis inducing agents used to treat a wide variety of ailments including IBD (Tuckermann et al., 2005). Indeed both elevated plasma levels of corticosterone and increased apoptosis in thymocytes (Concordet and Ferry, 1993; Quadrilatero and Hoffman-Goetz, 2005) and intestinal lymphocytes (Hoffman-Goetz and Quadrilatero, 2003) have been reported in mice following exercise.

At first glance it may appear that increased apoptosis of IL with acute exercise could offer protection against inflammation. However, during acute exercise there is also increased oxidative stress and intestinal ischemia that may cause death of the intestinal

epithelial cells concurrent with IL death. Indeed, alterations to the epithelial barrier are evident in studies demonstrating endotoxemia post exercise. For example, following strenuous exercise Ashton et al. (2003) and Bosenberg et al. (1988) reported increased concentrations of LPS in the blood of human volunteers. However, the results of these studies may be questioned given the limitations of the LPS assay. These include the difficulty in LPS isolation from other membrane phospholipids and cytoplasmic components as well as the hydrophobicity of the endotoxin (Cordwell, 2006). In addition, when examining the effects of exercise in humans it is important to record all details of the experimental conditions such as the environmental factors (e.g., temperature, humidity), exercise protocol (e.g., exhaustive vs. submaximal work), subject characteristics (e.g., age, weight) and whether the subjects were ingesting dietary supplements. The works cited here do not contain these potential shortcomings but it is important to ensure that when reviewing the literature, particularly with older studies, that they do not contain these experimental limitations. Nonetheless, as discussed in section 1.2.4.2, if bacterial translocation were to occur post exercise it may result in an inflammatory response, as the complex immune network of highly activated lymphocytes would encounter bacterial antigen. This interaction, and subsequent immune response, would ensue even after the exercise stress is removed. Thus, theoretically the net result could be activation of immune cells (including lymphocytes) in response to the bacterial infection.

1.5. Lactoferrin

The transferrin family of proteins is responsible for the regulation of iron in both the blood (serum transferrin) and tissues (Lactoferrin - Lf) (Wally and Buchanan, 2007). Iron is potentially detrimental to the host as it can either enhance the formation of tissue damaging free radicals or provide an important nutritional source to bacteria (Carrier et al., 2006). Lf is produced endogenously (human Lf - hLf) by glandular epithelial cells and is found in external (i.e., saliva, tears) and mucosal secretions as well as in secondary neutrophil granules (Baker and Baker, 2005; Ward et al., 2005). A major dietary source of Lf is cow (bovine) milk. Bovine lactoferrin (bLf) is an 80 kDa glycoprotein found at concentrations of 0.2 mg/mL and 1.5 mg/mL in milk and colostrums, respectively (Marshall, 2004). Upon pepsin digestion lactoferricin (Lfcin) is generated and this hydrolysate exhibits similar and, in some cases, enhanced biological properties to that of Lf (Gifford et al., 2005). hLf and bLf share 69% sequence homology (bLf and hLf contain 689 and 691 amino acids, respectively) (Pierce et al., 1991) and both contain cationic regions owing to the cellular binding ability of Lf (Legrand et al., 2006).

Historically, the immunological benefit of Lf was attributed to its ability to bind iron and prevent bacterial growth (Teraguchi et al., 1995). Recently, the biological functions of Lf have been expanded to also include effects on various immune parameters. For example, dietary feeding of Lf to weanling pigs increased mRNA production of the anti-microbial peptides 39-residue proline–arginine-rich peptide and protegrin-1 (Wang et al., 2006); these observations suggest a bacteriocidal role of Lf. Lf administration increased IgA and IgG concentrations in the saliva and intestinal secretions of mice; in vitro LPS stimulated splenocytes increased production of both IgG

and IgA in the presence of Lf (Debbabi et al., 1998). Similar findings were found by Shan et al. (2007) who reported that Lf supplementation in pigs increased serum IgG and IgA levels following stimulation of lymphocytes with the plant lectin, phytohemagglutinin.

The immunological properties of Lf are likely due to its cell binding ability (Legrand et al., 2006) and surface molecules have been identified that bind and internalize Lf (Suzuki et al., 2005; Legrand et al., 2006). Cells found to bind to Lf include neutrophils (Deriy et al., 2000; Birgens et al., 1984), mononuclear phagocytes (Britigan et al., 1991; Birgens et al., 1984), peripheral blood $CD4^+$ cells (Li et al., 2006), T and B-cells (Birgens et al., 1984), activated lymphocytes (Mincheva-Nilsson et al., 1997; Mazurier et al, 1989) and intestinal epithelial cells (Ashida et al., 2004). The role of Lf once inside the cell is unknown but researchers have suggested that Lf modulates DNA expression. In support of this hypothesis is the following evidence: 1) Lf is internalized in intestinal cells and is found concentrated around the nucleus (Ashida et al., 2004), 2) Lf receptors are found at greater concentrations within the cell on active lymphocytes (Mazurier et al., 1989) and, 3) Lf binds with distinct sequence specificity to DNA (He and Furmanski, 1995). Although the notion of signaling from receptor to DNA binding and transcriptional regulation is still controversial, the prevailing perspective is that the immunomodulatory effects of Lf are mediated by internalization via a specific cellular receptor (Ward et al., 2002; Legrand et al., 2005).

1.5.1. Inflammatory Properties

There are several lines of evidence that show Lf has anti-inflammatory properties. First, independent of its iron binding ability, Lf can bind and neutralize several bacterial components such as LPS and Lipid A (Appelmelk et al., 1994). This neutralization reduces bacterial activity and decreases the immune response including decreased production of pro-inflammatory cytokines (Fischer et al., 2006). In human CaCo-2 cells, Lf, co-cultured with invasive *E. coli* HB101 (pRI203), significantly attenuated the increase in levels of TNF-α and IL-6 (Berlutti et al., 2006). Valenti and colleagues (1999) demonstrated that bLf binding to *Listeria monocytogenes* prevented this bacterium from inducing intestinal tissue damage. Thus, interaction of Lf with bacterium or bacterial components may prevent or reduce inflammation

The anti-inflammatory role of bLf is also related to its ability to promote anti-inflammatory cytokine production or reduce pro-inflammatory cytokine production. In support of Lf as an anti-inflammatory protein is the following evidence: 1) Lf addition in culture reduced IL-2 production (important in T cell activation), in T_H1 but not T_H2 cells (Zimecki et al., 1996), 2) Lf administration reduced IFN-γ and increased IL-10 in the peripheral blood of patients suffering from systemic lupus (Li et al., 2006), 3) bLf addition by gavage attenuated the increase of TNF-α, IL-1 and IL-6 in the ears and joints of zymosan treated mice (Hartog et al., 2007), 4) oral administration of bLf reduced plasma levels of TNF-α and increased IL-10 in a rat model of rheumatoid arthritis (Hayashida et al., 2004), 5) bLf provided by gavage reduced TNF-α and increased IL-10 in the colonic tissue of rats subjected to DSS and TNBS induced colitis (Togawa et al.,

2002a; 2002b), and 6) hLf and bLf incubated with monocytes reduced TNF-α production following LPS stimulation (Choe and Lee, 1999).

However, Lf has also been shown to have pro-inflammatory effects. Evidence for the pro-inflammatory effects of bLf include: 1) reduced lung colonization of Co26Lu tumour cells following bLf administration in mice by gavage, which was attributed to increased IL-18, IFN-γ and IL-1 (T_H1 cytokines) as well as enhanced phagocytotic activity of splenocytes and augmented NK cell activity (Kuhara et al., 2000; Wang et al., 2000), 2) increased concentrations of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-1 as well as decreased IL-2, were reported in the small intestine of mice receiving bLf by gavage (Iigo et al., 2004), 3) increased expression of IL-10 and IFN-α in mouse lymphocytes following bLf administration (Takakura et al., 2006), 4) addition of bLf increased Type 1 interferon and TNF- α production and reduced viral load up to 100 fold in macrophages infected with the vesicular stomatitis virus (Puddu et al., 2007), 5) the phagocytic activity of neutrophils in healthy volunteers was increased following co-culture with bLf (Miyauchi et al., 1998), and 6) macrophages incubated with bLf were activated via upregulation of CD40 and increased production of the pro-inflammatory cytokine IL-6 (Curran et al., 2006).

The basis for these mixed effects of bLf (i.e., pro- and anti-inflammatory responses) is not fully understood. However; several factors such as the route of administration and the immune status of the individual may be critical. For example, Sfeir et al. (2004) demonstrated that bLf administered by gavage or by intramuscular injection resulted in a mixed T_H1/T_H2 (e.g., increased IFN-γ, IL-4; IgG_{2a}, IgG_1) response whereas dietary administration resulted solely in a T_H2 (e.g., increased IL-4, IL-5; IgG_1)

response. Indeed, Yamauchi et al. (2006) argue that mode of administration may alter bLf digestion thus affecting the nature of the peptide fragment available for absorption or cellular interaction. In addition, the immune status of the individual can influence the direction of the bLf response. For example, during carcinogenesis or viral challenge bLf appears to increase T_H1 cytokine production whereas during inflammatory conditions bLf favours T_H2 cytokine production (Fischer et al., 2006). Although the mechanism is not understood at this time, Fischer and colleagues speculate that bLf affects either T_H cell maturation, differentiation, or development after the initial stimulus. Once the T_H cell is committed along a path of development (i.e., post stimulus) the addition of bLf may enhance the specific pathway of development. Taken together, both the mode of bLf administration and immune status appear to influence its mechanism of action and may explain its ability to drive two separate (and opposite) responses.

1.5.2. Bovine Lactoferrin, Apoptosis and Proliferation

Among the roles of Lf, it may influence various parameters of cell death and cell proliferation. Addition of Lf to the diets of animals has been shown to increase apoptosis or the expression of apoptotic proteins within epithelial cells. Fujita et al. (2004a; 2004b) found that Fas expression is upregulated in the colonic mucosa of AOM treated mice fed bLf; expression of Bid, Bax, caspase 8 and caspase 3 (all pro-apoptotic proteins) were increased as a result of bLf enriched diets. Lee et al. (2008) observed increases in caspase 9 and caspase 3 in Jukat T leukemia cells following incubation with hLf. In human tongue-derived cancer cells and in human monocytic tumour cells increased apoptotic morphology (e.g., apoptotic bodies, chromatin condensation), increased DNA

fragmentation, and elevated caspase 3 levels were visualized following LFcin treatment (Sakai et al., 2005; Yoo et al., 1997). Therefore, Lf or its derivatives, may be able to induce apoptosis or influence apoptotic proteins to favour cell death.

On the other hand, Lf can alter epithelial cell (EC) proliferation. In vitro administration of Lf resulted in increased proliferation (as measured by ^{3}[H]-thymidine incorporation) in CaCo-2 cells (Buccigrossi et al., 2007) and in rat intestinal epithelial cells (Hagiwara et al., 1995). Similarly, Nichols et al. (1990) demonstrated increased ^{3}[H]-thymidine incorporation in rat enterocytes following in vitro hLf treatment. These studies indicate increased in vitro cellular proliferation in the presence Lf. The nature of this diverse response (apoptosis vs. cell proliferation) may be related to the type of cell involved: neoplastic vs. normal cell. Cell numbers decreased to below original values in human colon adenocarcinoma cells following Lf addition (Amouric et al., 1984). Cell viability was reduced by 50% in Jurkat T cells following incubation with hLf (Lee et al., 2008). Lf may thus decrease proliferation of atypical cells but promote the growth of normal healthy cells.

Chapter 2: Study Rationale and Conceptual Framework

Inflammatory bowel diseases (IBD), characterized by recurring and relapsing bouts of intestinal inflammation, affect millions of people worldwide (estimated 1.5 million Americans and 2.2 million Europeans) and are diseases of high morbidity (Loftus, 2004). A potential detrimental consequence of sustained intestinal inflammation is tumour formation and an approximate 10 fold increase in colon cancer incidence is observed in patients with ulcerative colitis, a form of IBD (Seril et al., 2003). Even in situations of less profound clinical pathology, such as gastrointestinal disturbances experienced by athletes, the treatment of intestinal inflammation has numerous applications and advantages.

Intestinal lymphocytes (IL) are integral in maintaining homeostasis as they participate in both immune effector and regulator functions. Apoptosis, or programmed cell death, is essential in regulating the population of these immune cells, as failure to remove activated lymphocytes can result in the onset of inflammatory disease (Levine and Fiocchi, 2001). In addition, cytokines play a central role in regulating the immune response. The balance between pro-inflammatory (e.g., TNF-α) and anti-inflammatory (e.g., IL-10) cytokines is critical in the regulation of immunological homeostasis. Therefore, the examination of both apoptosis and inflammatory cytokine levels in intestinal lymphocytes offers useful insight into mechanisms of intestinal immune regulation.

Bovine lactoferrin (bLf) is a common dietary protein found in dairy products and during inflammatory stress this protein has demonstrated anti-inflammatory properties.

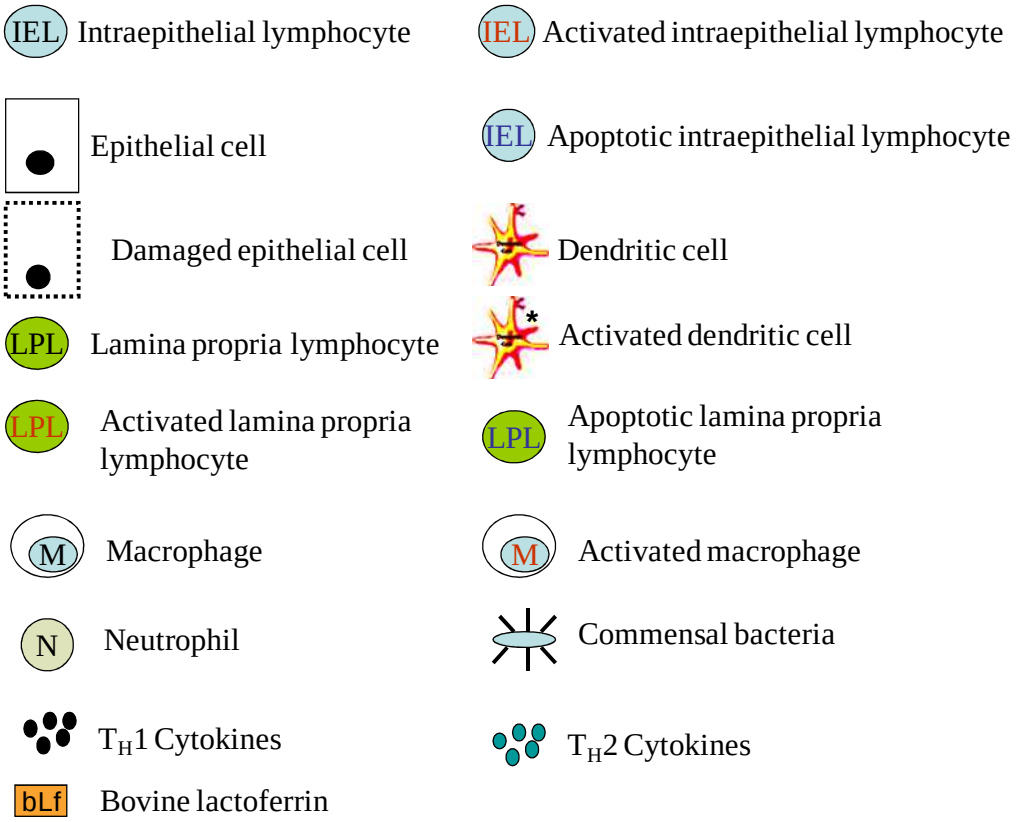
These immunomodulatory attributes of bLf are, in part, mediated by altering the environment in favour of anti-inflammatory cytokines (e.g., decreased pro- and/or increased anti-inflammatory cytokine levels). bLf has also been shown to affect apoptosis in epithelial cells and carcinoma cells in vitro. Although it has not been determined experimentally whether dietary bLf affects apoptosis of intestinal lymphocytes there is evidence to suggest a possible mechanism. Therefore, bLf is a potentially attractive agent for a variety of inflammatory disorders and, perhaps, carcinogenesis. Clinically, the benefits of bLf induced IL apoptosis and the generation of an anti-inflammation cytokine environment would include: alleviation of disease symptoms (in athletes and IBD patients), reduced tissue damage and the potential for reduced colon carcinogenesis.

The purpose of this research was to describe the effects of bLf in mice on: 1) IL apoptosis (using surface expression of phosphatidylserine, cell number and protein levels of pro-apoptotic caspase 3 and anti-apoptotic Bcl-2) and pro-(TNF-α) and anti-(IL-10) inflammatory cytokine levels in a healthy, non-inflamed state; 2) IL apoptosis (using surface expression of phosphatidylserine, cell number and protein levels of pro-apoptotic caspase 3 and cytochrome c and anti-apoptotic Bcl-2), pro- (TNF-α) and anti- (IL-10) inflammatory cytokine levels, and levels of the pro-inflammatory transcription factor NFκB under conditions of inflammation induced by a physiological stressor (i.e., repeated bouts of acute exercise (AE); 3) IL apoptosis (using surface expression of phosphatidylserine, cell number and protein levels of pro-apoptotic caspase 3, cytochrome c, and Bax and anti-apoptotic Bcl-2), pro- (TNF-α) and anti- (IL-10) inflammatory cytokine levels, and levels of the pro-inflammatory transcription factor NFκB under conditions of inflammation induced by the addition of a chemical irritant

(e.g., DSS); 4) imparting mucosal protection against DSS and/or AE induced intestinal alterations (using histological analysis); and 5) IL apoptosis (by measuring levels of pro-apoptotic caspase 3 and anti-apoptotic Bcl-2) and the formation of pre-cancerous colonic lesions (i.e., aberrant crypts) under conditions of inflammation (i.e., DSS or AE) following carcinogen (e.g., AOM) exposure.

Based on published research it was hypothesized that during inflammation bLf would act on IL to induce apoptosis and alter cytokine levels to either increase anti-inflammatory or reduce pro-inflammatory cytokines. The overall conceptual framework for the series of experiments described in the subsequent chapters is presented diagrammatically in Figures 2.1-2.3. Figure 2.1 illustrates the normal, non-inflamed state of the intestine with a focus on T cells. Figure 2.2 presents inflammation processes in the intestine, again with an emphasis on T cells. Figure 2.3 shows how bLf may influence the inflammatory process by altering both apoptosis of intestinal lymphocytes (i.e., LPL and IEL) and the T_H1/T_H2 cytokine balance.

Legend for figures 2.1, 2.2 and 2.3.



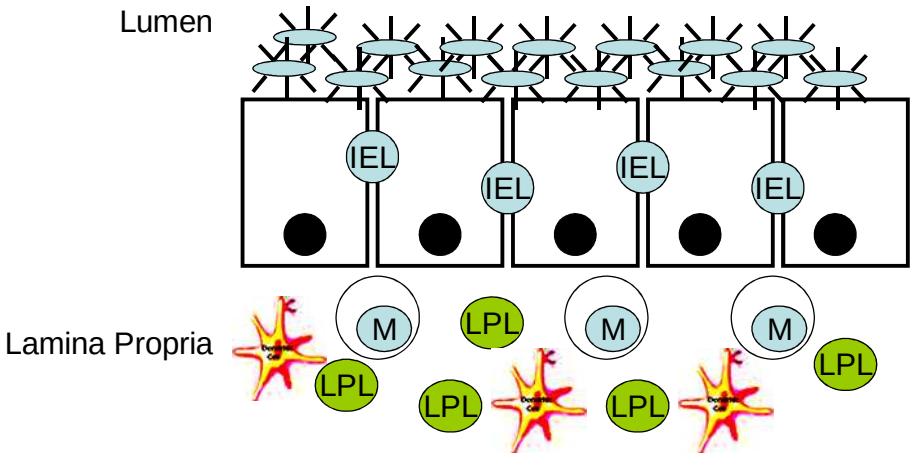


Figure 2.1: Schematic diagram showing leukocyte orientation within the colonic epithelium during a normal, non-inflammatory state (Focusing on T cells).

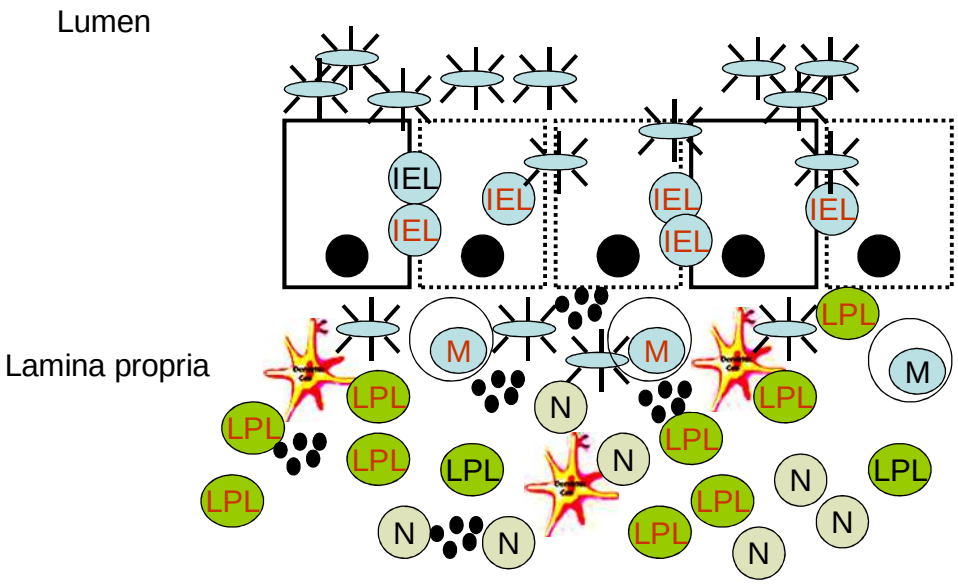


Figure 2.2: Schematic diagram showing leukocytes during inflammation. As the epithelium is damaged and commensal bacteria translocates into the lamina propria, the macrophages and dendritic cells (DC) become activated resulting in subsequent activation of lymphocytes. Activation results in production of T_H1 cytokines and recruitment of neutrophils.

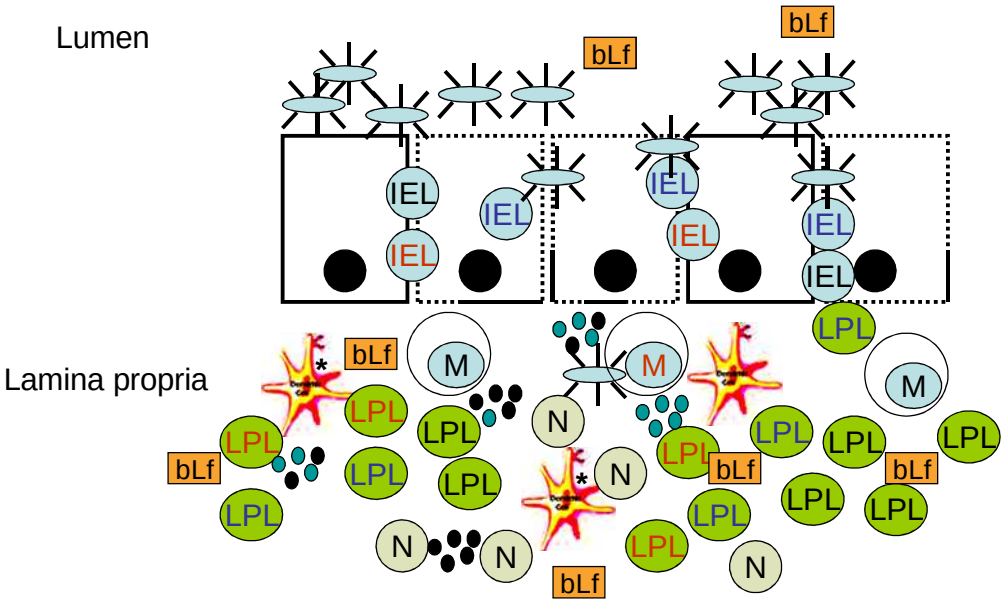


Figure 2.3: Schematic diagram showing the possible effects of bLf during inflammation. bLf may be able to induce apoptosis of activated IEL and LPL. In addition, a possible shift from T_H1 to T_H2 cytokine secretion could occur. The overall result is alleviation from inflammation and recovery of the epithelium.

The addition of bLf during inflammation may thus provide protection by: 1) inducing apoptosis of activated lymphocytes and 2) shifting the cytokine milieu to predominantly a T_H2 environment by either reducing TNF-α or increasing IL-10. It is expected that the bLf induced modifications in both these immunological parameters will result in mucosal protection from the external stimuli.

CHAPTER 3: Effect of Short Term Dietary Intake of Bovine Lactoferrin on Intestinal Lymphocyte Apoptosis in Healthy Mice

3.1. Objectives

To determine the effect of dietary bovine lactoferrin (bLf) administration on $CD4^+$ and $CD8^+$ intestinal lymphocyte (IL) apoptosis and IL cytokine levels of TNF-α and IL-10 in healthy female C57BL/6 mice.

3.2. Hypotheses

Hypothesis 1: bLf administration will decrease the levels of TNF-α and increase the levels of IL-10 in mouse IL relative to the levels of TNF-α and IL-10 in mice not given bLf supplementation.

Hypothesis 2: bLf administration will increase the percent apoptosis in $CD4^+$ and $CD8^+$ mouse IL relative to the percent apoptosis in mice not given bLf supplementation.

3.3. Study Design

This study was designed to provide baseline measurements on the effect of dietary bLf on TNF-α and IL-10 levels and the % $CD4^+$ and $CD8^+$ IL apoptosis during a normal (non-inflammatory) state. It was also the aim of this study to determine an effective bLf dose. Previous studies have determined that during an inflammatory state bLf provided by gavage is able to decrease TNF-α and increase IL-10 in the rat colonic epithelium (Togawa et al., 2002a; 2002b). However, whether this protective effect occurs in IL of healthy mice is unknown. This study also examined varying concentrations of bLf in the diet and determined if there were notable changes in IL apoptosis and cytokine measurements by dose of bLf. IL were isolated and enumerated and apoptosis of these cells determined by measurement of surface phosphatidylserine expression by flow cytometry. Furthermore, levels of the pro-apoptotic protein caspase 3, anti-apoptotic

protein Bcl-2 and the cytokines TNF-α and IL-10 were quantified in mouse IL by Western blot analysis. The study design for this experiment is given in Figure 3.1.

Study duration: Two week acclimation plus 4 d of dietary bLf administration.

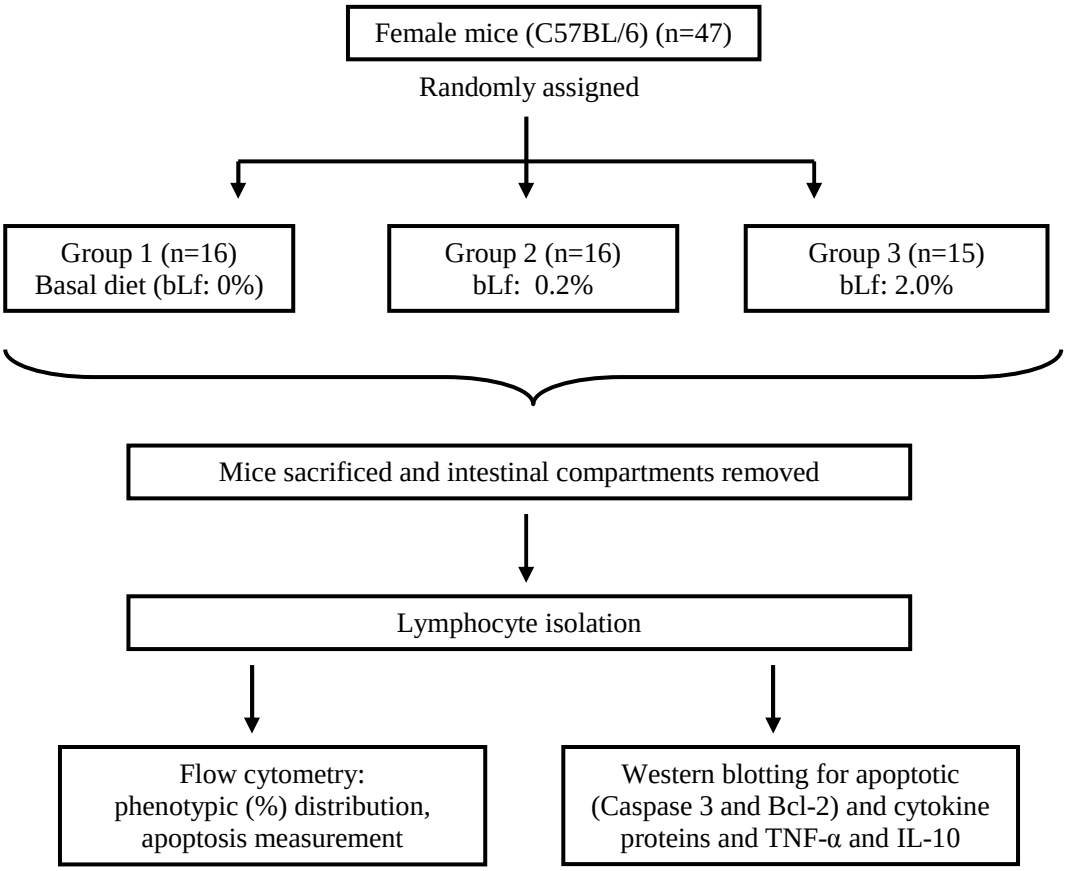


Figure 3.1: Experimental set up for study 1 outlining the outcome measures.

One-way analysis of variance (ANOVA) and post-hoc Tukey test was used to analyze the study data. The independent variable is diet condition (no bLf, 0.2% bLf, 2.0% bLf) and dependent variables included: % $CD4^+$ and % $CD8^+$ IL (as well as percent apoptotic IL), apoptotic markers in IL (caspase 3 and Bcl-2) and cytokine proteins (TNF-α and IL-10). The software package SPSS (version 15) was used to analyze the data and in all cases $p \leq 0.05$ was accepted as being significant.

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3.4. Overview

The objective of this study was to describe the effects of short term dietary exposure of bovine lactoferrin (Lf) on intestinal lymphocyte apoptosis and expression of tumour necrosis factor (TNF)-α in healthy mice. Female C57BL/6 mice were randomly assigned to 3 treatment groups; 0% Lf (n=16), 0.2% Lf (n=16) and 2.0% Lf (n=15). Bovine Lf was administered orally, as part of the diet, for 4 consecutive days. Intestinal lymphocytes (IL) were isolated and analyzed for % CD4, % CD8, % apoptotic CD4, and % apoptotic CD8 cells using flow cytometry. Pro- (caspase 3) and anti- (Bcl-2) apoptotic protein expression and TNF-α expression in IL were determined by Western blotting. There was a significant increase in the % CD4 ($p=0.02$) and % apoptotic CD4 ($p=0.02$) IL in bovine Lf fed compared with control mice. The % CD8, % apoptotic CD8 and the expression of caspase 3 and Bcl-2 in IL did not differ significantly by diet group. In contrast, the expression of TNF-α was significantly lower in Lf fed relative to control mice ($p=0.01$). Short term dietary Lf decreased TNF-α expression in IL and increased apoptosis of CD4 IL in healthy mice.

3.5. Introduction

Bovine lactoferrin (Lf), an iron binding glycoprotein found in they whey fraction of mammalian milk and colostrums, has immune modulatory properties. Lf and lactoferricin (Lfcin), a result of pepsin digestion of Lf, increase apoptosis (programmed cell death) of colonic tumours in rats (Fujita et al., 2004a; 2004b), leukemia (Jurkat), breast cancer (MCF-7), colon cancer (Colo-35) and human cell lines (Mader et al., 2005); Lf is also associated with an increase in the population of NK cells in mice and humans (Kuhara et al., 2000; Shau et al., 1992), an increase in the population of CD8 T cells in mice (Kuhara et al., 2000; Wang et al., 2000), and a decrease in blood concentrations of the T_H1 cytokines, TNF-α and IFN-γ, during inflammation in rats (Hayashida et al., 2004; Togawa et al., 2002a; 2002b). CD4 T lymphocytes, crucial to immune regulation through the production of cytokines, are also affected by Lf. For example, after Lf administration to rodents, CD4 T cells increase in peripheral blood and decrease in the spleen (Wakabayashi et al., 2006), and increase in mucosal lymphoid tissue (Wang et al., 2000)

Most experimental studies on the immune modulating effects of exogenous Lf have focused on pathological conditions including carcinogenesis (Fujita et al., 2004a; 2002b; Kuhara et al., 2002; Wang et al., 2000; Sekine et al., 1997a; 1997b) and inflammation (Togawa et al., 2002a; 2002b; Wakabayashi et al., 2006). Few studies describe how (or if) Lf acts on lymphocytes in non-diseased states (Wakabayashi et al., 2006; Takakura et al., 2006; Debabbi et al., 1998; Sfeir et al., 2004). Moreover, experimental studies (Kuhara et al., 2000; Wang et al., 2000; Hayashida et al., 2004; Togawa et al., 2002a; 2002b; Wakabayashi et al., 2006; Takakura et al., 2006; Debabbi et al., 1998) typically use gastric intubation to deliver Lf, a modality which is clearly non-

physiological. Indeed, there is virtually no research on Lf administration through physiological routes (i.e., oral feeding) in non-disease states. This absence is striking given that Sfeir et al. (2004) reported mucosal immune responsiveness varies according to the route of Lf administration.

The purpose of this pilot study was to describe the effects in healthy animals of short term dietary exposure to bovine Lf on intestinal lymphocyte apoptosis (Annexin V and caspase 3 expression) and the expression of tumour necrosis factor (TNF)-α. Because Lf induces apoptosis under pathological conditions and in tumour cell lines *in vitro*, our hypothesis was that dietary bovine Lf would also increase apoptosis in IL obtained from healthy animals.

3.6. Materials and Methods

Animals and Diets: Female C57BL/6 mice (n=47) (Harlan Sprague Dawley, Indianapolis, IN, USA), 3-4 weeks of age, were housed individually at 21±1^{o}C on a 12/12h reversed light/dark cycle, with *ad libitum* access to a maintenance diet (Laboratory Rodent Chow, PMI feeds Richmond, IN, USA) and tap water for 2 weeks. After this acclimation period, mice were randomly assigned based on weight, to 3 diet groups: control [0% bovine Lf] (n=16), low dose [0.2% bovine Lf] (n=16), or high dose [2.0% bovine Lf] (n=15). Diets were based on a semi-purified AIN 76A standard diet and formulated to contain either: 0%, 0.2% or 2.0% bovine Lf (Erie Foods International Inc, Erie IL), while maintaining similar overall protein concentrations (20% total protein in all groups) (Table 3.1). Diets were individually prepared and pelleted to resemble the maintenance diet and no change was observed in feeding behaviour (i.e., amount, pattern

or timing of feeding) compared to the maintenance diet. Bovine Lf was 16% iron saturated and greater than 90% pure. Mice were given *ad libitum* access to the Lf containing diets for 4 consecutive days prior to sacrifice. All protocols with live animals conformed to the established ethical guidelines of the Canadian Council on Animal Care and were approved by the University Animal Ethics Committee.

Table 3.1: Composition of the semi-purified AIN 76A diets

Modified AIN 76 A Diet			
Component	**Control (%)**	**0.2% Lf (%)**	**2.0% Lf (%)**
Sucrose	49.9	49.9	49.9
Casein	20.0	19.8	18.0
Bovine Lf	0.0	0.2	2.0
Corn Stach	15.0	15.0	15.0
Cellulose	5.0	5.0	5.0
Corn Oil	5.0	5.0	5.0
Mineral Mix	3.5	3.5	3.5
Vitamin Mix	1.0	1.0	1.0
Choline	0.2	0.2	0.2
L-cysteine	0.3	0.3	0.3
TBHQ	0.1	0.1	0.1

Intestinal Lymphocyte Preparation: Intestinal lymphocytes (IL) were isolated according to Lefrancois (1993) and modified by Hoffman-Goetz and Quadrilatero (2003). The intestinal compartment was immediately removed, Peyer's patches and visible fat dissected out, intestinal lymphocytes (IL) isolated over a column of 0.3 g of nylon wool, washed, and layered over a density gradient medium (Lympholyte-M; Cedarlane Laboratories, Hornby, Ont.) to remove epithelial cells and debris. IL were collected and counted manually by microscopy after staining with Turk's solution (Hoffman-Goetz and Spagnuolo, 2007b). IL included both intraepithelial and lamina propria lymphocytes.

IL Apoptosis and Phenotypes: Apoptosis was assessed by FITC-Annexin V^+ staining which detects the expression of phosphatidylserine on cell surface membranes. Externalization of phosphatidylserine is an early apoptotic event and occurs as a result of the loss of plasma membrane lipid asymmetry (Vermes et al., 2002). Propidium iodide (PI) is a non-specific DNA dye that becomes incorporated in non-viable cells but is excluded from living cells with intact plasma membranes (Lecoeur et al., 2001). PI was used to assess late apoptotic or dead cells. Briefly, 1 x 10^5 IL were incubated with 100µL Annexin V binding buffer (Pharmingen, Mississauga, ON) and 2.5 µL Annexin V-FITC monoclonal antibody and 2.5µL of PI (Sigma Chemical) for 15 min at room temperature in the dark. 400µL Annexin V binding buffer was added to each sample before analysis by flow cytometry (Epics XL Flow Cytometer, Beckman-Coulter, Hialeah, FL) for apoptotic and necrotic cells.

For T cell phenotypes, 1 x 10^5 IL were incubated for 45 min at 4ºC in the dark with 2.5 µL of anti-mouse monoclonal antibodies (mAb): FITC-conjugated CD45 (anti-

CD45, clone: 30F11), PE-conjugated CD4 (anti-CD4, clone: GK1.5), or PE-conjugated CD8α (anti-CD8a, clone: 53-6.7) (Pharmingen). Details about preparation of cell suspensions for determination of lymphocyte phenotypes are reported elsewhere (Hoffman-Goetz and Quadrilatero, 2003; Davidson and Hoffman-Goetz, 2006). The initial acquisition gate was created based on the forward and side scatter properties of a population shown to collect >90% $CD45^+$ cells (leukocytes).

Western Blot Analysis of Caspase 3, Bcl-2 and TNF-α: Lysis buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100] and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) were added to IL on ice for 45 min. Lysates were centrifuged, the supernatant collected, and protein was determined by the BCA assay. Forty micrograms of protein and molecular weight markers (Full Range Rainbow and Biotin Markers, Amersham Biosciences, Buckinghamshire, U.K.) were separated by electrophoresis on a 12% SDS PAGE gel and transferred onto a PVDF membrane (Sigma Chemical). Following transfer, membranes were stained with Ponceau S (Sigma Chemical) to confirm quality of transfer and equal loading. Membranes were blocked overnight in 10% milk-TBST (Tris-Buffered Saline Tween-20) at 4^oC and incubated with primary antibody for 1 h. The concentration of antibodies to Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG_1, MW=28kDa), caspase 3 (clone: H-227; rabbit anti-human clone IgG, MW=35kDa) and TNF-α (clone: N-19; goat anti-human polyclonal IgG, MW=17kDa) were 1:200 (in 10% milk-TBST) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by incubation for 1 h with secondary antibody: horseradish peroxidase-conjugated anti-mouse (Bcl-2), anti-rabbit (caspase 3) or anti-goat (TNF-α) IgG at a concentration of

1:2000 in 10% milk-TBST. Protein was determined using ECL Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging System (Cambridge, UK). Samples from the three treatment groups were run together on the gels.

Statistical Analysis: All data were analyzed with a one-way analysis of variance by group using SPSS (Version 15; Chicago, IL, USA). Post hoc analysis was performed with Tukey HSD test to determine difference between the three diet groups. For Tukey's post hoc analysis, $p \leq 0.05$ was accepted as being significantly different from chance alone. All values are expressed as group means ± SEM.

3.7. Results

There was no significant change in body weights over the 4 days of the experiment (average start weights: 0% Lf: 17.20 ± 0.40g; 0.2% Lf: 17.58 ± 0.46g; 2.0% Lf: 17.25 ± 0.27g; average end weights: 0% Lf: 17.20 ± 0.64g; 0.2% Lf: 17.80 ± 0.45g; 2.0% Lf: 17.48 ± 0.37g), or in food intake (0% Lf: 10.45 ± 0.45g; 0.2% Lf: 10.86 ± 0.46g; 2.0% Lf: 10.42 ± 0.48g) between the dietary groups.

Table 3.2: Percent apoptosis of IL from mice given dietary bovine lactoferrin

Group	Sample Size (n)	Ann^+ (%) "Early Apoptotic"	Ann^+/PI^+ (%) "Late Apoptotic"	PI^+ (%) "Dead"
0% Lf	16	24.73 ± 1.42	16.57 ± 1.32	17.75 ± 1.36
0.2% Lf	16	31.63 ± 1.94*	22.07 ± 1.89*	23.07 ± 1.89
2.0% Lf	15	31.7 ± 1.77*	24.35 ± 1.66*	25.34 ± 1.66*

*$p \leq 0.05$ compared to control group, using Tukey's post hoc analysis

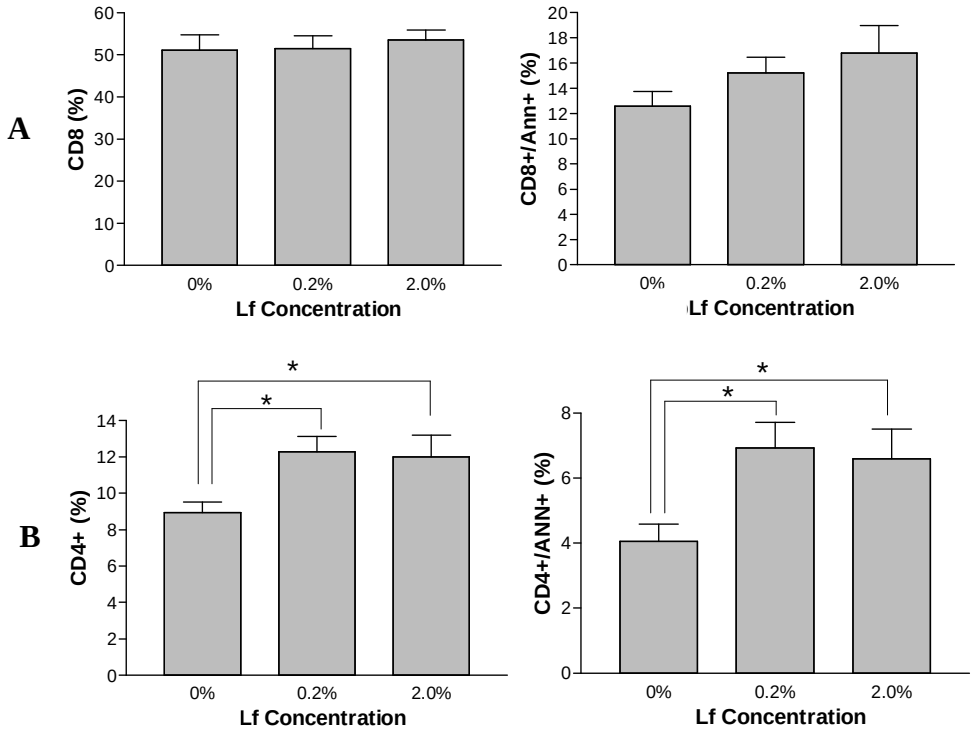

Figure 3.2: Percent T cell phenotypes and apoptosis in IL of mice given bovine Lf in the diet. A: % CD8 IL and % $CD8/Ann^+$ IL; B: % CD4 IL and % $CD4/Ann^+$ IL. All values are means ± SEM. 0% = 0% Lf (n=16), 0.2% = low dose (0.2% Lf) (n=16); 2.0% = high dose (2.0% Lf) (n=15). * $p \leq 0.05$ compared to the control (0% Lf).

There was no significant effect of short term dietary bovine Lf exposure on total IL counts in mice (0% Lf: $4.32x10^7 \pm 0.29$ cells; 0.2% Lf: $3.46x10^7 \pm 0.35$ cells; 2.0% Lf: $3.90 \times 10^7 \pm 0.30$ cells). In contrast, the % Annexin V^+ (early apoptotic) and % Annexin V^+/PI^+ (late apoptotic) IL were significantly higher in the Lf fed relative to control mice ($F_{2,44}$=5.47, P=0.008 and $F_{2,44}$=5.91, P=0.005, respectively) (Table 3.2). The % PI^+ (necrotic) IL was also significantly higher in the 2.0% Lf fed mice relative to the group not supplemented with Lf ($F_{2,44}$=5.57, P=0.007) (Table 3.2).

Figure 3.2 shows the flow cytometry results for the IL T cell subsets $CD8^+$ and $CD4^+$ and for the expression of apoptosis in these subsets using the Annexin V marker. No significant change was observed in the % of $CD8^+$ IL or in the % $CD8^+$/Annexin V^+ as a function of diet group (Figure 3.2A). In contrast, the % $CD4^+$ ($F_{2,44}$=4.38, P=0.02) and the % $CD4^+$/Annexin V^+ ($F_{2,44}$=4.45, P=0.02) were both significantly higher in the Lf groups relative to the control (Figure 3.2B).

Figure 3.3 presents the concentrations of caspase 3, Bcl-2 and TNF-α, as determined from Western blot analysis, in response to Lf or control diets in mouse IL. Short term dietary Lf did not significantly alter the expression of caspase 3 or Bcl-2 in mouse IL. However, the expression of TNF-α (Figure 3.3C) was significantly affected by diet condition ($F_{2,23}$=5.26, P=0.01) with the two Lf groups having lower concentrations than the control fed group. Figure 3.4 shows representative Western blots for caspase 3, Bcl-2 and TNF-α from the three diet groups together with biotin standards.

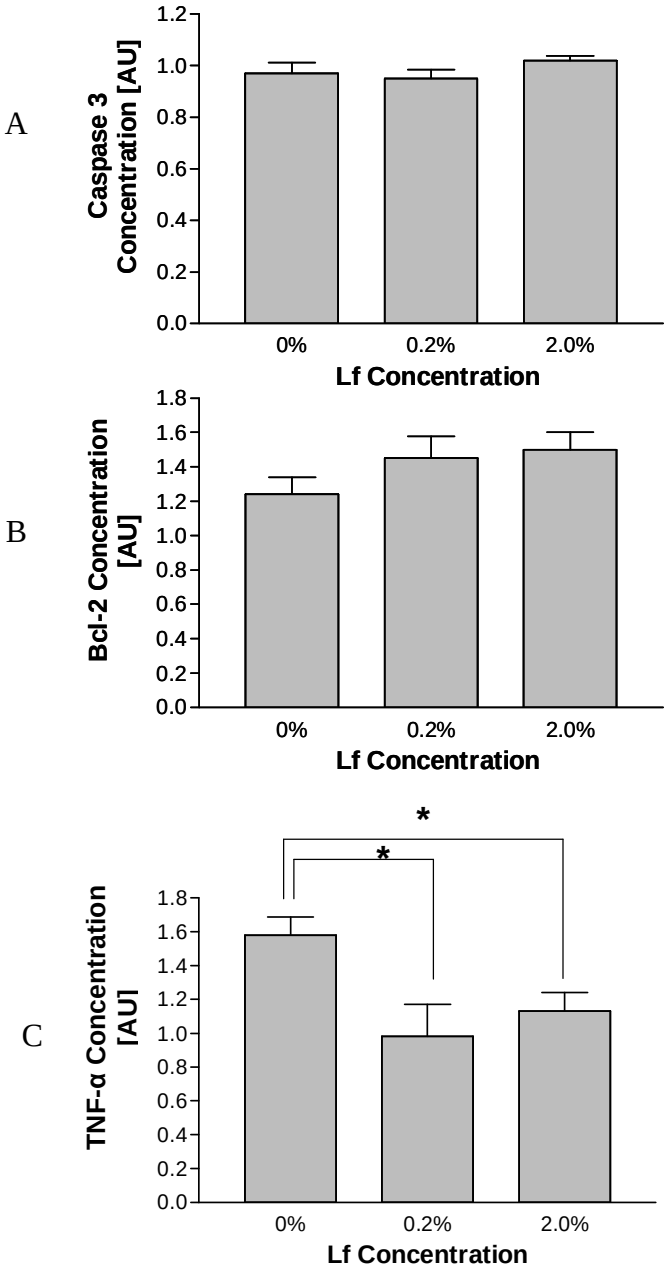

Figure 3.3: Concentration of apoptotic proteins in IL of mice given bovine Lf in the diet. (A) Caspase 3, (B) Bcl-2 and (C) TNF-α. All values are means ± SEM. AU= arbitrary densitometric units; 0% = 0% Lf (n=16); 0.2% = low dose (0.2% Lf) (n=16); 2.0% = high dose (2.0% Lf) (n=15). * $p \leq 0.05$ compared to the control (0% Lf).

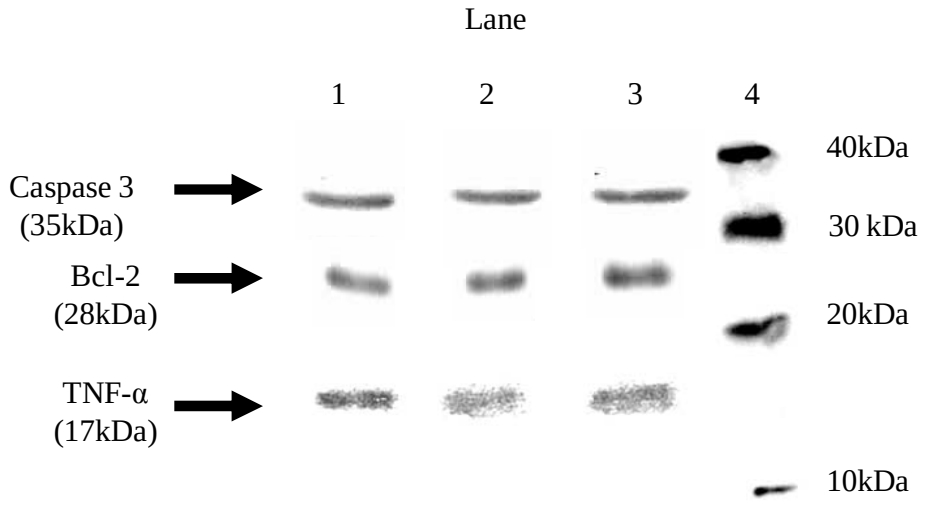

Figure 3.4: Representative Western blots of apoptotic proteins in IL of mice given bovine Lf in the diet. Lane 1 is 0% Lf, Lane 2 is 0.2% Lf, Lane 3 is 2.0% Lf, and Lane 4 is the molecular weight (biotin) standards.

3.8. Discussion

To our knowledge, this is the first study to describe the effects of short term exposure of Lf administered in the diet on intestinal lymphocytes from healthy animals. A novel finding was that four days of bovine Lf feeding in healthy mice was associated with increased apoptosis of CD4 (but not CD8) IL. Our research also confirms and extends previous observations that Lf modifies CD4 lymphocytes in different situations. A second important observation was that dietary Lf was associated with decreased expression of TNF-α in IL from healthy animals.

We investigated whether Lf triggered IL apoptosis by influencing the expression of the pro-apoptosis (caspase 3) and anti-apoptosis (Bcl-2) intracellular proteins. There was no difference in the expression of these proteins between the dietary treatment

groups. Although others have shown that Lf increases the expression of pro-apoptotic proteins, these effects have been documented only in pathological states (e.g., cancer) or when Lf is administered through non-physiological routes (e.g., gavage) (Fujita et al., 2004a; 2004b; Mader et al., 2005; Chandra Mohan, 2006). In this present study (during a normal physiological state), the small increases in IL apoptosis without a corresponding increase in caspase 3 expression may reflect a number of possible factors including 1) apoptosis occurring through caspase 3 independent pathways and 2) the timing of caspase 3 expression in relation to other apoptotic markers. Although activation of caspase 3 is central to apoptosis, caspase-independent cell death also occurs, with pathways involving cathepsin and calpain proteases and mitochondrial derived apoptosis inducing factor (AIF) (Jaattela and Tschopp, 2003; Chwieralsk et al,. 2006). Whether Lf induces IL apoptosis through caspase-independent pathways has not been determined. Moreover, given the kinetics of the sequence in apoptosis, (i.e., phosphatidylserine externalization occurs early in the apoptotic pathway prior to caspase 3 activation (Huppertz et al., 1999), we cannot rule out the expression of caspase 3 in IL if samples were obtained at other time points. Similar factors may be involved in the apparent lack of effect on Bcl-2 expression (e.g., activation of apoptosis through other pathways, involvement of other proteins upstream from Bcl-2, kinetics of protein expression). Indeed, an important limitation of this study is that protein expression in IL was determined at only one time point (i.e., after 4 days of Lf exposure) and future experiments will be necessary to determine whether longer (or shorter) exposures to Lf affect the expression of apoptotic proteins in IL of healthy mice.

Concurrent with an increase in the percent of apoptotic CD4 IL there was also an increase in the percent of this T cell subpopulation. This finding suggests that Lf not only stimulates greater apoptosis of IL but also their replacement. There is evidence that administration of Lf increases the mobilization of CD4 T lymphocytes from various tissue sites. Wakabayashi et al. (2006), after a single dose of Lf (2.5 g/kg body weight) given by gavage, found increased blood CD4 cells and decreased splenic CD4 cells in Balb/c mice as well as a small increase in CCR9, a chemokine receptor involved in the recruitment of intestinal lymphocytes. CCR9 and other cell adhesion molecules (e.g., $\alpha_4\beta_7$) are known for their specific role in intestinal homing of primed IL after exposure to oral antigen as well as for controlled intestinal migration of naïve $CD8^+\alpha\beta$ T cells (Mowat et al., 2003; Kunisawa and Kiyono, 2005; Sato and Iwasaki, 2005). Therefore, the increase in the % of CD4 IL observed in the present study may be due to increased mobilization to the intestine from other lymphoid tissues (e.g., spleen) to replace apoptotic cells. Further studies will be needed to determine whether CD4 lymphocytes home to the gut in response to Lf feeding in healthy mice.

An important result of this study was that the expression of TNF-α was lower in IL of healthy mice fed Lf compared to the control. TNF-α is produced by a variety of immune cells, including $CD4^+$ cells (Mosmann and Sad, 1996), and affects a variety of physiological processes such as cell proliferation, differentiation and apoptosis (Dinarello, 2000). This cytokine also contributes to tissue injury and decreased levels are associated with the resolution of inflammation and less inflammatory damage in the intestine (Togawa et al., 2002a; 2002b; Baert et al., 1999). Other investigators have identified a link between exogenous Lf and TNF-α. Hayashida et al. (2004) found that

subcutaneous injection of Lf decreased LPS induced TNF-α production in a rat model of rheumatoid arthritis. Togawa et al. (2002a) observed an approximate 4 fold decrease in TNF-α expression in the colon of male rats after 3 days of Lf administration by gavage. Our findings agree with these earlier reports of Lf reducing TNF-α production and show this effect after only 4 days of oral feeding. The mechanism by which short term feeding of Lf reduces the expression of TNF-α in IL cannot be directly determined from our results. However, since CD4 cells produce TNF-α (Mossman and Sad, 1996) and given the increase in the percent of apoptotic CD4 IL in this study, it is tantalizing to suggest that simple loss of TNF-α producing CD4 cells may account for the observed reduction in TNF-α expression. Although this remains speculative, in support of this hypothesis, others have shown similar effects. For example, the co-polymer glatiramer acetate decreases the population of TNF-α producing CD4 T cells in patients with multiple sclerosis (Kantengwa et al., 2007). In addition, Li et al. (2007b) reported glucocorticoid treatment of IFN-γ stimulated murine microglial cells resulted in reduced TNF-α secretion and partially suppressed anti-CD3 induced proliferation of CD4 T cells. Taken together, the mechanism of decreased TNF-α, as a result of short-term oral Lf feeding, may be a consequence of increased apoptosis of TNF-α producing CD4 IL.

Previous research has shown increases in intestinal CD8 lymphocytes as a result of Lf administration (Kuhara et al., 2000; Wang et al., 2000). In contrast, we did not find a significant impact of short term Lf administration on the percent or apoptosis of CD8 IL in healthy mice. Possible explanations for this discrepancy include dose and mode of Lf administration. These earlier studies provided Lf by gavage and at much higher concentrations (~900mg/3 days compared to ~ 200mg/4days in the present study). The

resistance to Lf-associated apoptosis of CD8 IL may represent another mechanism of host protection as these cells have prominent roles in tumour surveillance and defense against bacterial pathogens. We have shown elsewhere a dichotomous response of mouse IL T cell subpopulations to physiological stress (Davidson and Hoffman-Goetz, 2006; Hoffman-Goetz and Spagnuolo, 2007a): CD4 IL were more sensitive to repeated exercise stress induced apoptosis than CD8 IL, possibly due to differential of expression of antioxidant and heat shock proteins. In addition, Murosaki et al. (1997) reported that in mice following water immersion stress, intraepithelial intestinal CD8 cells were more resistant to apoptosis compared to CD4 cells. This highlights the possibility of a differential response of IL to external stimuli and may explain why short term oral feeding of Lf influences apoptosis of only one subset.

In this study two different concentrations of Lf had significant effects on IL apoptosis and cytokine production but there was no observable linear dose dependency (i.e., no stepwise increase in % CD4 or % $CD4^+/Ann^+$ IL or decrease in TNF-α expression with increasing Lf concentrations). It is not clear from our data why there was no dose response for Lf on the IL immune parameters. However, Lf receptors are found on numerous immune cells including neutrophils (Deriy et al., 2000) and lymphocytes (Mazurier et al,. 1989). Therefore, the absence of a dose response may be due to the number and availability of Lf receptors on IL; once these receptors are saturated, any additional Lf would have no effect and likely would be excreted.

3.9. Conclusion

In summary, Lf given by diet for four days was associated with a small but significant increase in the percent and apoptosis of CD4, but not CD8, IL and decreased the expression of TNF-α in IL of healthy mice. Additional studies will be needed to determine whether the reduction in TNF-α expression in IL occurs specifically in CD4 lymphocytes and what the physiological consequences are of these small changes in IL apoptosis.

3.10. Additional Findings

The concentration of IL-10 in intestinal lymphocytes was also quantified by Western blotting, similar to the methods outlined in section 3.6, using an antibody specific for IL-10 (clone: M-18; goat anti-mouse IgG, MW=15kDa, Santa Cruz, California). There was no significant effect ($F_{2,20}$ = 0.571, p=0.574) of bLf on IL-10 levels in the IL of healthy mice (0% Lf: 1.2 ± 0.1 AU, 0.2% Lf: 1.1 ± 0.08 AU, 2.0% Lf: 1.1 ± 0.1).

These results suggest that bLf does not affect levels of IL-10 in the IL of healthy mice. Although there are no published studies on the effects of bLf given in the diet on IL-10 concentrations, others (Togawa et al., 2002a; 2002b; Hayashida et al., 2004; Takakura et al., 2006) have demonstrated that IL-10 is increased in plasma or colonic tissue following bLf treatment. Reasons for the differences between the IL-10 results presented here and those of the Togawa et al. (2002a; 2002b), Hayashida et al., (2004) and Takakura et al. (2006) are not known, however; considerable variation in how bLf was administered occurs across the studies and may be important in the result outcome.

For example, Togawa et al. (2002a; 2002b) and Takakura et al. (2006) provided bLf via gastric intubation, and Hayashida et al. (2004) orally administered bLf. Evidence to support this notion are the observations by Sfeir et al. (2004) who reported that the cytokine response varied based on the route of bLf administration. Sfeir and colleagues demonstrated that dietary administration increased IL-4 (anti-inflammatory) levels whereas gastric intubation resulted in IFN-γ (pro-inflammatory) production. Although no change in IL-10 levels were observed in this study the decrease in TNF-α levels in mouse IL following dietary bLf feeding supports the hypothesis that bLf favours a T_H1/T_H2 balance in favour of anti-inflammatory cytokine responses in the bowel.

CHAPTER 4: Lactoferrin Effect on Lymphocyte Cytokines and Apoptosis is Independent of Exercise

4.1. Objectives

To determine the effects of dietary bovine lactoferrin (bLf) on intestinal lymphocyte (IL) levels of TNF-α and IL-10 and on $CD4^+$ and $CD8^+$ IL apoptosis in female C57BL/6 mice following repeated bouts of acute exercise.

4.2. Hypotheses

Hypothesis 1: bLf administration will influence (increase or decrease) the number and/or percent of apoptotic intestinal $CD4^+$ and $CD8^+$ lymphocytes in mice subjected to repeated bouts of acute exercise relative to the percent of apoptotic intestinal $CD4^+$ and $CD8^+$ lymphocytes in mice not receiving bLf supplementation**.**

Hypothesis 2: bLf administration will decrease TNF-α and increase IL-10 levels in IL of mice subjected to repeated bouts of acute exercise relative to the levels of TNF-α and IL-10 in mice not receiving bLf supplementation**.**

4.3. Study Design

This study was designed to determine if dietary bLf influenced the inflammatory cytokines, TNF-α and IL-10, and the percentage of apoptotic $CD4^+$ and $CD8^+$ intestinal lymphocytes during physiological stress induced by repeated bouts of acute exercise. The findings of the first experiment (Chapter 3) determined that 2.0% bLf in the diet was associated with reduced TNF-α levels and increased apoptosis of $CD4^+$ IL. Given the observations that athletes experience gastrointestinal disturbances following strenuous exercise (Peters et al., 2001b), possibly due to increased intestinal inflammation, it was aim of this study to determine if dietary bLf increased anti-inflammatory responses following exercise challenge in mice. An exercise protocol of three repeated bouts of exhaustive exercise was chosen as it has previously been shown to induce oxidative stress

and intestinal lymphocyte apoptosis in mice (Hoffman-Goetz and Spagnuolo, 2007a; Quadrilatero and Hoffman-Goetz, 2005; Hoffman-Goetz and Quadrilatero, 2003). Mice were sacrificed immediately following cessation of the final exercise bout (IMM) or 24 h after cessation of the final bout (24 h POST) and these were compared to control animals not subjected to exercise (SED). The experimental groups are outlined in Table 4.1 and the study design for experiment 2 is outlined in Figure 4.1.

Table 4.1: Summary of study 2 treatment groups with panel A showing the 3x2 factorial design and panel B showing the individual treatment groups.

A

Diet		**Exercise**		
		SED	IMM	24 h POST
Lactoferrin	No	1	3	5
	Yes	2	4	6

B

Group	Treatment
1	No bLf/ SED
2	bLf/SED
3	No bLf/ IMM
4	bLf/IMM
5	No bLf/ 24 h POST
6	bLf/24 h POST

Study duration: Two week acclimation plus 12 d of dietary bLf administration.

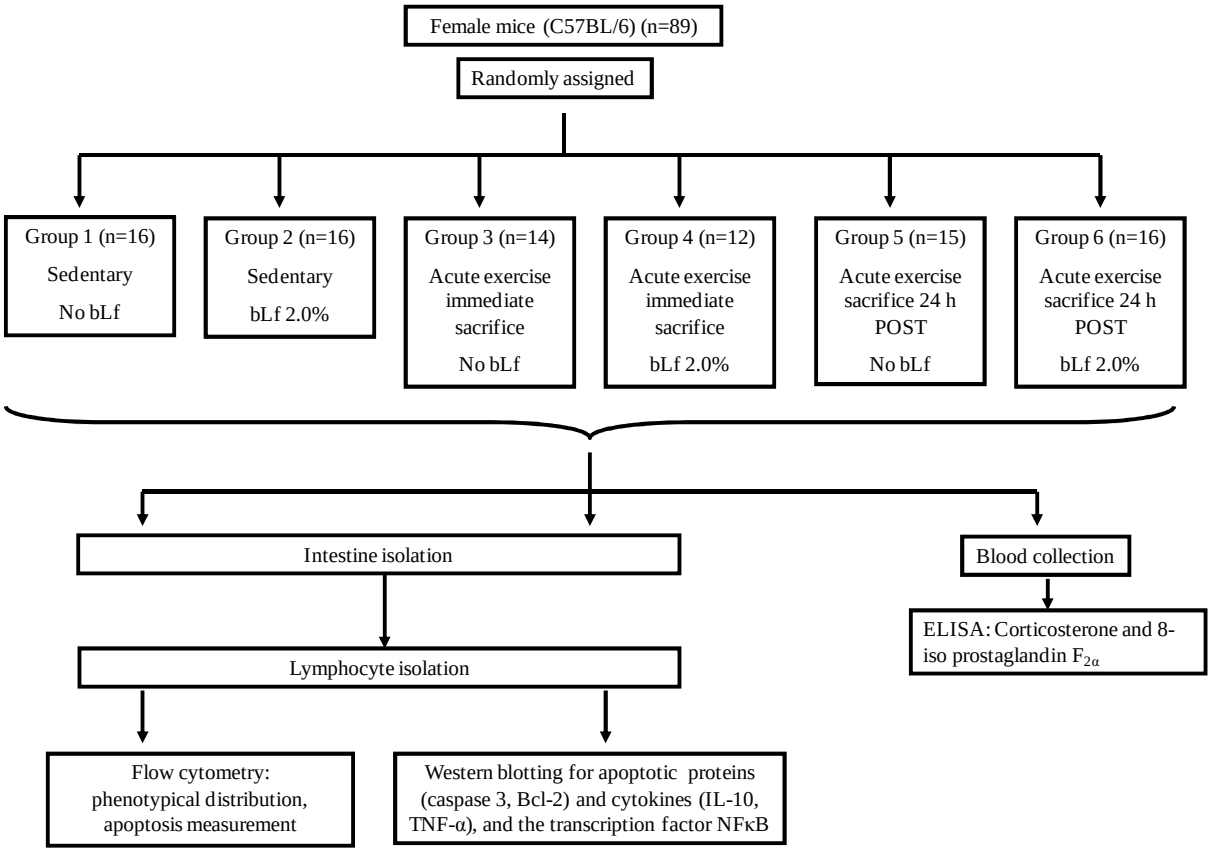

Figure 4.1: Schematic outline of the study design for experiment 2.

A two way ANOVA (3x2 factorial design) and post-hoc Tukey tests were used to analyze the study data. In this study the independent variables are diet condition (0% bLf or 2.0% bLf) and exercise treatment (SED, IMM or 24 h POST) (Table 4.1A). Dependent variables included: % $CD4^+$ and % $CD8^+$ IL (including apoptotic determination), apoptotic markers in intestinal lymphocytes (caspase 3 and Bcl-2), cytokine levels (TNF-α, IL-10), NFκB, plasma concentrations of 8-iso prostaglandin $F_{2\alpha}$, and plasma corticosterone concentrations.

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4.4. Overview

Strenuous exercise increases apoptosis of intestinal lymphocytes (IL). Bovine lactoferrin (bLf), a protein found in milk products, affects lymphocyte apoptosis and the expression of T_H1 and T_H2 cytokines. The purpose of this experiment was to determine if bLf affects apoptosis and T_H1 (TNF-α) and T_H2 (IL-10) cytokine expression in IL of mice given strenuous exercise. Female C57BL/6 mice (n=89), given 3 bouts of treadmill exercise, were sacrificed immediately, or 24 h after the last bout, or prior to initiation of exercise; within exercise conditions mice were fed a control (0% bLf) or bLf supplemented (2% bLf) diet for 12 days until sacrifice. IL were enumerated and apoptosis and cytokine expression were determined by Western blotting; markers of stress (corticosterone; iso-prostanes) were measured in plasma by radioimmunoassay and direct immunoassay. Exercise increased IL loss ($p<0.05$) and the expression of caspase 3 ($p<0.001$), HSP 70 ($p<0.01$) and IL-10 ($p< 0.05$) in mouse IL; bLf did not alter these responses. However, bLf reduced TNF-α expression in mouse IL ($p<0.05$), possibly through decreased NFκB expression ($p<0.05$) in the supplemented group. Dietary bLf does not affect IL apoptosis following exercise but may confer intestinal protection through changes in cytokine expression, independent of exercise.

4.5. Introduction

Intestinal lymphocytes (IL) comprised of lamina propria and intraepithelial lymphocytes, are important in the protection of the gut mucosal barrier against infection and tumour growth (Abreu-Martin and Targan, 1996). Apoptosis of IL affects gastrointestinal equilibrium not just during normal physiological states but also during times of antigen activation and inflammation (Bu et al., 2001). For example, breakdown of the mucosal barrier results in chronic exposure of IL to commensal bacteria, with resultant excessive activation of IL and, potentially, ongoing inflammation (Sartor, 1997). Apoptosis of these activated T cells can provide protection against mucosal damage (Lugering et al., 2006) whereas inappropriate IL apoptosis during quiescent states could leave the GI tract vulnerable to infection or tumour growth.

Aerobic exercise increases IL loss and apoptosis following a single bout (Hoffman-Goetz and Quadrilatero, 2003) and after repeated bouts (Hoffman-Goetz and Spagnuolo, 2007a) of exhaustive treadmill running in mice. Oxidative stress (from increased whole body and cellular respiration) is one mechanism whereby lymphocyte apoptosis could occur since administration of the antioxidant N-acetyl-cysteine decreases the extent of mouse IL loss following exercise (Quadrilatero and Hoffman-Goetz, 2005). Oxidant stress could also occur from ischemia-reperfusion injury due to changes in splanchnic blood flow with exercise; indeed, ischemia-reperfusion injury is associated with damage of the mucosal epithelium, bacterial translocation, and inflammation (Cerqueira et al., 2005). Glucocorticoids (GC) are another potential mechanism contributing to exercise-induced IL apoptosis and cell loss. GC concentrations in blood increase with long duration exercise (Li et al., 2007a). In support of the role of GC in

exercise-induced apoptosis are the following observations: 1) in vitro exposure of mouse lymphocytes to corticosterone increased cell death (Hoffman-Goetz and Zajchowski, 1999) and 2) repeated treadmill exercise, with associated elevated GC, was linked with greater expression of protein markers of apoptosis in mouse IL (Hoffman-Goetz and Spagnuolo, 2007a).

Strenuous exercise alters the production and balance of pro- and anti-inflammatory cytokines, leading to perpetuation or resolution, of intestinal inflammation. For example, TNF-α, a pro-inflammatory T_H1 cytokine responsible for inducing tissue damage, is increased in circulation following exercise at or greater than 75% VO_{2max} in humans (Ostrowski et al., 1999). On the other hand, anti-inflammatory (T_H2) cytokines, such as IL-10, also have a putative role in the resolution of inflammation. IL-10 knockout mice spontaneously develop intestinal colitis (Kuhn et al., 1993) and alleviation of this condition occurs upon IL-10 administration (Lindsay et al., 2004). Therefore, the balance between pro- and anti-inflammatory cytokines is vital for intestinal homeostasis. The impact of exercise on the maintenance of the balance between T_H1 and T_H2 cytokines in the gastrointestinal tract has not been well characterized. In humans, increases in plasma IL-10 and TNF-α following intense exercise have been reported (Ostrowski et al., 1999); increased IL-10 expression in IL after repeated bouts of treadmill exercise in mice has recently been described (Hoffman-Goetz et al., 2008).

Bovine lactoferrin (bLf) is an 80 kDa iron binding protein found in milk products (Lonnerdal and Iyer, 1995). It interacts with many immune cells and receptors for Lf have been found on activated lymphocytes (Mazurier et al., 1989). Among the reported immunomodulatory properties of Lf is alteration of cytokine production (Togawa et al.,

2002a; 2002b) and induction of apoptosis (Mader et al., 2007; Spagnuolo et al., 2007). Togawa et al. (2002a; 2002b) found that bLf reduced TNF-α and increased IL-10 levels during experimentally-induced intestinal inflammation in rats. Decreased NFκB activity leading to decreased TNF-α production was also evident following bLf administration (Haversen et al., 2002). It is not known if bLf supplementation affects T_H1 or T_H2 cytokine expression in lymphocytes in response to exercise. However, given altered cytokine expression during exercise, the interaction between exercise and bLf may be beneficial by reducing pro-inflammatory and increasing anti-inflammatory cytokines.

How Lf influences apoptosis is unclear since Lf administration increases cell death (Spagnuolo et al., 2007) and cell viability (Shoji et al., 2007). Opposing actions of Lf during oxidative stress have also been reported with decreased oxidative damage in intestinal epithelial cells following H_2O_2 treatment (Shoji et al., 2007) and increased mitochondrial ROS production resulting in cell death (Lupetti et al., 2002). Thus, how bLf affects IL apoptosis following strenuous exercise (associated with oxidant stress) is not obvious. The purpose of this study was to determine the effects of dietary Lf administration in mice on 1) TNF-α and IL-10 expression in IL and 2) cell loss and apoptosis of IL in conjunction with repeated bouts of aerobic exercise. We hypothesized that dietary Lf given prior to strenuous exercise would result in a shift to a T_H2 cytokine response in IL and further that bLf would affect exercise-induced IL apoptosis (either enhance or reduce).

4.6. Materials and Methods

Animals and Diets: Female C57BL/6 mice (n=89) (Harlan Sprague Dawley, Indianapolis, IN, USA), 3-4 weeks of age, were individually housed on a 12/12h reversed light/dark cycle at 21±1^{o}C. Mice had *ad libitum* access to a maintenance diet (Laboratory Rodent Chow, PMI feeds Richmond, IN, USA) and tap water for 2 weeks. Following acclimation, mice were randomly assigned by weight to 2 dietary conditions: bovine lactoferrin (bLf; n=44) or control diet (no bLf; n=45). Diets were formulated based on a semi-purified AIN 76A standard diet to contain either: 0% or 2.0% bovine Lf (Erie Foods International Inc, Erie IL), while maintaining similar overall protein concentrations (20% total protein in both groups). Concentrations of dietary bLf were based on our previous work indicating that 2.0% dietary lactoferrin resulted in apoptotic and cytokine responses in mouse IL (Spagnuolo et al., 2007). bLf was 16% iron saturated and greater than 90% pure. Mice were given *ad libitum* access to the diets for 12 consecutive days prior to sacrifice. All protocols with live animals were approved by the University Animal Research Committee and conformed to the ethical principles of the Canadian Council on Animal Care and the American College of Sports Medicine.

Exercise Protocol: Within diet conditions, mice were randomized to one of three exercise groups: three bouts of intensive treadmill running (each bout separated by a 24 h rest interval) with sacrifice immediately upon completion of the final exercise session (IMM, n=26), or with sacrifice 24h after completion of the final exercise session (24 h POST, n=31), or to a no exercise sedentary control condition (SED, n=32). Thus, there were a total of 6 groups: no bLf/SED (n=16); bLf/SED (n=16); no bLf/IMM (n=14);

bLf/IMM (n=12); no bLf/24 h POST (n=15); and bLf/24 h POST (n=16). The treadmill protocol, described in detail elsewhere (Hoffman-Goetz and Spagnuolo, 2007a), consisted of a 10 min warm-up, 90 min continuous exercise (30 min at 22 $m \cdot min^{-1}$, 2°slope; 30 min at 25 $m \cdot min^{-1}$, 2°slope; 30 min at 28 $m \cdot min^{-1}$, 2°slope) and 5 min deceleration. Mice were motivated to run (Omni-Max metabolic treadmill, Omni Tech Electronics, Columbus, OH) during the dark cycle by occasional gentle prodding using a soft nylon brush.

Intestinal Lymphocyte Preparation: IL were isolated as described by Hoffman-Goetz and Quadrilatero (2003). Briefly, the intestinal compartments were immediately removed and Peyer's patches and visible fat dissected out. IL were prepared as single suspensions by isolation over a column containing 0.3 g of pre-washed nylon wool, washed and layered over a density gradient medium (Lympholyte-M; Cedarlane Laboratories, Hornby, Ont.) to exclude epithelial cells and remove cellular debris. A sample of IL, consisting of both intraepithelial and lamina propria lymphocytes, was stained with Turk's solution and enumerated manually by microscopy.

Flow Cytometry: Flow cytometry was used to determine IL phenotype distribution and extent of apoptosis. In order to discern IL populations an initial acquisition gate was created based on the forward and side scatter properties of a population shown to collect >90% $CD45^+$ cells (leukocytes) using a CD45 (common leukocyte antigen, clone: 30F11) FITC-conjugated monoclonal antibody (mAb; Miltenyi Biotec, Auburn, CA). Apoptosis was determined by Annexin V and PI staining. Annexin V detects the cell membrane

expression of phosphatidylserine, which is an early marker of apoptosis, whereas PI is a non-specific DNA dye that indicates late apoptotic or necrotic cells. 1.0 x 10^5 cells were incubated with 2.5 μL of both Annexin V (Pharmingen) and PI (Sigma Chemical) in 100 μl of Annexin binding buffer (Pharmingen) for 15 min at room temperature in the dark, followed by addition of binding buffer (400μl) and analysis by flow cytometry. Individual IL phenotypes were determined by suspending 5 x10^5 cells in 100μl of PBS and with the addition of 2.5μl of PE-conjugated mAbs (PharMingen, San Diego, CA, USA) for CD4 (anti-CD4, clone: GK1.5) and CD8α (anti-CD8α, clone: 53-6.7) in the dark at 4ºC for 45 min. Cells were washed and centrifuged (5 min, 450 x *g*), the pellet resuspended and analyzed for phenotype on a two color flow cytometer (Epics XL Flow Cytometer, Beckman-Coulter, Hialeah, FL) with an excitation wavelength of 488nm and emission filters of 525nm (green fluorescence) and 575nm (red fluorescence).

Corticosterone and 8-iso Prostaglandin $F_{2\alpha}$: Mice were sacrificed by sodium pentobarbital (0.06-0.08 ml) overdose. Blood was immediately collected by cardiac puncture into a 1 ml syringe containing 0.08ml of heparin. Following centrifugation (6 min, 1500*g*) plasma was collected and frozen at -20°C for corticosterone and 8-iso prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) analysis.

Plasma corticosterone was quantified using a commercially available double antibody ^{125}I radioimmunoassay kit (ICN Biomedicals, Inc., Costa Mesa, CA) according to the manufacturer's protocol. Plasma samples were diluted 1:200 with steroid dilutant and radioactivity was measured with a Gamma 5500 counter (Beckman Coulter, Hialeah, FLA). 8-iso-$PGF_{2\alpha}$ in plasma was quantified using a direct enzyme immunoassay (EIA)

kit (Assay Designs, Ann Arbor, Michigan, USA). Samples were hydrolyzed (25 µl 10N NaOH: 100 µl sample) at 45°C for 2 h and neutralized to pH 6-8 with 12N HCl. Samples were centrifuged (5 min, 14000*g*), and incubated with 8-iso-$PGF_{2\alpha}$ antibody for 24 h at 4°C. Absorbance was determined at room temperature at 405 nm.

Western Blot Analysis of Caspase 3, Bcl-2, NFκB, HSP 70, IL-10 and TNF-α: Cytoplasmic protein samples were separated by electrophoresis on a 12% SDS PAGE gel and transferred onto a PVDF membrane (Sigma Chemical) (Quadrilatero and Hoffman-Goetz, 2005). For quantification of NFκB, nuclear fractions were isolated; following cytoplasmic fractionation the pellet was resuspended in phosphate buffered saline, incubated 1 h with high salt lysis buffer, samples centrifuged (15 min, 15000*g*), and the supernatant recovered as the nuclear fraction. Membranes containing the separated proteins (cytoplasmic or nuclear) were incubated for 1 h with primary antibody (1:200 in 10% milk-TBST): Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG_1, MW=28kDa), caspase 3 (clone: H-227; rabbit anti-human clone IgG, MW=35kDa), TNF-α (clone: N-19; goat anti-human polyclonal IgG, MW=17kDa), NFκB (clone: C-20; rabbit anti-human polyclonal IgG, MW=65kDa), IL-10 (clone: M-18; goat anti-mouse IgG, MW=15kDa) or HSP 70 (clone: C92F3A-5; mouse anti-mouse monoclonal IgG_1, MW=70kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by incubation for 1 h with secondary antibody: horseradish peroxidase-conjugated anti-mouse (Bcl-2, HSP 70), anti-rabbit (caspase 3, NFκB) or anti-goat (TNF-α, IL-10) IgG at a concentration of 1:2000 in 10% milk-TBST. Protein was determined using ECL or ECL Plus Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK)

and the ChemiGenius 2 Bio-imaging System (Cambridge, UK). Samples from each treatment group were run together along with a biotinylated protein ladder to identify the molecular weight of the immunoblotted protein (Cell Signaling Technology, Beverly, MA, USA). For IL-10 and TNF-α blots, recombinant IL-10 and TNF-α standards (mouse IL-10, CL9310R, mouse TNF-α, CL9300TR; Cedarlane Laboratories) were run on each gel.

Statistical Analysis: All data were analyzed as a 2x3 analysis of variance design with diet (two levels: no bLf, bLf) and exercise (three levels: SED, IMM, 24 h POST) as the independent factors using SPSS (Version 15; Chicago, IL, USA). Post hoc analysis was performed with Tukey HSD test to determine differences between exercise groups and for interactions between diet and exercise groups. $P \leq 0.05$ was accepted as being significantly different from chance alone. All values are expressed as group means ± SEM.

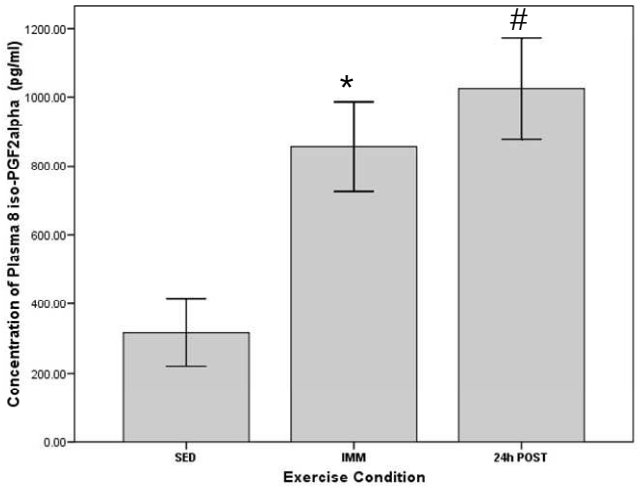
4.7. Results

Effect of Exercise and bLf on Food Intake and Body Weights of Mice: There were no significant main or interaction effects of diet or exercise treatments on total food intake over the course of the 12 d experiment (results are presented for the diet x exercise groups): no bLf/SED 33.1 ± 1.0g; bLf/SED 34.0 ± 0.7 g; no bLf/IMM 31.7 ± 0.8 g; bLf/IMM 33.5 ± 1.0 g; no bLf/24h POST 32.5 ± 0.8 g; bLf/24h POST 33.6 ± 0.1 g. Similarly, there were no significant main or interaction effects of diet or exercise treatments on animal weights prior to sacrifice: no bLf/SED 20.1 ± 0.5 g; bLf/SED 20.4

± 0.3 g; no bLf/IMM 19.8 ± 0.3 g; bLf/IMM 19.8 ± 0.5 g; no bLf/24 h POST 20.0 ± 0.5 g and bLf/24 h POST 20.8 ± 0.6 g.

Effect of Exercise and Lactoferrin on Plasma Isoprostanes and Corticosterone in Mice: Figure 4.2 shows the plasma concentrations of 8-iso-$PGF_{2\alpha}$ (Panel A) in mice. There was a significant main effect of exercise on plasma concentrations of 8-iso-$PGF_{2\alpha}$ ($F_{2,63} = 6.94$, $p<0.01$). This was due to increased concentrations of plasma 8-iso-$PGF_{2\alpha}$ in the immediate (IMM) ($p<0.05$) and 24 h POST group ($p<0.01$) compared to the SED mice. There was no effect of dietary bLf on plasma 8-iso-$PGF_{2\alpha}$ concentrations. Not unexpectedly, exercise significantly increased plasma corticosterone levels in mice ($F_{2,73} = 12.49$, $p<0.0001$) (Panel B). This was due to the higher levels in the IMM group relative to the SED animals ($p<0.0001$). There was a smaller main effect of diet on plasma corticosterone in mice ($F_{1,73} = 6.07$, $p<0.05$); plasma corticosterone was decreased in the bLf (398.6 ± 17.2 ng/ml) compared to the no bLf (473.3 ± 23.6 ng/ml) fed group. There was no significant diet x exercise interaction effect for 8-iso-$PGF_{2\alpha}$ or corticosterone.

A



B

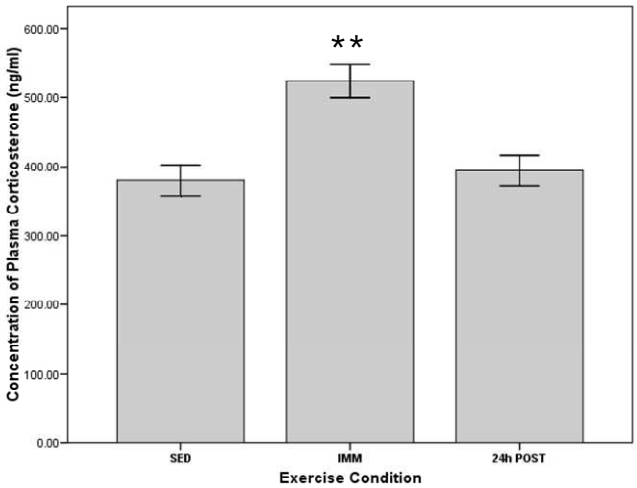


Figure 4.2: Plasma concentrations in mice of common markers of exercise induced stress. Panel A: 8-iso prostaglandin $F_{2\alpha}$ (pg/ml); *$p<0.05$, #$p<0.01$ vs. SED; Panel B: Corticosterone (ng/ml); **$p<0.0001$ vs. SED and 24 h POST. All values are means ± 1 SEM. SED = no treadmill exercise; IMM = three bouts of treadmill exercise each separated by 24 h rest and sacrifice immediately after the third bout; 24 h POST= three bouts of exercise each separated by 24 h rest and sacrifice 24 h after the third bout.

Effect of Exercise and Lactoferrin on IL Counts, Apoptosis and Phenotypes in Mice: A shown in Figure 4.3, there was a significant main effect of exercise on total IL numbers in mice ($F_{2,83}$ = 3.09, p=0.05). This effect was due to a decrease in IL counts at 24 h POST exercise relative to the SED group (p<0.05). There was a non-significant trend (p=0.06) for higher IL counts in mice fed bLf (5.3 ± 0.3 x 10^7 cells) compared with the no bLf group (4.5 ± 0.3 x 10^7 cells). No interaction effect was observed for IL counts between diet and exercise. IL loss following exercise has been attributed to increased apoptosis as measured by Annexin V expression (Quadrilatero and Hoffman-Goetz, 2005). We found a small effect of exercise on the % Annexin V^+/PI^+ in IL, a difference which approached significance (P=0.07; SED: 18.7 ± 1.6%, IMM: 22.5 ± 2.6%, 24 h POST 16.9 ± 1.0 %). Neither diet nor diet x exercise significantly affected the % Annexin V^+/PI^+ IL.

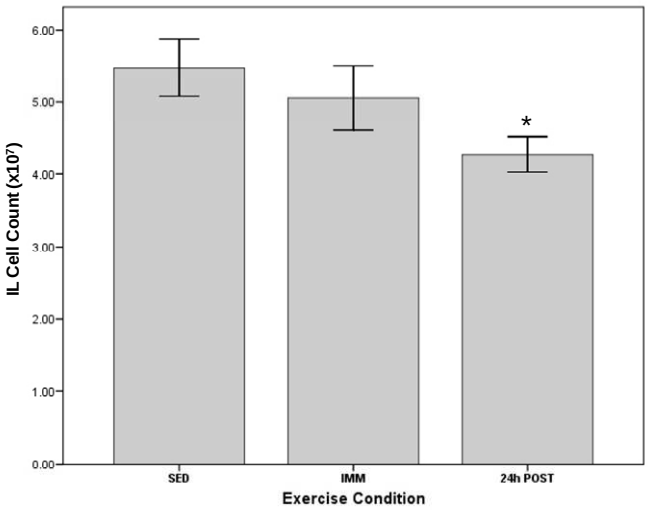
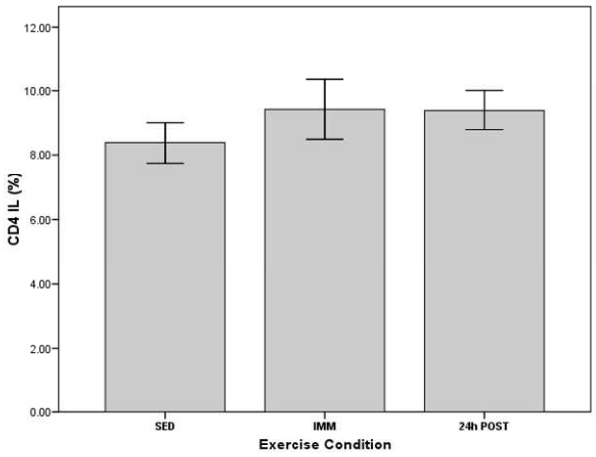


Figure 4.3: Intestinal lymphocyte (IL) counts in mice given repeated treadmill exercise. *p<0.05 vs. SED. Values are means ± SEM. SED = no exercise; IMM = 3 repeated bouts of exhaustive exercise and immediate sacrifice; 24 h POST = 3 repeated bouts of exhaustive exercise and sacrifice 24 h after the third bout.

A



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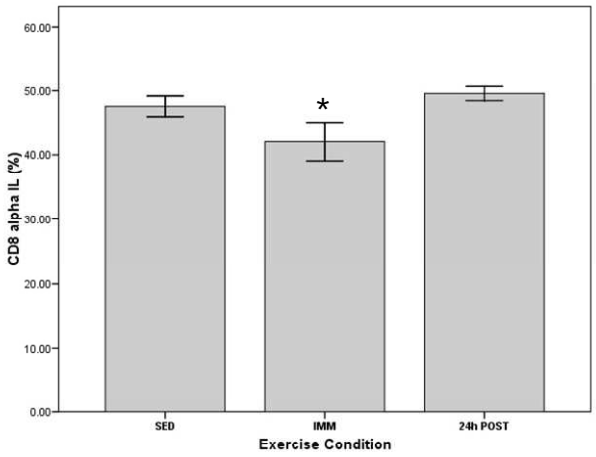

Figure 4.4: Intestinal lymphocyte (IL) phenotype distribution in mice following repeated bouts of strenuous exercise. Panel A: Percent CD4; Panel B: Percent CD8α; *p<0.05 vs. 24 h POST. Values are means ± SEM. SED = no exercise; IMM = 3 repeated bouts of exhaustive exercise and immediate sacrifice; 24 h POST = 3 repeated bouts of exhaustive exercise and sacrifice 24 h after the third bout.

Figure 4.4 gives the phenotypic distribution for the IL T cell subsets CD4 and CD8α. There were no significant main effects of exercise or diet, nor was there an interaction effect between these factors, in the percent of CD4 IL. In contrast, there was a significant main effect of exercise ($F_{2,83}$ = 3.91, $p<0.05$) on the percentage of CD8α IL in mice, an effect due to an increase between IMM and 24 h POST groups ($F_{2,83}$ = 3.91,

$p<0.05$). There was no significant interaction or main effect of diet on the percentage of CD8α mouse IL.

Effect of Exercise and Lactoferrin on Bcl-2, Caspase 3, NFκB and HSP 70 in Mouse IL: Exercise significantly affected the expression of the anti-apoptotic protein Bcl-2 in mouse IL ($F_{1,82} = 3.85$, $p<0.01$) with decreased concentrations in the IMM compared to the SED group (IMM: 1.54 ± 0.09 vs. SED: 2.02 ± 0.17; $p<0.01$). There was a main effect of bLf on Bcl-2 concentrations in mouse IL ($F_{1,82} = 7.35$, $p<0.01$) with the supplemented group having lower levels (bLf: 1.58 ± 0.16 AU) compared with the control group (no bLf: 1.95 ± 0.07 AU). There was a significant interaction ($F_{2,82} = 4.52$, $p<0.01$) between diet and exercise on the expression of Bcl-2 in mouse IL (Figure 4.5A). SED mice given the control diet (no bLf) had higher Bcl-2 expression relative to the other treatment groups ($p<0.05$) which did not differ from each other. Figure 4.5B shows the exercise effect on the expression of the pro-apoptotic protein caspase 3 in mouse IL ($F_{2,71} = 8.09$, $p<0.001$). Repeated exercise stress increased caspase 3 expression IMM after compared to the SED and 24 h POST group ($p<0.05$). There was no diet x exercise or diet effect on the expression of caspase 3.

There was no effect of exercise on the expression of NFκB. In contrast, bLf affected NFκB concentration in mouse IL ($F_{1,43} = 5.74$, $p<0.05$) with the bLf group having lower levels of NFκB (Figure 4.5C). A significant interaction occurred in the expression of NFκB in mouse IL ($F_{2,\ 43} = 4.57$, $p<0.05$). The expression of NFκB in IL was decreased in the immediate exercise group fed bLf (0.80 ± 0.14) relative to the immediate exercise group fed the control diet (1.50 ± 0.16, $p<0.05$). Figure 4.5D shows the main effect of exercise on HSP 70 in mouse IL ($F_{2,81} = 1.54$, $p<0.01$). HSP 70 protein

expression in mouse IL was higher in the IMM exercise vs. the SED condition ($p<0.05$) which then returned to levels not different from SED (i.e., SED vs. 24 h POST). There was no diet or interaction effect on the expression of HSP 70.

Effect of Exercise and Lactoferrin on IL-10 and TNF-α Expression in Mouse IL: Figure 4.6A describes the concentration of the T_H2 cytokine, IL-10, in mouse IL as a function of exercise ($F_{1,27} = 3.96$, $p<0.05$), an effect due to the differences between the IMM and 24 h POST exercise groups ($p<0.05$). There was no exercise x diet or diet effects on IL-10 expression in mouse IL. The T_H1 cytokine, TNF-α, was significantly affected by dietary bLf ($F_{1,61} = 3.85$, $p<0.05$, Figure 4.6B), with the bLf fed group having lower TNF-α expression compared to the control (no bLf) group. TNF-α expression in mouse IL did not vary as a function of exercise condition nor was there a diet x exercise effect.

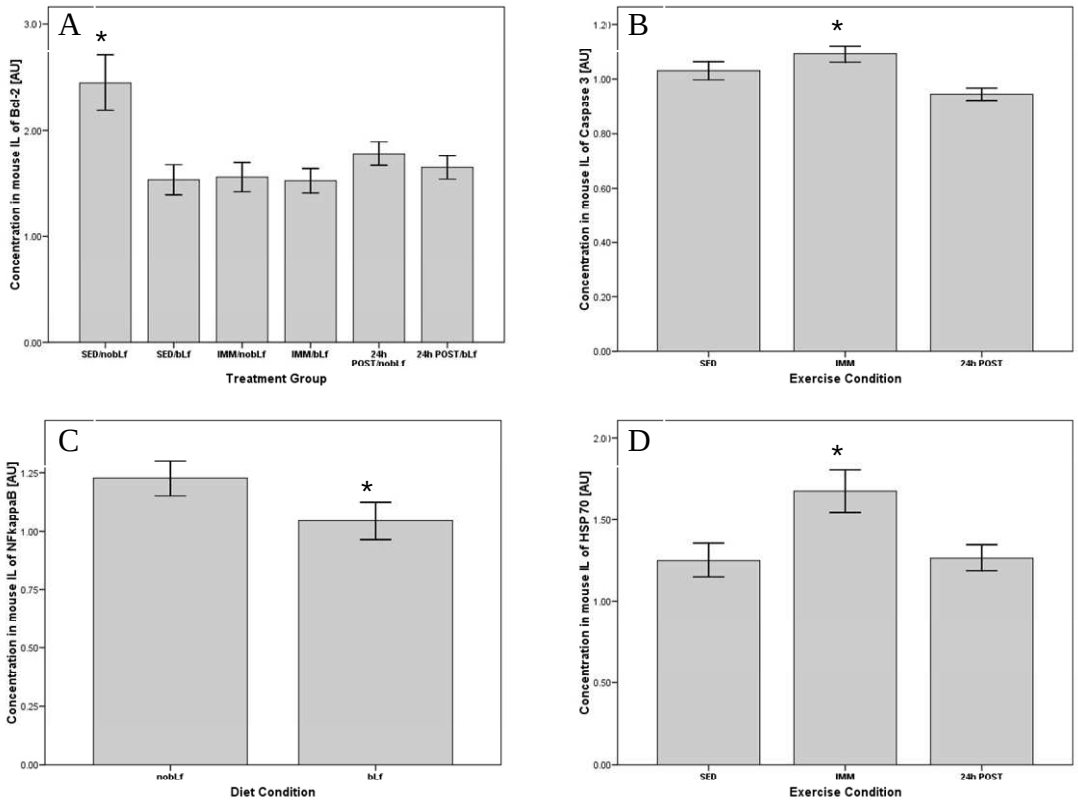

Figure 4.5: Concentrations of apoptotic and stress proteins in mouse IL. Panel A: Anti-apoptotic Bcl-2 in arbitrary densitometric units [AU]); *p<0.05 vs. all other treatment groups. Panel B: Pro-apoptotic protein caspase 3 [AU]; *p<0.05 vs. SED and 24 h POST; Panel C: NFκB [AU]; *p<0.05 vs. no bLf; Panel D: HSP 70 [AU], *p<0.05 vs. SED and 24 h POST conditions. Values are means ± SEM

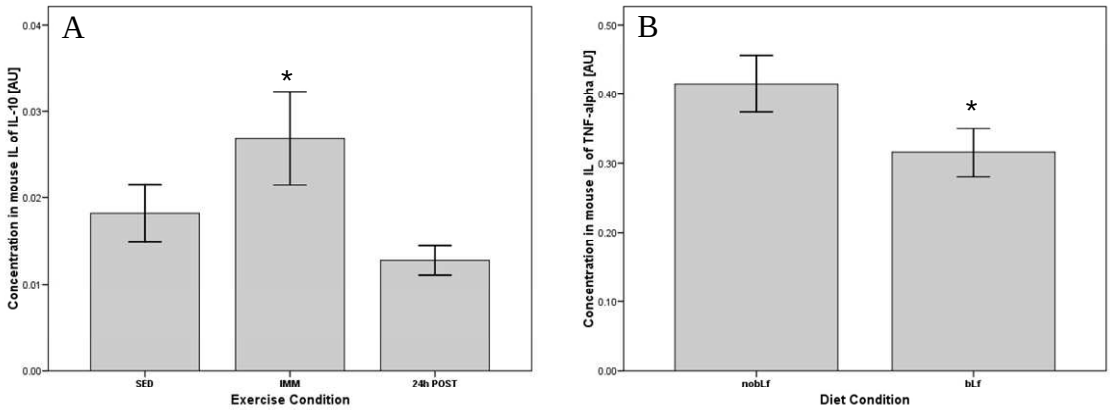

Figure 4.6: Effect of exercise and diet conditions on concentrations of the cytokines, IL-10 and TNF-α, in mouse IL. Panel A: Concentration of IL-10 [AU] as a function of exercise (*p<0.05 IMM vs. 24 h POST). Panel B: Concentration of TNF-α [AU] as a function of dietary bLf (*p<0.05). Values are means ± SEM.

4.8. Discussion

One main objective of this study was to determine if dietary Lf affected cell loss and apoptosis of IL in response to acute exercise in mice. A second related objective was to determine whether bLf given during exercise altered the expression of the cytokines IL-10 and TNF-α in IL, since these cytokines have regulatory roles in intestinal inflammation. We observed that dietary bLf did not ameliorate (nor did it exacerbate) the loss of T lymphocytes from mouse intestine under conditions of repeated exercise stress; moreover, bLf did not affect the increase in the pro-apoptotic protein caspase 3 or the expression of the heat shock protein HSP 70 in response to exercise. However, bLf feeding resulted in lower levels of the pro-inflammatory cytokine, TNF-α, in mouse IL.

Exhaustive exercise stress was associated with IL loss 24 hours after cessation of exercise, an observation which has been reported for single (Hoffman-Goetz and Quadrilatero, 2003) and repeated exercise (Hoffman-Goetz and Spagnuolo, 2007a) challenge. Cell loss has been related to oxidative stress induced apoptosis since provision of antioxidants reduced the exercise-related apoptosis of IL (Quadrilatero and Hoffman-Goetz, 2005). Others have shown decreased mitochondrial membrane potential (MMP) and increased apoptosis of human monocytes, neutrophils and lymphocytes following repeated bouts of exhaustive (85% VO_{2max}) exercise (Tuan et al., 2007).

bLf administration for 12 days in the diet did not alter mouse IL apoptosis and cell loss associated with aerobic exercise. The absence of a bLf effect under conditions of oxidant stress may reflect the magnitude of ROS production by exercise since ROS generated by bLf would likely be negligible compared to the generation of free radicals by exercise. Indeed, the repeated exercise challenge used in this study increased ROS

(measured by 8-isoprostanes) for up to 24 h after cessation of exercise. Elevated blood iso-prostanes have been reported for up to 6 days post race in ultramarathoners (Mastaloudis et al., 2004). The limited research on ROS generation by bLf suggests a mitochondrial pathway: addition of bovine lactoferricin (bLfcin- a pepsin derivative of bLf) resulted in MMP disruption with consequent caspase activation (Mader et al., 2007) and the bactericidal ability of human Lf to *Candida albicans* was due to increased mitochondrial ROS production (Lupetti et al., 2002). Both caspase activation and mitochondrial ROS production with bLfcin and Lf were reversed by antioxidant administration. Moreover, there are multiple pathways whereby exercise could induce IL loss and apoptosis apart from mitochondrial derived ROS arising from increased cellular respiration. These would include increased secretion of glucocorticoid (GC) hormones and ischemia/reperfusion injury. Even within mitochondria, ROS can be generated by several enzyme systems: mitochondrial electron transport chain, xanthine oxidase, NADPH oxidase, lipoxygenase, cyclooxygenase, and peroxisomes (Fruehauf and Meyskens, 2007). Given multiple mechanisms of ROS generation during exercise, the contribution of bLf induced ROS would be minimal and likely not add to observed increases in IL apoptosis with exercise.

Lactoferrin has also been associated with protection against ROS generation. The iron binding property of bLf is credited to its capacity to decrease metal (iron) catalyzed hydroxyl radical formation. Indeed this property of Lf has been linked to decreased hydroxyl radical formation and decreased intracellular oxidative damage in neutrophils, macrophages and intestinal epithelial cells (Shoji et al., 2007). Although bLf can reduce metal catalyzed hydroxyl radical formation, there is no evidence that bLf attenuates ROS

production through other mitochondrial pathways (such as MMP or cytochrome c oxidase) which would be more typical of aerobic exercise. Therefore, the multiple pathways of apoptosis and ROS production initiated by exercise would be larger than any bLf attributed reduction in metal catalyzed hydroxyl radical generation and thus not prevent IL loss.

Repeated bouts of exhaustive exercise resulted in greater loss of IL likely due to apoptosis of CD4, but not CD8α, T cells; apoptosis as the mechanism leading to IL loss is suggested by marginally higher % Annexin V^+/PI^+ and significantly higher caspase 3 expression. Nevertheless, because the % Annexin V^+/PI^+ IL were not non-significantly different across exercise groups ($p=0.07$), it is not possible to unequivocally conclude that IL loss was due to apoptosis. Other factors (for example, hemodynamic effects) may have contributed to IL loss with exercise. Dietary Lf did not alter the overall apoptotic index or the distribution in individual phenotypes of mouse IL following the repeated exercise challenge. There are, however, conflicting data about how bLf affects intestinal lymphocyte subsets. Wang et al. (2000) found increased CD4 and CD8 expression in mouse intestine following three consecutive days of bLf administration by gavage. CD4, but not CD8α, mouse IL were increased in response to four days of dietary bLf feeding (Spagnuolo et al., 2007). No change in any IL phenotype was observed following 3 doses of bLf by gavage (Takakura et al., 2006). Discrepancies among studies may reflect differences in duration, dose and mode of bLf administration; moreover, none combined lactoferrin with exercise. In the present study bLf was given by diet whereas others (Takakura et al., 2006; Wang et al., 2000) used gastric intubation to deliver lactoferrin.

Additionally, these studies used shorter durations and smaller cumulative doses of bLf than reported here.

Dietary Lf did not affect the expression of the pro-apoptotic protein, caspase 3, or the anti-apoptotic protein, Bcl-2, in mouse IL in response to exercise. IL loss following exercise has been attributed to apoptotic mechanisms including increased caspase 3 and reduced Bcl-2 expression (Hoffman-Goetz and Quadrilatero, 2003; Hoffman-Goetz and Spagnuolo, 2007a). Although there was no significant effect of bLf on expression of caspase 3 in mouse IL, overall Bcl-2 levels were lower in the bLf fed mice suggestive of a possible role of bLf in altering MMP. However, an important limitation is that no other mitochondrial pro- or anti-apoptotic proteins (e.g., Bax, Bid) were quantified and there are many proteins which regulate MMP. Nonetheless, bLf was insufficient to alter Bcl-2/Bax ratios in human tongue squamous carcinoma cells (Chandra Mohan et al., 2006) and the observed Bcl-2 decrease with bLf supplementation may be minimal in the overall regulation of the MMP.

Immediately following exercise, there was an increased expression of HSP 70 in mouse IL. Elevated HSP 70 after exercise has been demonstrated in rat blood leukocytes (Antunes-Neto et al., 2006) and the current findings extend these observations to a repeated exercise model in mice IL (Hoffman-Goetz and Spagnuolo, 2007a). Given that the increase in HSP 70 expression under conditions of heat and other physiological stressors serves to protect cells from damage (van Eden et al., 2005), the finding of increased levels with repeated exercise challenge was expected. Concentrations of plasma 8-iso-$PGF_{2\alpha}$ were increased after exercise. 8-iso-$PGF_{2\alpha}$ is produced from arachidonic acid, a plasma membrane lipid, through a free-radical catalyzed mechanism

(Patrono et al., 1997) and elevated plasma levels are a common indicator of exercise induced oxidative stress in humans (Steensberg et al., 2002) and mice (Hoffman-Goetz and Quadrilatero, 2003). Lf did not affect either stress marker which we attribute to the magnitude of the exercise induced oxidant stress response and the various methods of exercise induced ROS production. Given the inability of Lf, at the concentrations provided in the diet, to significantly influence exercise induced IL apoptosis, it was not surprising that Lf did not influence the expression of stress proteins or protect against oxidant stress generation.

Dietary bLf was associated with reduced expression of TNF-α in mouse IL. Although the mechanism of decreased concentrations of TNF-α in mouse IL as a result of dietary Lf administration is unknown, it may be linked to the observed decrease in levels of NFκB a transcription factor responsible for the expression of pro-inflammatory cytokines (e.g., TNF-α) (Bours et al., 2000). Exercise did not affect the levels of NFκB in mouse IL but concentrations were lower in the Lf group. A potential benefit of decreased NFκB in mouse IL is the subsequent decreased production of pro-inflammatory cytokines. Togawa et al. (2002b) showed that in rat colon following trinitrobenzenesulfonic acid (TNBS) induced colitis bLf decreased concentrations of TNF-α and phosphorylation of Iκ, indicative of decreased levels of activated NFκB. Lf administration decreased the extent of LPS induced binding of NFκB to the TNF-α promoter (Haversen et al., 2002). These results suggest that bLf may protect against gut inflammation by reducing the production of TNF-α through modulation of NFκB. Nevertheless, there is also evidence that bLf has little effect on inflammatory cytokine synthesis. bLf given to newborn Balb/c mice did not increase TNF-α levels in Peyer's

patches (Griffiths et al., 2004). The reason for the differences between our findings and those of Griffiths and colleagues (Griffiths et al., 2004) may be due to duration of exposure to Lf (12 vs. 28 days of exposure), age of the mice (~8 weeks vs. 4 weeks at sacrifice), immunological mucosal 'maturity' (adult vs. weaning mice), or mouse strain (C57BL/6 vs. Balb/c) with different immunological profiles.

Two cytokines involved in the pathogenesis of intestinal inflammation are TNF-α and IL-10. TNF-α is elevated in patients with inflammatory bowel diseases and symptom amelioration occurs with anti-TNF-α treatment whereas IL-10, a potent anti-inflammatory cytokine, inhibits immune cells from producing T_H1 cytokines (Papadakis and Targan, 2000). Thus, the balance between these pro- and anti-inflammatory cytokines (T_H1/T_H2) is crucial in maintaining homeostasis within the intestine. Our results show that bLf administration does not significantly increase the concentration of IL-10 but is associated with reduced concentrations of TNF-α, which could bias the balance towards an anti-inflammatory response. Others have shown bLf to favour a T_H2 response during stress. Togawa et al. (2002a, 2002b) reported decreased TNF- α and increased IL-10 following DSS and TNBS induced inflammation in the colon of rats fed bLf by gavage. Similarly, bLf suppressed TNF-α and increased IL-10 concentrations of LPS stimulated peripheral blood lymphocytes (Hayashida et al., 2004). In cultured IEL bLf for 3 days prior to stimulation with anti-CD3 antibodies increased production of IL-10 and decreased production of IFN-γ, a T_H1 cytokine (Takakura et al., 2006). Thus, bLf may shift the balance from T_H1 to T_H2 responses under periods of stress. Although we did not find that bLf increased IL expression of IL-10, the decrease in TNF-α levels partially supports the hypothesis that bLf affects cytokine balance to favour a T_H2 response in the

bowel. Further studies will be needed to determine whether the expression of other anti-inflammatory T_H2 cytokines, such as IL-4 and IL-7 which are produced by intraepithelial intestinal lymphocytes, are influenced by dietary bLf and under conditions of exercise stress.

Repeated treadmill exercise increased plasma corticosterone concentrations in mice, a response which was not unexpected since GC levels increase after exercise of long duration (Li et al., 2007a). What was unexpected was that bLf was associated with lower levels of plasma corticosterone in mice. Whereas classical stress induced ACTH-GC responses have been well described, GC synthesis is also influenced by the pro-inflammatory cytokines, IL-1β and TNF-α (John and Buckingham, 2003). Lactoferrin reduces the secretion of TNF-α and IL-1β in a variety of cells including THP-1 cells (Haversen et al., 2002) and lymphocytes (Crouch et al., 1992). Thus, it is possible that the lower levels of TNF-α seen in the current study also contributed to the lower plasma corticosterone levels in bLf fed mice. Nevertheless, the actual physiological significance of this effect remains to be determined since bLf did not reduce GC levels in response to exercise.

4.9. Conclusion

Repeated bouts of treadmill exercise in mice resulted in increased oxidative stress, as measured by 8-iso prostaglandin $F_{2\alpha}$ and concentrations of corticosterone, which contributed to IL loss probably via apoptosis. Although dietary bLf reduced plasma corticosterone levels and Bcl-2 expression in IL, there was no effect of this dietary supplement on IL loss either alone or with exercise challenge. Similarly, the oxidant

stress effects of exercise on increased caspase 3 and HSP 70 were not moderated by dietary bLf. The inability of bLf to affect the loss of IL or reduce caspase 3 expression in IL may be due to the magnitude of the stress response induced by exercise. Nevertheless, bLf may provide some limited protection against oxidant stress-induced inflammation in the bowel by decreasing IL expression of TNF-α, possibly through a mechanism involving decreased NFκB, to favour a T_H2 response. Further studies will be needed to determine if higher concentrations of bLf in the diet and for longer time periods affect T_H1/T_H2 balance in the gastrointestinal tract at rest and after exercise.

CHAPTER 5: Dietary Lactoferrin Does Not Prevent Dextran Sulfate Sodium Induced Murine Intestinal Lymphocyte Death

5.1. Objectives

To determine the effects of dietary bovine lactoferrin (bLf) on intestinal lymphocyte (IL) levels of TNF-α and IL-10 and on $CD4^+$ and $CD8^+$ IL apoptosis in female C57BL/6 mice following dextran sulfate sodium treatment.

5.2. Hypotheses

Hypothesis 1: bLf administration will influence (increase or decrease) the number and/or percent of apoptotic intestinal $CD4^+$ and $CD8^+$ lymphocytes in mice subjected to DSS treatment relative to the percent of apoptotic intestinal $CD4^+$ and $CD8^+$ lymphocytes of mice not receiving bLf supplementation.

Hypothesis 2: bLf administration will decrease levels of TNF-α and increase levels of IL-10 in the IL of mice subjected to DSS treatment relative to the IL levels of TNF-α and IL-10 in mice not receiving bLf supplementation.

5.3. Study Design

This study was designed to determine the possible physiological benefit of dietary bLf on levels of the cytokines, TNF-α and IL-10, and on the % of $CD4^+$ and $CD8^+$ intestinal lymphocyte apoptosis during DSS induced intestinal inflammation. Since DSS is the most commonly method used in the study of intestinal inflammation, it was important to use this model in the study of dietary bLf effects on inflammation. During DSS induced inflammation, Togawa et al. (2002a) provided bLf by gavage and demonstrated a decrease in the extent of colonic tissue damage as well as a reduction in the concentration of TNF-α and NFκB in the colonic tissue of rats. The previous study (Chapter 4) determined that inclusion of bLf at 2.0% in the diet for 12 d was effective at reducing TNF-α and NFκB in mouse IL following exercise. It was, therefore, a

reasonable argument that these effects on TNF-α and NFκB would also occur within the IL following dietary bLf and DSS administration to mice. The study design for experiment 3 is outlined in Figure 5.1.

Study duration: Two week acclimation plus 12 d of dietary bLf administration.

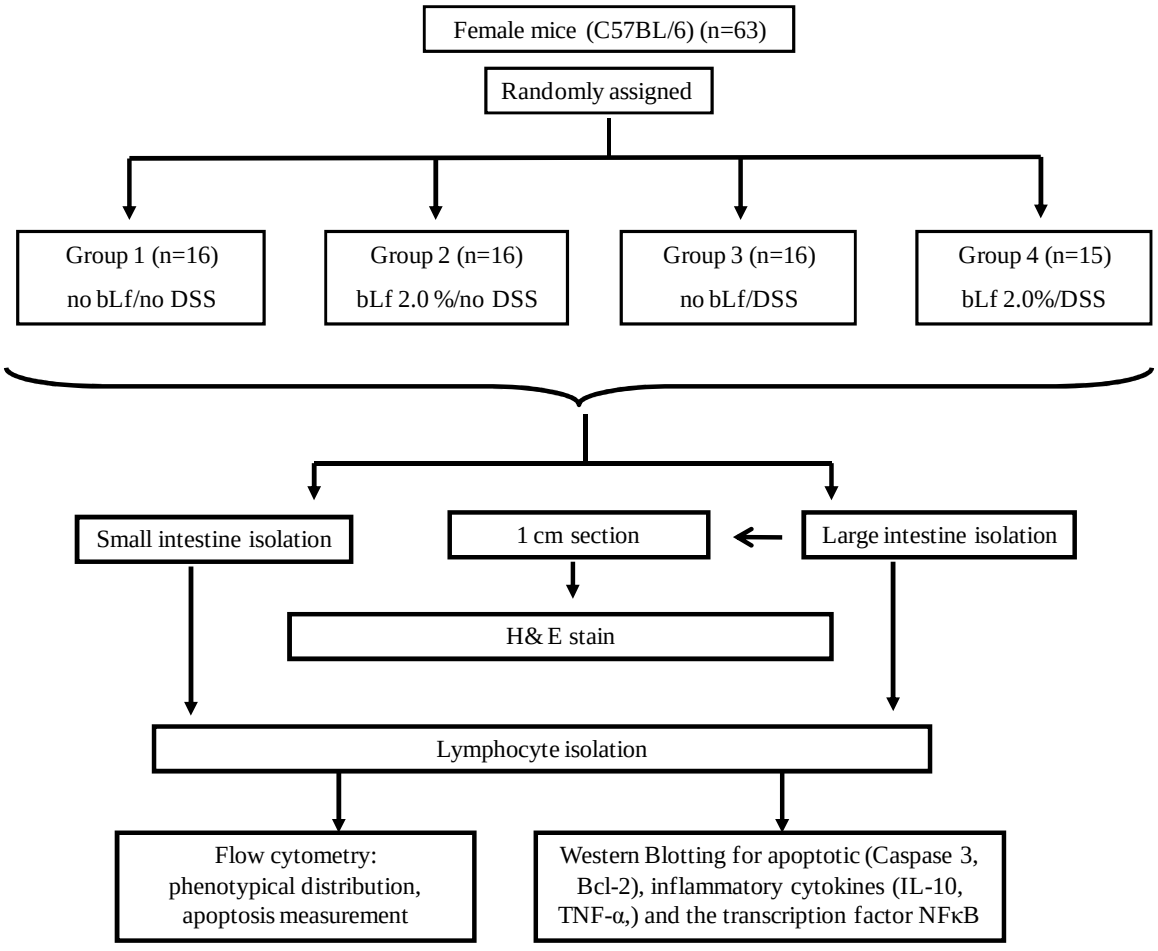

Figure 5.1: Schematic outline of the study design for experiment 3.

A two way ANOVA (2x2 factorial design) was used to analyze the study data. In this study the independent variables are diet condition (0% bLf or 2.0% bLf) and DSS treatment (no DSS or DSS). Dependent variables include: % $CD4^+$ and % $CD8^+$ IL (including apoptotic determination), apoptotic markers in intestinal lymphocytes (caspase 3, Bax and Bcl-2), cytokine proteins (TNF-α, IL-10) and the pro-inflammatory transcription factor NFκB.

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5.4. Overview

Dextran sulfate sodium (DSS) induced intestinal inflammation is characterized by pronounced mucosal and epithelial cell damage. Bovine lactoferrin (bLf), a common dietary protein, influences inflammatory cytokines and intestinal lymphocyte (IL) apoptosis. The objectives of this study were to determine if 1) DSS induces IL necrotic or apoptotic death, 2) dietary bLf affects DSS induced IL death and, 3) if bLf alters cytokine profiles during DSS induced inflammation. Female C57BL/6 mice were randomized to 2% or 0% bLf diets for 12 d and within diets to 5% or 0% DSS in the drinking water for 4 d after which intestinal histology, IL number, % IL apoptosis/necrosis, IL phenotypes, protein levels of pro- inflammatory cytokine (TNF-α) and transcription factor (NFκB), apoptotic (caspase 3, Bax) proteins, anti-inflammatory cytokine (IL-10) and anti-apoptotic (Bcl-2) protein levels in IL were evaluated. DSS treatment resulted in shortened intestinal length, decreased body weight and widespread mucosal damage as well as increased IL death as determined by a decreased percentage of viable (PI^-/ANN^-, $p<0.005$) and increased percentage of necrotic/late apoptotic (PI^+/ANN^+, $p<0.05$) and necrotic (PI^+/ANN^-, $p<0.05$) IL. DSS exposure increased caspase 3 ($p<0.05$) and decreased Bcl-2 ($p<0.01$) protein levels in mouse IL. Dietary bLf did not influence these cell death outcome measures. However, bLf reduced protein levels of the pro-inflammatory transcription factor, NFκB, in IL ($p<0.05$) and was associated with a 34%, albeit non-significant, reduction in TNF-α relative to non-bLf fed mice. DSS treatment increased apoptosis and necrosis of mouse IL and elevated pro-apoptotic and reduced anti-apoptotic protein levels in these cells. Dietary bLf did not influence necrosis or

apoptosis of IL but may provide limited protection in the intestine by affecting the pro-inflammatory transcription factor NFκB, and potentially, cytokine expression.

5.5. Introduction

Inflammatory bowel diseases (IBD) are characterized by pronounced destruction of the gastrointestinal mucosa and the epithelial cell barrier (Brandtzaeg et al., 1997). Although the etiology of IBD is unknown, alterations in T_H1 and T_H2 cytokine balance coupled with prolonged or excessive immune cell activation to dietary or bacterial antigens are believed to contribute to IBD pathogenesis (Podolsky, 2002). Inflamed tissue of patients with active IBD contains large quantities of activated immune cells including neutrophils, macrophages and lymphocytes as well as increased concentrations of pro-inflammatory cytokines (Brandtzaeg et al., 1997; MacDermott, 1996)

Oral administration of dextran sulfate sodium (DSS) is a common method of inducing intestinal colitis in animal models. The mechanism by which DSS induces inflammation likely involves multiple biological pathways including direct cytotoxic effects on epithelial cells (Cooper et al., 1993) and indirect damage due to changes in resident bacteria (Okayasu et al., 1990), upregulation of lymphocyte adhesion molecules on intestinal epithelial cells (Ni et al., 1996) and activation of gut macrophages and T cells (Okayasu et al., 1990; Takizawa et al., 1995a; 1995b). DSS induced mucosal injury is characterized by nitrosative damage and, similar to human IBD, increased tissue concentrations of neutrophils, macrophages and activated T cells (Cooper et al., 1993; Takizawa et al., 1995a; 1995b)

Concentrations of inflammatory cytokines (IL-1, IL-6, TNF-α) are increased in patients with active IBD. For example, Isaacs et al. (1992) showed elevated mRNA expression of IL-1 and IL-6 in colonic mucosal biopsies of patients with IBD; increased intestinal mRNA of TNF-α was also found in children with active IBD (MacDonald et al., 1990). The sources of most cytokines in inflamed tissue are activated macrophages and lymphocytes (MacDermott, 1996). Similar to human IBD, DSS induced intestinal inflammation in mice results in higher concentrations of pro-inflammatory cytokines within the colonic mucosa (Takizawa et al., 1995a, 1995b; Egger et al., 2000). Moreover, the DNA-binding activity of NFκB is increased with DSS treatment (Herfarth et al., 2000). NFκB is a transcription factor found inactive in the cytoplasm, which upon activation (e.g., stress signals, pathogens and cytokines such as TNF-α) is relieved of its cytoplasmic inhibitor (IκB) and translocates to the nucleus where it binds to DNA and activates various pro-inflammatory genes (Li et al., 2002). NFκB is elevated in mice given DSS; addition of oligonucleotides ('NFκB decoys') that suppress transcription of NFκB reduces the extent of DSS induced inflammatory damage (De Vry et al., 2007). Thus, decreases in NFκB could potentially lead to decreased pro-inflammatory (T_H1) cytokine synthesis and less damage to the mucosal barrier.

Cell death can be characterized by two processes distinguished by different cellular morphologies and immunological consequences. Apoptosis, involving cellular shrinking, chromatic condensation, nuclear fragmentation and budding of the plasma membrane, is a genetically coded form of cell suicide with resultant apoptotic bodies engulfed by phagocytes (Vermes et al., 2002). Cell death by apoptosis normally does not recruit lymphocytes or neutrophils, key characteristics of inflammation (Lu et al., 2006).

In contrast, necrotic cell death involves cellular swelling, membrane disruption and release of intracellular contents and involves recruitment of inflammatory cells (Hoffman-Goetz et al., 2005). In DSS induced intestinal inflammation both apoptotic and necrotic damage have been shown to occur for epithelial cells (Renes et al., 2002); whether DSS induces either or both mechanisms of cell death in other cell populations resident in the gut, such as intestinal lymphocytes (IL), is not known.

Lactoferrin (Lf) is an 80 kDa iron binding glycoprotein found in neutrophilic granules and various mucosal secretions including milk and colostrums as well as commercially available dairy and whey protein containing products. During periods of physiological stress dietary Lf may alter cytokine levels with apparent bias to a T_H2 (anti-inflammatory) cytokine profile. For example, dietary administration of bovine lactoferrin (bLf) reduced TNF-α and NFκB expression in mouse IL following oxidant stress arising from repeated bouts of acute exercise (Spagnuolo and Hoffman-Goetz, 2008a). Similarly, Togawa et al. (2002a) showed reduced TNF-α and NFκB during DSS induced colitis in the colon of rats fed bLf by gavage. Oral administration of human Lf (hLf) to mice reduced the number of TNF-α producing $CD4^+$ T lymphocytes in the colon following DSS treatment (Haversen et al., 2002). Whether dietary bLf alters cytokine profile following DSS treatment in mouse IL is not known. Moreover, bLf has also been shown to influence intestinal cell turnover with reports of increased apoptosis (Spagnuolo et al., 2007) and increased cell viability (Shoji et al., 2007). However, the influence of dietary bLf on IL turnover (apoptosis, necrosis, viability) during periods of DSS induced inflammation has not been empirically characterized.

The purpose of this study was to determine the effect of bLf supplementation in mice given DSS on IL apoptotic and necrotic death and cytokine profile. We hypothesized that DSS induces IL death by both apoptotic and necrotic pathways and that dietary bLf given prior to DSS treatment affects cell death either by enhancing or reducing IL apoptosis and necrosis. We also addressed whether bLf given during DSS induced inflammation in mice decreases T_H1 (TNF-α) or increases T_H2 (IL-10) cytokine protein levels in IL.

5.6. Materials and Methods

Animals and Diets: Sixty three, 3-4 week old, female C57BL/6 mice, obtained from Harlan Sprague Dawley (Indianapolis, IN, USA), were individually housed on a 12/12h reversed light/dark cycle at 21±1^{o}C. Mice had *ad libitum* access to tap water and maintenance diet (Laboratory Rodent Chow, PMI feeds Richmond, IN, USA) for 2 weeks prior to the start of the study. Following acclimation, mice were randomly assigned by weight to two dietary conditions: bovine lactoferrin (bLf; n=31) or control diet (no bLf; n=32). Diets were prepared to contain either 0% or 2.0% bLf (Erie Foods International Inc, Erie IL) while maintaining similar overall protein concentrations (20% total protein in both groups) and were formulated based on a semi-purified AIN 76A standard diet. Concentrations of bLf were based on previous studies showing that 2.0% bLf was sufficient to influence apoptosis as well as cytokine production (Spagnuolo et al., 2007; Spagnuolo and Hoffman-Goetz, 2008a). bLf was 16% iron saturated and greater than 90% pure. Mice had free access to the diets for 12 days prior to sacrifice. All protocols

with live animals were approved by the University Animal Ethics Committee according to the principles of the Canadian Council on Animal Care.

DSS Protocol: Within diet conditions, mice were randomized to one of two treatment conditions: 0% DSS (n=31) or 5.0% DSS (n=32) in drinking water. There were a total of 4 groups: no bLf/no DSS (n=16); no bLf/DSS (n=16); bLf/no DSS (n=15) and bLf/DSS (n=16). DSS (MP Biomedicals, Solon, OH; mol wt=36-50 kDa) was solubilized in tap water at room temperature; mice had *ad libitum* access to DSS containing drinking water for 4 days prior to sacrifice.

Intestinal Lymphocyte Preparation: Mice were sacrificed by sodium pentobarbital (0.06-0.08 ml) overdose, the intestinal compartment, which includes both the small and large intestine, was removed and Peyer's patches and visible fat dissected out. IL were isolated as previously described (Spagnuolo and Hoffman-Goetz, 2008a). Briefly, IL were prepared as single cell suspensions by isolation over a column containing 0.3 g of pre-washed nylon wool, washed and layered over a density gradient medium (Lympholyte-M; Cedarlane Laboratories, Hornby, Ont.) to exclude epithelial cells and remove cellular debris. A sample of IL, which contains both intraepithelial and lamina propria lymphocytes, was stained with Turk's solution and counted manually by microscopy.

Flow Cytometry: Flow cytometry was used to determine IL apoptosis/necrosis and lymphocyte phenotype distribution. Due to equipment constraints, only a sample of 6-8

mice per treatment condition was included in the flow cytometry analysis. To distinguish between IL populations, an initial acquisition gate was created based on the forward and side scatter properties of a population shown to collect >90% $CD45^+$ cells using a CD45 (common leukocyte antigen, clone: 30F11) FITC-conjugated monoclonal antibody (mAb; Miltenyi Biotec, Auburn, CA). Apoptosis and necrosis were determined using FITC-conjugated Annexin V and PI staining, respectively. Annexin V detects externalization of cell membrane associated phosphatidylserine, an early marker of apoptosis whereas PI binds to DNA following cell membrane disruption and is indicative of necrotic cells (Vermes et al. 2002). Briefly, 1.0 x 10^5 cells were incubated with 2.5 µL of both Annexin V-FITC (Pharmingen) and PI (Sigma Chemical) in 100 µl of Annexin binding buffer (Pharmingen) for 15 min at room temperature in the dark, followed by addition of binding buffer (400µl) and analysis by flow cytometry. Individual IL phenotypes were determined by suspending 5 x10^5 cells in 100µl PBS and incubated with 2.5µl of PE-conjugated mAbs (PharMingen, San Diego, CA, USA) for CD4 (anti-CD4, clone: GK1.5) and CD8α (anti-CD8α, clone: 53-6.7) in the dark at 4ºC for 45 min. Cells were washed and centrifuged (5 min, 450*g*) and the pellet resuspended in 50µl Annexin V binding buffer and incubated with 2.5µl of Annexin V-FITC for 15 min at room temperature in the dark. Following incubation, 400 µl of binding buffer was added and analyzed for phenotype and apoptosis on a flow cytometer (FACSVantage SE).

Western Blot Analysis of Caspase 3, Bcl-2, Bax, NFκB, IL-10 and TNF-α: Cytoplasmic and nuclear mouse IL protein samples were prepared and separated by electrophoresis on a 12% SDS PAGE gel and transferred onto a PVDF membrane (Sigma

Chemical). Following transfer, membranes were stained with Ponceau S (Sigma Chemical) to confirm quality of transfer and equal loading. Membranes containing the separated proteins (cytoplasmic or nuclear) were incubated for 1 h with primary antibody (1:200 in 10% milk-TBST): Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG_1, mol wt =28kDa), caspase 3 (clone: H-227; rabbit anti-human clone IgG, mol wt =35kDa), TNF-α (clone: N-19; goat anti-human polyclonal IgG, mol wt =17kDa), NFκB (clone: C-20; rabbit anti-human polyclonal IgG, mol wt=65kDa), IL-10 (clone: M-18; goat anti-mouse IgG, mol wt =15kDa) or Bax (clone: 5B7; mouse anti-mouse monoclonal IgG_1, mol wt =23kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by 1 h incubation with secondary antibody: horseradish peroxidase-conjugated anti-mouse (Bcl-2, Bax), anti-rabbit (caspase 3, NFκB) or anti-goat (TNF-α, IL-10) IgG at a concentration of 1:2000 in 10% milk-TBST. Protein was determined using ECL or ECL Plus Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging System (Cambridge, UK). Each gel contained samples from all 4 treatment groups along with a biotinylated protein ladder to identify the molecular weight of the immunoblotted protein (Cell Signaling Technology, Beverly, MA, USA). For IL-10 and TNF-α blots, recombinant IL-10 and TNF-α standards (mouse IL-10, CL9310R, mouse TNF-α, CL9300TR; Cedarlane Laboratories) were run on each gel.

Histology: Approximately 1 cm sections of distal colon were excised, cleaned of fecal matter, and rinsed with PBS. Samples were fixed in 10% formalin and embedded in paraffin wax. Following sectioning, samples were stained with Hematoxylin and Eosin

(H&E) and viewed under light microscopy at 40X magnification. Samples were masked as to group allocation during visualization.

Statistical Analysis: Data were analyzed as a 2x2 analysis of variance design with diet (two levels: no bLf, bLf) and DSS (two levels: no DSS and DSS) as the independent factors using SPSS (Version 15; Chicago, IL, USA). $P \leq 0.05$ was accepted as being significantly different from chance alone. Significant interaction effects were further examined by t test analysis within the treatment conditions. All values are expressed as group means ± SEM.

5.7. Results

Effect of DSS and bLf on Mouse Body Weight and Food Intake: Mice did not differ by treatment condition (diet x DSS) in their average body weights at the start of the experiment: no bLf/no DSS 18.8 ± 0.5g; no bLf/DSS 18.9 ± 0.5g; bLf/no DSS 18.1 ± 0.5g; bLf/DSS 19.6 ± 0.5g. In contrast, there was a significant effect of DSS, but not diet, on the weight of animals at sacrifice ($F_{1,59} = 12.45$, $p<0.001$; Table 5.1). Mice given DSS had significantly lower body weights than those not given DSS ($p<0.05$). Cumulative food intake did not differ among the treatment groups over the 12 days of feeding: no bLf/no DSS 40.9 ± 1.4g; no bLf/DSS 40.0 ± 1.3g; bLf/no DSS 42.5 ± 0.9g; bLf/DSS 40.1 ± 1.1g.

Table 5.1: Dextran sulfate sodium and bovine lactoferrin effects on mouse intestinal inflammatory indicators (final body weight and intestinal compartment length) and lymphocyte counts.

Treatment Condition	Final Body Weight (g)	Intestine Length (cm)	IL count (10^7)
no DSS	19.93 ± 0.25	42.92 ± 0.38	4.29 ± 0.77
DSS	18.45 ± 0.32**	41.47 ± 0.41$^{\#\#}$	4.41 ± 0.78
no bLf	19.05 ± 0.32	41.73 ± 0.48	3.71 ± 0.35
bLf	19.31 ± 0.31	42.65 ± 0.47	5.01 ± 0.42*

$^{**}p < 0.001$ vs. no DSS, $^{\#\#}p<0.05$ vs. no DSS, $^{*}p<0.05$ vs. no bLf.

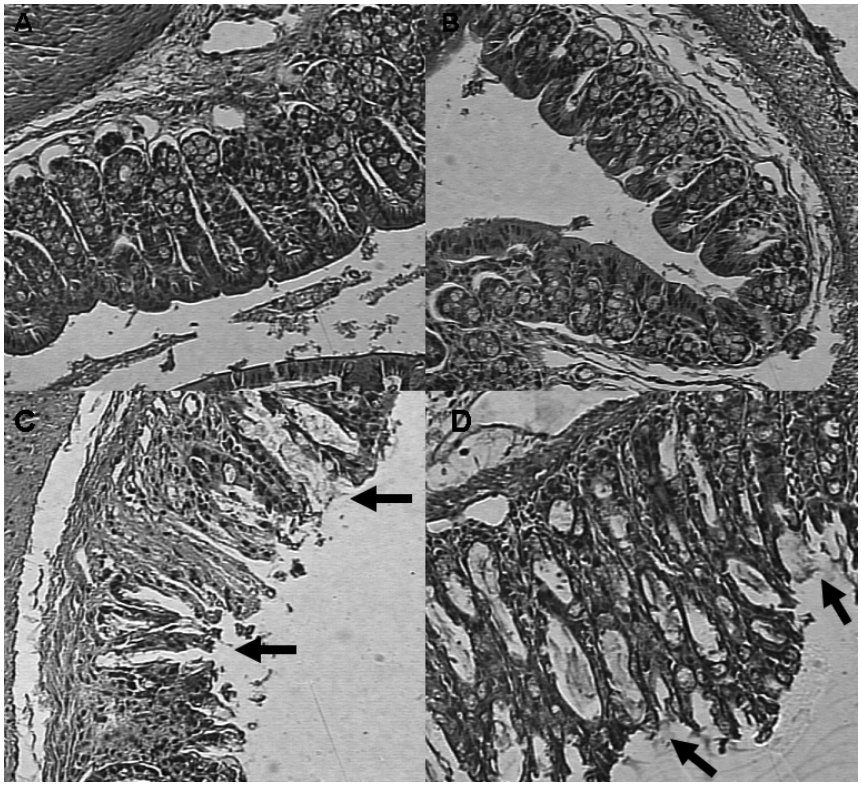


Figure 5.2: Hematoxylin and Eosin stained tissue sections of mouse colon viewed by light microscopy under 40x. A: no bLf/ no DSS, B: bLf/ no DSS, C: no bLf/DSS, D: bLf/DSS. Arrows indicate crypt disruption.

Effect of bLf and DSS on Mouse Intestinal Morphology and Disease Indicators: There was a significant effect of DSS on the length of the intestinal compartment (both the small and large intestine) ($F_{1,59} = 6.61$, $p<0.01$; Table 5.1) with reduced length in DSS treated mice. As a result of DSS treatment 30 of 32 mice had visual rectal bleeding and all animals had visual blood within the intestine. No rectal bleeding was observed in mice not given DSS and bLf did not decrease any bleeding. H&E stained colon sections (Figure 5.2) indicated structural damage arising from DSS treatment. Figure 5.2C and 5.2D illustrate mucosal damage and crypt disruption following DSS treatment compared to the controls not given DSS (Figure 5.2A and 5.2B). Further, in mice given DSS, ablation of crypts was observed whereas intact crypts characterized mice not challenged with DSS.

Effect of DSS and bLf on Mouse IL Counts, Cell Death and Phenotypes: There was a significant diet effect on IL counts ($F_{1,59} = 5.49$, $p<0.05$; Table 5.1). This was due to higher cell numbers in mice fed bLf compared with controls. There was no significant main effect of DSS nor was there an interaction between diet and DSS on mouse IL counts.

Figure 5.3 shows the apoptosis and necrosis flow cytometry results for mouse IL. There were significant effects of DSS on the percentage of PI^+/ANN^- (necrotic) ($F_{1,27} = 7.65$, $p<0.05$; Figure 5.3A), PI^+/ANN^+ (late apoptotic) ($F_{1,27} = 4.35$, $p<0.05$; Figure 5.3B) and PI^-/ANN^- (viable) ($F_{1,27} = 11.34$, $p<0.005$; Figure 5.3C) mouse IL. Diet condition did not significantly affect these outcome measures.

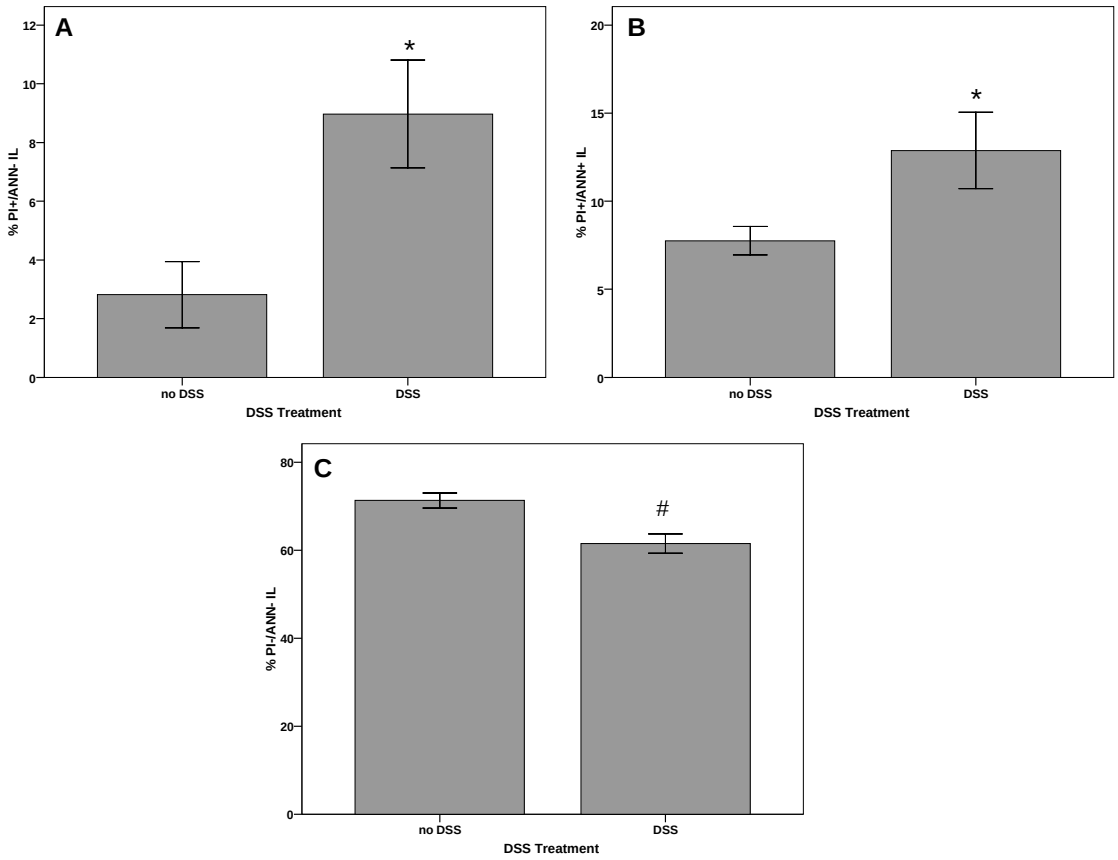


Figure 5.3: % PI and Annexin V^+ mouse IL by DSS treatment condition. *$p<0.05$, $^{\#}p<0.005$. See text for details of statistical analysis.

Figure 5.4 shows the flow cytometric results for $CD4^+$ IL and for expression of the apoptotic marker Annexin V within this subset. There was a significant interaction effect between DSS treatment and diet in the % $CD4^+$ IL ($F_{1,23} = 4.46$, $p<0.05$; Figure 5.4A). This interaction effect was due to a decrease in the percentage of $CD4^+$ IL in the bLf/DSS vs. the no bLf/DSS groups ($t_9 = 2.41$, $p<0.05$, Figure 5.4A) and a decrease in the bLf/no DSS vs. the no bLf/no DSS groups ($t_{11} = 2.92$, $p<0.01$, Figure 5.4A).

There was a similar interaction effect between DSS and diet on the percentage of mouse $CD4^+/ANN^+$ IL (apoptotic CD4) ($F_{1,23} = 5.03$, $p<0.05$; Figure 5.4B). This interaction reflected a decrease in the percentage of $CD4^+/ANN^+$ IL in the bLf/DSS vs. the no bLf/DSS groups ($t_9 = 1.79$, $p<0.05$, Figure 5.4B). Subsequently, there was also a significant main effect of DSS on the percentage of $CD4^+/ANN^-$ (viable CD4) ($F_{1,23} = 14.04$, $p<0.001$; Figure 5.4C), as these cells decreased following DSS treatment.

Figure 5.5 illustrates flow cytometric results for $CD8\alpha^+$ IL and for expression of the apoptotic marker Annexin V within this subset. There were no main or interaction effects of diet and DSS on the percentage of $CD8\alpha^+$ IL. There was, however, a trend toward an increase in the percentage of $CD8\alpha^+$ IL as a result of DSS treatment (no DSS: 53.77 ± 1.85%, DSS: 60.86 ± 3.75%, $p=0.085$). There was a significant main effect of DSS on $CD8\alpha^+/ANN^+$ IL (apoptotic CD8α) ($F_{1,23} = 11.67$, $p<0.01$; Figure 5.5A): mice given DSS had a lower percentage of $CD8\alpha^+/ANN^+$ IL as compared to those not receiving DSS. There was also a significant main effect of DSS on the percentage of non-apoptotic $CD8\alpha^+$ IL (i.e., $CD8\alpha^+/ANN^-$) ($F_{1,24} = 4.96$, $p<0.05$; Figure 5.5B) with a higher percentage observed in mice receiving DSS.

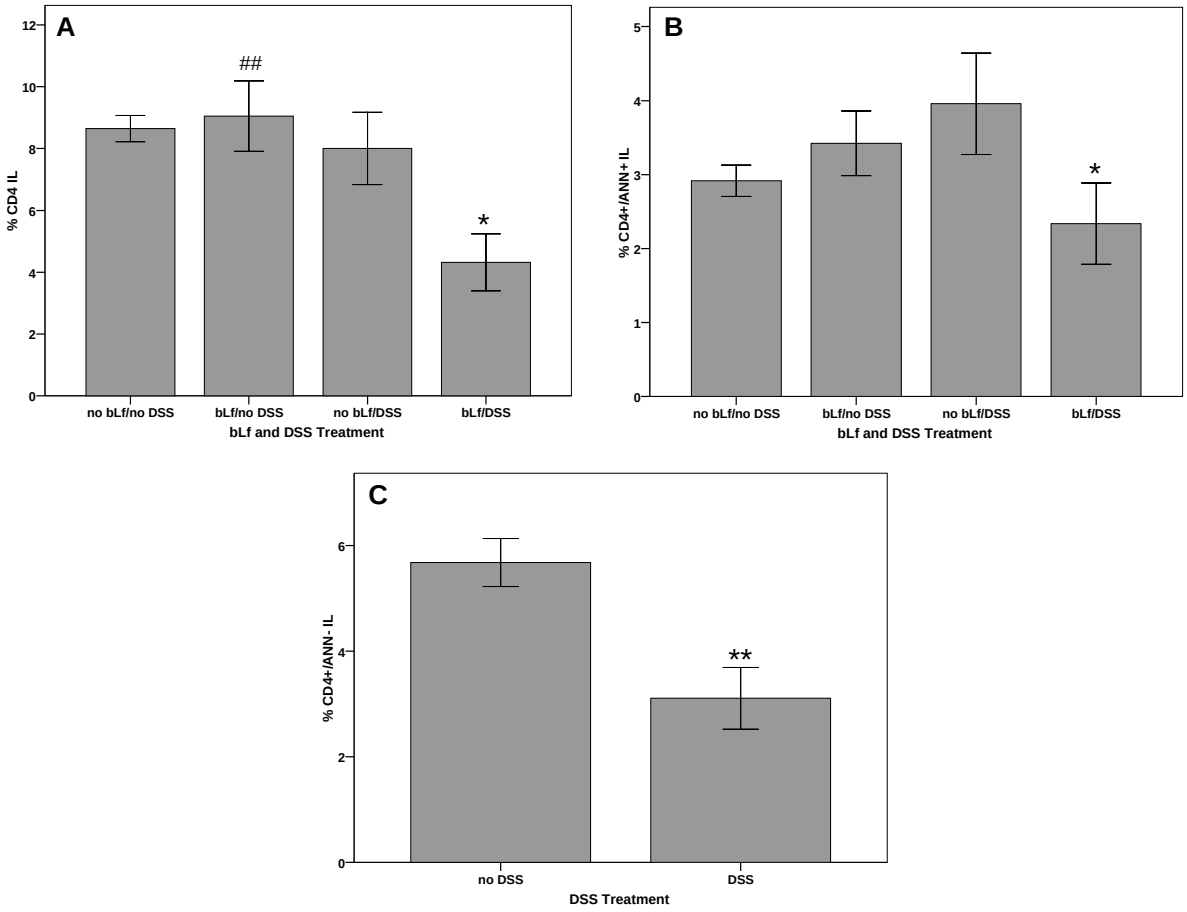


Figure 5.4: (A) %CD4 IL, *p<0.05, ##p<0.01, (B) %CD4^{+}/Annexin V^{+}, *p<0.05, and (C) %CD4^{+}/Annexin V^{-}, **p<0.001, of mice by DSS and bovine lactoferrin treatment conditions,. See text for details of statistical analysis.

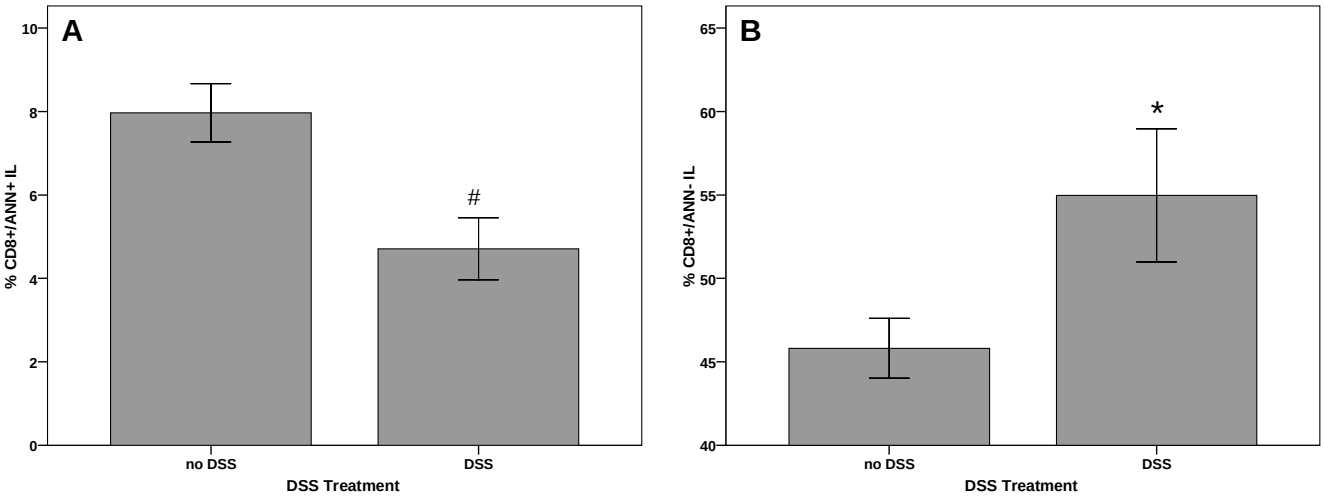


Figure 5.5: %CD8$^+$/Annexin V$^+$ (A), and %CD8$^+$/Annexin V$^-$ (B) of mouse IL by DSS treatment conditions. $^{\#}p<0.01$, $^{*}p<0.05$. See text for details of statistical analysis.

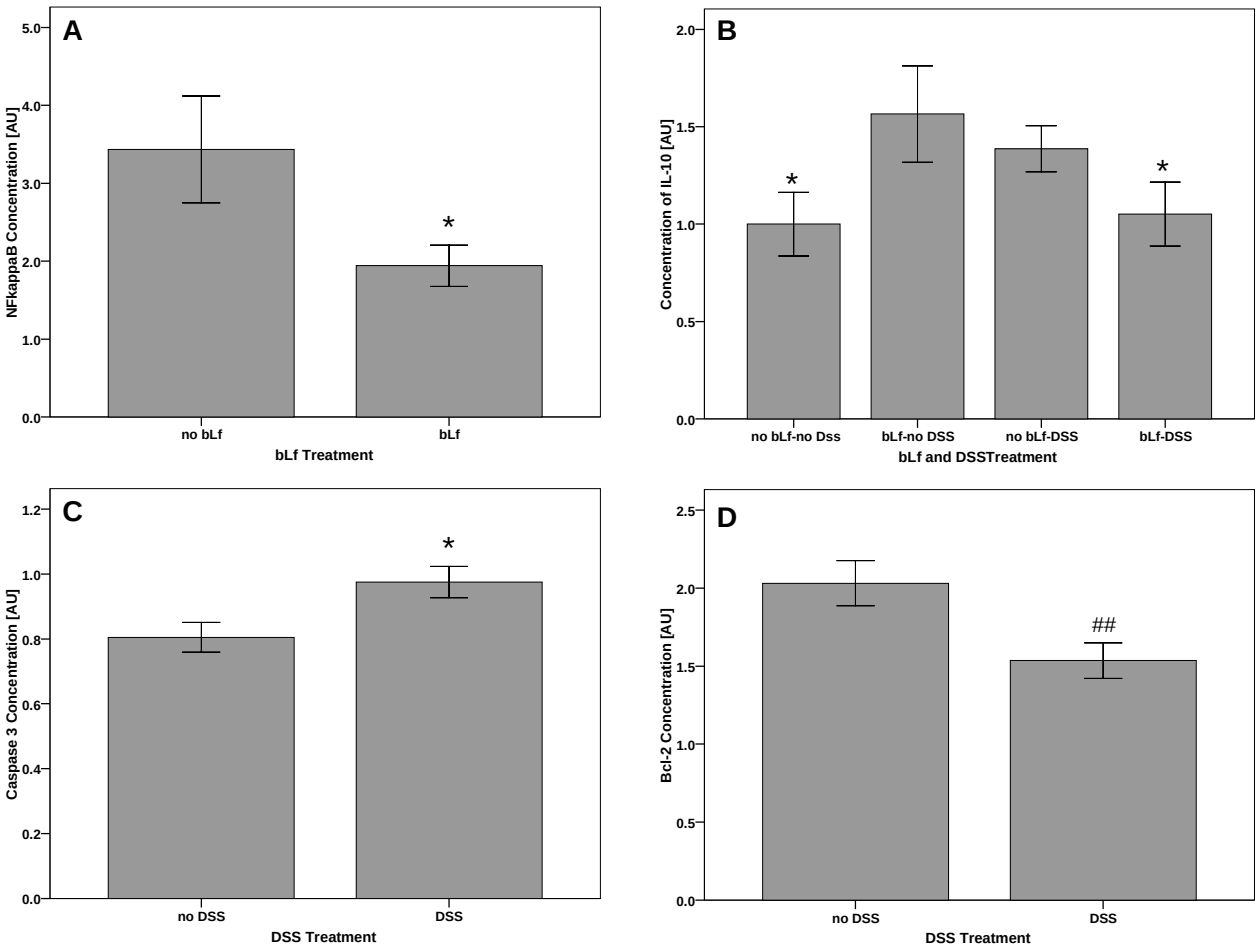


Figure 5.6: Concentration of apoptotic and inflammatory proteins in mouse IL by DSS and bovine lactoferrin treatment. (A) NFκB, $^{*}p<0.05$, (B) anti-inflammatory IL-10, $^{*}p<0.05$, (C) pro-apoptotic caspase 3, $^{*}p<0.05$, (D) anti-apoptotic Bcl-2, $^{\#\#}p<0.01$. Values are expressed as arbitrary densitometric units [AU]. See text for details of statistical analysis.

Effect of DSS and bLf on NFκB, IL-10, TNF-α: Western blotting results for NFκB, IL-10 and TNF-α concentrations in mouse IL are shown in Figure 5.6. There was a significant effect of diet on NFκB protein levels in mouse IL ($F_{1,43} = 5.37$, $p<0.05$; Figure 5.6A), an effect due to lower NFκB levels in mice fed bLf supplemented diets. There was no significant effect of DSS or interaction between diet and DSS on NFκB levels in mouse IL. Protein levels of TNF-α, a pro-inflammatory cytokine, in mouse IL was not affected by diet or DSS. Nevertheless, we observed a 34% decrease in TNF-α levels in IL of mice fed bLf compared to no bLf controls (no bLf: 1.3 ± 0.18, bLf: 0.91 ± 0.090). Protein levels of IL-10, an anti-inflammatory cytokine, in mouse IL was significantly affected as an interaction of diet x DSS exposure ($F_{1,33} = 6.78$, $p<0.05$; Figure 5.6B). This interaction reflected increased IL-10 concentrations in the no bLf/DSS ($t_{17} = -1.95$, $p<0.05$) and bLf/no DSS ($t_{15} = -1.95$, $p<0.05$) groups compared with the no bLf/no DSS control group and a decrease in IL-10 in the bLf/DSS group ($t_{16} = 1.79$, $p<0.05$) compared to the bLf/no DSS group.

Effect of DSS and bLf on Bax, Bcl-2, and Caspase 3: Figure 5.6C shows the effects of DSS on the concentrations of the pro-apoptotic protein caspase 3 in mouse IL ($F_{1,37} = 6.37$, $p<0.05$), an effect due to higher caspase 3 expression in DSS challenged animals relative to no DSS controls. bLf, either alone or interacting with DSS, did not significantly influence protein levels of caspase 3 in mouse IL. As shown in Figure 5.6D, there was a significant effect of DSS on the concentration of the anti-apoptotic protein Bcl-2 ($F_{1,54} = 7.60$, $p<0.01$), with decreased concentrations following DSS treatment. There was no change in the concentration of the pro-apoptotic protein, Bax, as a function

of diet, DSS treatment or as an interaction between diet and DSS in mouse IL. (data not shown).

5.8. Discussion

The objectives of this study were threefold: 1) to characterize the effects of DSS, an inducer of inflammatory damage in gut mucosa and epithelium, on mouse IL death, 2) to determine if bLf affected mouse IL death during DSS challenge and 3) to determine if bLf affected protein levels of mouse pro- and anti-inflammatory cytokines (TNF-α and IL-10) and pro- and anti-apoptotic proteins (caspase 3, Bax, Bcl-2) in IL during DSS exposure. A novel finding of this study was that mice subjected to 5% DSS for 4 days had increased death of IL as measured by the expression of Annexin V and PI as well as increased pro-apoptotic caspase 3 and decreased anti-apoptotic Bcl-2 protein levels in IL. Our study is also the first to report that bLf reduced the IL concentration of NFκB, an important transcription factor in the synthesis of pro-inflammatory cytokines such as TNF-α.

Administration of 5% DSS in drinking water for 4 consecutive days resulted in marked structural intestinal damage, intestinal and rectal bleeding, shortened intestinal length and reduced body weights in mice. These findings suggest active inflammation in the bowel and agree with those structural (reduced colon length, crypt destruction, epithelial cell ulceration) and disease indicators (rectal bleeding, lower final body weight) induced by DSS in mice reported by others (Cooper et al., 1993; Okayasu et al., 1990; Egger et al., 2000; Naito et al., 2003). In this present study, dietary bLf did not affect disease activity or reduce structural damage in the intestine. These observations are in

contrast with those of Haversen et al. (2003) showing that mice given 2 mg of human lactoferrin (hLf) twice daily for 7 d during 5% DSS treatment had less colonic length shortening, mucosal damage and fecal occult blood. The reasons for this discrepancy between our findings and those of Haversen and colleagues are not known but may reflect differences in: the type of Lf (bovine vs. human), the total concentration of Lf to which the mice were exposed (800mg vs. 28mg), the duration of Lf exposure (12 d vs. 7 d), or mode of administration (diet vs. sublingual).

IL isolated from animals given DSS exhibited high degrees of necrotic cell death. The percentage of late apoptotic/necrotic (ANN^+/PI^+) IL and necrotic (PI^+/ANN^-) IL increased whereas viable (ANN^-/PI^-) IL decreased following DSS treatment. DSS induced necrotic death of IL has not been previously reported. However, erosion of the intestinal mucosa has been suggested to occur as a result of necrosis (Renes et al., 2002). Dieleman et al. (1994) demonstrated that DSS reduced the viability and growth of a colonic epithelial cell line as measured by PI staining and [^{3}H] thymidine incorporation. Thus, given the anatomic proximity of IL to the intestinal epithelium, it seems likely that DSS acts in a direct cytotoxic manner on IL similar to epithelial cells.

Dietary bLf did not affect DSS induced IL death, which may be related to the mechanism of action and magnitude of inflammatory damage of DSS. First, if DSS is directly cytotoxic to IL a primary mechanism by which bLf could be protective would be by interaction and neutralization of DSS. There is, however, no evidence in the literature to support a neutralization role for bLf on DSS. Second, the inflammatory response in the bowel induced by DSS is extensive: any potential benefit provided by bLf (at least in the concentration and exposure provided in this study) may be insufficient to compensate

for the large pathophysiological changes resulting from DSS exposure. Although bLf primes toward an anti-inflammatory cytokine profile (Spagnuolo and Hoffman-Goetz, 2008a; Togawa et al. 2000a, 2002b) and decreases the extent of oxidative stress (Shoji et al., 2007), this benefit may not be able to overcome the array of DSS induced disease sequelae including disruption in membrane barrier function and potential exposure of commensal bacteria, immune activation, infiltration of neutrophils, macrophages and lymphocytes, increased nitrosative and oxidative stress and increased pro-inflammatory cytokine production (Cooper et al., 1993; Okayasu et al., 1990; Takizawa et al., 1995a; 1995b; Egger et al., 2000; Naito et al., 2001).

Death of IL by DSS was largely necrotic; nevertheless, DSS induced apoptosis cannot be completely dismissed given the increased expression of PI^+/ANN^+, the increased protein levels of caspase 3, and the decreased levels Bcl-2 in mouse IL. Indeed, apoptotic cells that are not recognized by phagocytes undergo secondary (or apoptotic) necrosis, subsequent to apoptosis, a process characterized by the appearance of the PI^+/ANN^+ phenotype (Vermes et al., 2002; van Cruchten and Van Den Broeck, 2002). Given the dynamic kinetic process of apoptosis and the cross-sectional nature of the study, we were unable to fully differentiate between apoptotic and necrotic IL death induced by DSS. Increases in both measurement parameters indicate that both processes were likely occurring in IL and is consistent with Renes et al. (2002), who observed apoptosis and necrosis within the colonic epithelium of DSS treated rats. Future studies, such as sampling at different time points and morphological analysis of IL, will be needed to further clarify the mechanism of DSS induced IL death.

Dietary bLf administration did not affect the concentration of apoptotic related proteins, a finding which is consistent with its inability to influence IL death during DSS induced inflammation. Although bLfcin has been shown to affect apoptotic related parameters such as mitochondrial membrane permeability (Mader et al., 2005; 2007) and apoptotic proteins such as Bcl-2 (Spagnuolo and Hoffman-Goetz, 2008a), the inability of bLf to affect either caspase 3 or Bcl-2 in the present study likely reflects the extent and severity of the DSS induced inflammatory response.

Administration of dietary bLf in mice for 12 days resulted in an increase in the total number of IL, similar to other reports (Spagnuolo and Hoffman-Goetz, 2008a; Wang et al., 2000). However, DSS treatment did not influence overall IL numbers despite an increase in IL death. We suggest that this can be explained by the increase in the percentage of $CD8^+$ IL with DSS treatment. Increases in $CD3^+$ T cells (Melgar et al., 2006), T and B lymphocytes (subsets not identified) (Cooper et al., 1993) and activated T lymphocytes (Takizawa et al., 1995a; 1995b) in the colonic mucosa of mice following DSS treatment have been reported. In this study, the observed percent increase in CD8 cells, with no change in IL number, may reflect mobilization of this subpopulation from other lymphoid compartments in response to the DSS induced intestinal inflammation. In support of this hypothesis are the following observations: 1) there is a decreased number of $CD8\alpha^+$ T cells in the spleen coupled with an approximate three fold increase in peripheral blood lymphocytes following DSS treatment in mice (Da Silva et al., 2006), 2) there is an increased number of 'mature phenotype' thymocytes following DSS treatment in mice, a phenomenon attributed to preparation for mobilization to other compartments (Fritsch Fredin et al., 2007) and 3) there is an increased expression of IL

adhesion molecules in mouse intestinal epithelial cells following incubation with DSS (Ni et al., 1996). Taken together, the percent increase in CD8α T cells in mouse intestine may reflect lymphocyte recruitment from other pools in the periphery and may contribute to the maintenance of total IL numbers following DSS treatment. Alternatively, the percent increase in CD8α cells may only reflect a loss of another lymphocyte population (e.g., B cells). However; given the consistent total IL numbers between DSS and non DSS treated mice this interpretation seems less likely (i.e., a cell population must replace the dying cells).

During inflammation cytokines play central roles in dictating the extent, duration and direction of the immune response. Our results suggest a shift towards an anti-inflammatory response with increased IL-10 (independent of DSS exposure environment) and a trend of decreased TNF-α following bLf administration. bLf given through the diet alters the ratio of TNF-α/IL-10 in mouse IL to favour anti-inflammatory responses following a known physiological stressor (repeated bouts of exhaustive exercise with associated oxidant stress) (Spagnuolo and Hoffman-Goetz, 2008a). Togawa et al. (2002a; 2002b) reported that bLf administered by gavage increased IL-10 and decreased TNF-α following DSS and TNBS treatment in rat colon, respectively. Thus, a potential benefit of bLf supplementation may involve a shift in the T_H1 and T_H2 cytokine balance to favour anti-inflammatory responses.

Treatment of mice with DSS was associated with increased IL-10 concentrations in IL (no bLf/no DSS vs. no bLf/DSS), a finding which may seem paradoxical given the T_H1 suppressing, anti-inflammatory role of this cytokine. However, increased IL-10 production following DSS exposure may be an adaptive response to reduce the extent of

inflammation. In support of this hypothesis are the findings of Egger et al. (2000) who demonstrated increased mRNA of several pro-inflammatory cytokines (e.g., TNF-α, IFN-γ, IL-1) as well as increased IL-10 mRNA in the colonic mucosa of DSS treated mice. Increased IL-10 mRNA was also found in mucosal biopsies of patients with active Celiac Disease (Forsberg et al., 2007). Haversen et al. (2003) reported increases in IL-10 producing cells in the colonic mucosa of mice subjected to DSS, which were decreased following oral hLf administration; this reduction was related to a hLf associated down-regulation of DSS induced inflammation. We report a decrease in IL-10 following dietary bLf administration in mouse IL within DSS treatment (bLf/no DSS vs. bLf/DSS), which may reflect a lessening of the inflammatory stress. Therefore, increased IL-10 production may be a physiological compensatory mechanism in response to DSS and the addition of bLf may attenuate the need for this compensation.

Dietary bLf was associated with decreased protein levels of the pro-inflammatory transcription factor NFκB in mouse IL. Reduced NFκB may subsequently result in less pro-inflammatory cytokine production by immune cells. In support of this are the following observations: first, following LPS stimulation, binding of NFκB to the TNF-α promoter in monocytes was inhibited by Lf (21). Second, Togawa et al. (2002b) demonstrated reduced phosphorylation of IκB indicating decreased NFκB activation in rat colon following gavage administration of bLf in rats with TNBS induced colitis; third, dietary bLf reduced both NFκB and TNF-α expression in mouse IL following oxidant stress accompanying acute, repeated exercise (Spagnuolo and Hoffman-Goetz, 2008a). These findings suggest that bLf may reduce the extent of inflammation by altering the T_H1/T_H2 balance acting through decreased NFκB protein levels.

5.9. Conclusion

In summary, 5% DSS treatment for 4 days in female C57BL/6 mice was associated with marked inflammatory damage within the intestinal compartment. DSS resulted in necrotic death of IL as well as increased protein levels of the apoptotic protein caspase 3 and decreased expression of the anti-apoptotic Bcl-2 protein. DSS treatment also induced a non-significant increase in the percent of CD8α IL indicative of an activated immune response. Finally, dietary bLf did not provide any observable benefit in disease activity measurements nor did it influence DSS induced IL death. Nevertheless, bLf may offer some limited intestinal protection by decreasing the levels of the pro-inflammatory transcription factor NFκB. However, the physiological impact of lower NFκB levels in IL on the actual synthesis of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) will need to be determined experimentally.

Chapter 6: Effect of Dextran Sulfate Sodium and Acute Exercise on Mouse Intestinal Inflammation and Lymphocyte Cytochrome c levels

6.1. Objectives

To determine the effects of dietary bovine lactoferrin (bLf) on small and large intestinal histology and on cytochrome c levels in mouse intestinal lymphocytes in female C57BL/6 mice following dextran sulfate sodium or repeated bouts of acute exercise.

6.2. Hypotheses

Hypothesis 1: bLf administration will increase cytochrome c levels in mouse IL following repeated bouts of strenuous exercise and DSS challenge relative to the IL levels of cytochrome c in mice not receiving bLf supplementation.

Hypothesis 2: Mice receiving repeated bouts of strenuous exercise will exhibit similar intestinal histology relative to mice receiving DSS treatment.

Hypothesis 3: bLf administration will provide protection, as determined by histological scores, against both DSS and AE induced intestinal damage relative to the histological scores of mice not receiving bLf supplementation.

6.3. Study Design

This study was designed to provide direct evidence about the possible benefits of dietary bLf on small and large intestinal morphology following DSS and AE challenge. In addition, it was the aim of this study to determine the histological effects of AE and if any alterations were similar to that induced by DSS. Previous experiments (Chapter 4 and 5) demonstrated that bLf had small, albeit, significant effects on decreasing the levels in mouse IL of the pro-inflammatory cytokine TNF-α, the pro-inflammatory transcription factor NFκB, and the anti-apoptotic protein Bcl-2. It was the aim in this experiment to determine if these bLf induced modifications were associated with direct mucosal protection against AE and DSS challenge. In addition, the cell death marker cytochrome

c, located in the mitochondria, was measured within the IL by Western blotting. Since the previous studies determined that dietary bLf given to mice was able to decrease the expression of the anti-apoptotic protein Bcl-2 (involved in regulation of mitochondrial outer membrane permeability), it was hypothesized that there would also be an increase in cytochrome c expression in mouse IL. The study design for experiment 4 is outlined in Figure 6.1.

Study duration: Two week acclimation plus 12 d of dietary bLf administration.

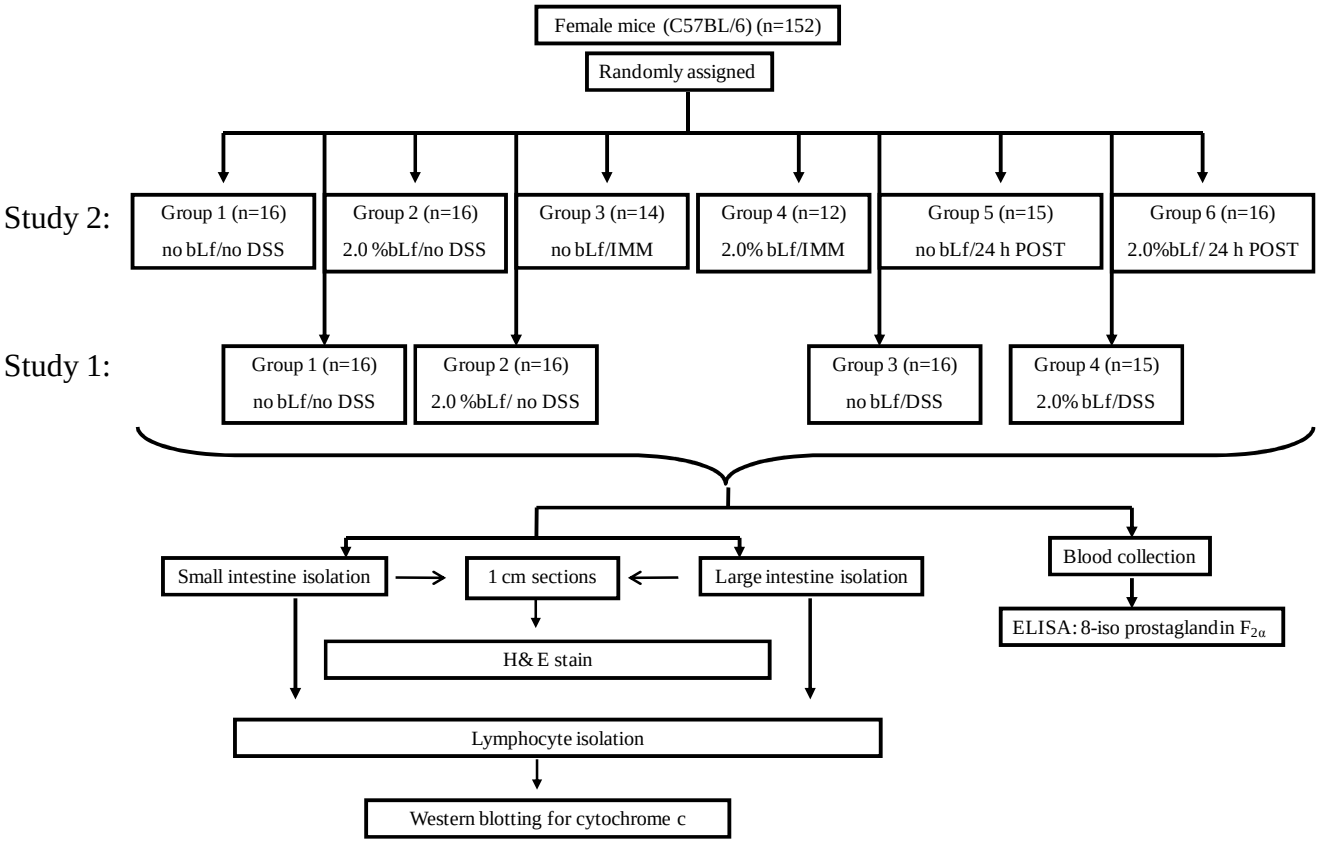


Figure 6.1: Schematic outline of the study design for experiment 4.

A two way ANOVA and post-hoc Tukey tests were used to analyze the study data. In the DSS study, a two way ANOVA (2x2 factorial design) was used to analyze the data. In this study the independent variables are diet condition (0% bLf or 2.0% bLf) and DSS treatment (no DSS or DSS). In the AE study, a two way ANOVA (2x3 factorial design) and post-hoc Tukey tests was used to analyze the data. In this experiment, the independent variables were diet condition (0% bLf or 2.0% bLf) and exercise treatment (SED, IMM, 24 h POST). For both experimental studies, the dependent variable was the cell death marker cytochrome c. Small and large intestinal histology was graded according to the inflammatory and crypt score outlined in Table 6.1 and verified by two independent veterinary pathologists.

Table 6.1: Details of the inflammatory and crypt scoring system used to grade small and large intestinal histology.

Grade	Inflammatory Score (Melgar et al., 2005)	Crypt Score (Cooper et al., 1993)
0	normal,	intact crypt
1	slight inflammation	loss of basal one-third of crypt
2	moderate inflammation and/or edema	loss of basal two-thirds
3	heavy inflammation and/or ulceration and/or edema.	loss of entire crypt with no alteration to the epithelium
4	N/A	loss of entire crypt and epithelium.

6.4. Overview

Dextran sulfate sodium (DSS) is commonly used to induce intestinal inflammation in animal models. Acute exercise (AE) is associated with oxidative stress and ischemia-reperfusion injury that may result in intestinal tissue damage. Bovine lactoferrin (bLf), an anti-inflammatory protein, may provide protection during DSS or AE inducing intestinal tissue damage. The purpose of this study was to investigate the influence of DSS and AE challenge in mice on: 1) tissue inflammatory changes within small and large intestine, 2) extent of cell death as measured by cytochrome c protein levels in intestinal lymphocytes (IL) and, 3) the effects of dietary bLf on these parameters. Tissue inflammation was determined by histology and cytochrome c levels in murine IL by Western blotting. Cytochrome c levels were elevated following bLf ($p<0.01$) and DSS ($p<0.05$) treatment whereas exercise had no significant effect. DSS, but not AE, resulted in histological alterations indicative of inflammation, with no attenuation by bLf, in the small and large intestine. The novel findings of this study were that both DSS and bLf induce cell death as suggested by increased cytochrome c expression in mouse IL. Further, the results show that DSS, but not AE, elicits intestinal inflammation and that bLf does not protect against DSS-induced tissue damage.

6.5. Introduction

Athletes commonly suffer from gastrointestinal disturbances such as abdominal pain and bleeding following strenuous exercise (Peters et al., 2001b; Bi and Triadafilopoulos, 2003). These gastrointestinal problems may be related to the induction of intestinal inflammation as a result of exhaustive exercise. A mechanism by which acute strenuous exercise could trigger intestinal inflammation is through oxidative stress and/or ischemia-reperfusion injury. Marshal (1998) suggested that acute exercise results in bacterial translocation following ischemia/reperfusion injury to the intestine post exercise. Choi et al. (2006) reported that 22 of 24 long distance runners had upper GI mucosal lesions post exercise. Bloody diarrhea was reportedly due to colonic lesions caused by ischemia following long distance running (Heer et al., 1987). Jeukendrup and colleagues (2000) reported mild endotoxemia due to gut ischemia-associated leakage of endotoxins in 68% of athletes completing a long-distance triathlon event. Further support for intense exercise as a model of inducing intestinal inflammatory damage comes from: 1) the high levels of bacterial lipopolysaccharide in human plasma following maximal aerobic exercise suggestive of increased intestinal permeability (Ashton et al., 2003), 2) the increase in the pro-inflammatory cytokine TNF-α in human plasma (Ostrowski et al., 1999) and mouse intestinal lymphocytes (Hoffman-Goetz et al., 2008) following acute exercise, 3) the mild increases in cellular infiltrates in the mouse small intestine following repeated bouts of acute exercise (Rosa et al., 2008), and 4) the increase in oxidative stress (measured by plasma levels of lipid metabolites) in mice following single (Hoffman-Goetz and Quadrilatero, 2003) and repeated bouts of strenuous exercise (Spagnuolo and Hoffman-Goetz, 2008a).

Sampling gastrointestinal tissue from athletes (or other human populations) is problematic for ethical reasons. Rodents have been used to examine the effects of intense exercise on various immunological and physiological parameters within the gastrointestinal tract. Among the methodological advantages of using an animal model of exercise-induced intestinal inflammation is the ability to directly analyze tissue alterations. However, to determine whether exercise actually induces intestinal inflammation it is important to compare the physiological alterations to a model known to elicit such effects. The "gold standard" for induction of experimental intestinal inflammation is dextran sulfate sodium (DSS), a sulfated polysaccharide, which is administered in the drinking water (Strober et al., 2002). Exposure of mice to DSS results in pathological alterations to the intestine including crypt destruction, intestinal shortening and bleeding, epithelial erosion and recruitment of immune cells (Okayasu et al., 1990; Cooper et al., 1993). DSS-induced intestinal inflammation is, in part, believed to occur by eliciting direct mucosal injury (Strober et al., 2002). Several studies (Okayasu et al., 1990; Cooper et al., 1993; Egger et al., 2000; Melgar et al., 2005; Renes et al,. 2002; Takizawa et al., 1995a; 1995b; Vetuschi et al., 2002) examined the effects of DSS on the intestinal epithelium and demonstrated epithelial cell death following treatment. There is, however, only limited research on whether these effects of DSS occur similarly in intestinal lymphocytes (IL) (Spagnuolo and Hoffman-Goetz, 2008b).

Given the potential of gastrointestinal disturbances (at least in part) related to oxidative stress that accompanies exercise, it is not surprising that athletes adjust their diets to improve antioxidant defenses. Froiland et al. (2004) reported 89% of Division 1 athletes used dietary supplements to improve performance and of these 12.6% ingested

whey protein, a natural by-product of cheese manufacturing. Whey protein administration also reduced the extent of oxidative damage as shown by decreased lipid peroxidation markers and decreased ratios of GSH/GSSG in untrained animals following a bout of exhaustive exercise (Elia et al., 2006). An important constituent of whey proteins is lactoferrin (Lf). Indeed, bovine lactoferrin (bLf) has demonstrated anti-inflammatory effects including increased apoptosis (Mader et al., 2007) and cytokine alterations (Togawa et al., 2002a; 2002b). In addition, dietary bLf reduces TNF-α levels in mouse IL through reductions in NFκB expression following acute exercise and DSS challenge (Spagnuolo and Hoffman-Goetz, 2008a, Spagnuolo and Hoffman-Goetz, 2008b). However, a direct role of dietary bLf in protecting the intestinal epithelium in response to repeated bouts of AE or DSS damage in the bowel has not been tested.

Apoptosis and necrosis are two terms used to describe cell death. Apoptosis involves a series of events resulting in DNA degradation, cellular shrinking and the formation of apoptotic bodies that are removed by local phagocytes to prevent the induction of inflammation. Necrosis occurs following direct cellular injury resulting in swelling, membrane rupture and the release of cellular contents and inflammation (Hoffman-Goetz et al., 2005). We reported that mice exposed to DSS had significant IL death characteristic of both apoptosis and necrosis (Spagnuolo and Hoffman-Goetz, 2008b). To investigate that DSS causes IL death and that AE induces a similar response to that of DSS, the cell death associated protein cytochrome c was quantified. Cytochrome c, located on the mitochondrial inner membrane and involved with electron transport, corresponds with both apoptosis and necrosis (Garrido et al., 2006; Jemmerson et al,. 2002). Mitochondrial dysfunction results in cytochrome c release into the

cytoplasm, the formation of an apoptosome complex consisting of caspase 9, ATP and APAF 1, and activation of caspase 3, the primary executor of apoptotic cell death (van Loo et al., 2002). Cytochrome c release from the mitochondria has also been associated with necrotic cell death. Jemmerson et al. (2002) demonstrated that cellular injury resulted in the presence of cytochrome c attributed to direct insult to the mitochondria. Thus, an increase in cytochrome c is a reasonable marker of cell death, either by apoptotic or necrotic mechanisms.

The purpose of this study was twofold. The first aim was to compare DSS and AE as modalities of inducing direct (histological) damage within mouse small and large intestine, both independently and in combination with dietary bLf. A second aim was to determine cytochrome c levels in mouse IL following DSS or AE exposure alone or in combination with dietary bLf. We hypothesized that if intestinal damage arises from AE than this protocol would induce intestinal alterations similar to those caused by DSS. We also hypothesized if DSS or AE exposure resulted in apoptotic or necrotic cell death, then cytochrome c levels would be increased in mouse IL.

6.6. Materials and Methods

Animals. A total of 152 female C57BL/6 mice, 3-4 weeks old, were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA) and individually housed at 21±1 $^{\circ}$C on a 12/12h reversed light/dark cycle. The purchase of animals, the husbandry and the experiments conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and to the Canadian

Council on Animal Care. All protocols with live animals were approved by the University Animal Ethics Committee.

Diet. Mice had *ad libitum* access to tap water and maintenance diet (Laboratory Rodent Chow, PMI feeds Richmond, IN, USA) for 2 weeks prior to the start of the studies. Following acclimation, mice were randomly assigned by weight into treatment conditions for Studies I and II (see below). Diets were formulated to contain 20% total protein which included either 0% or 2.0% bLf (Erie Foods International Inc, Erie IL); bLf concentrations were determined based on previous studies which showed 2.0% was sufficient to influence apoptosis and cytokine production (Spagnuolo et al., 2007). No bLf mice had 0% bLf in the diet; bLf mice had 2.0% bLf in the diet. Mice had free access to the diets for 12 d prior to sacrifice.

Study I: DSS Exposure. Mice (n=63) were randomized into 4 treatment conditions: no bLf/no DSS (n=16); no bLf/DSS (n=16); bLf/no DSS (n=15) and bLf/DSS (n=16). DSS (MP Biomedicals, Solon, OH; mol wt=36-50 kDa), concentration of 5% w/v, was solubilized in tap water at room temperature and mice had *ad libitum* access to DSS containing drinking water for 4 d prior to sacrifice.

Study II: Acute Exercise (AE) Exposure. Mice (n=89) were randomized to one of six treatment groups: no bLf/SED (n=16); bLf/SED (n=16); no bLf/IMM (n=14); bLf/IMM (n=12); no bLf/24 h POST (n=15); and bLf/24 h POST (n=16). SED were sedentary animals; IMM were animals that had three sessions of treadmill running, each separated

by a 24 h rest interval and with sacrifice upon completion of the third exercise bout; 24 h POST were animals given repeated treadmill running and sacrificed 24 h after the final exercise session (Spagnuolo et al., 2008a). The treadmill protocol described elsewhere (Hoffman-Goetz and Quadrilatero, 2003) consisted of a 10 min warm-up, 90 min continuous exercise (30 min at 22 $m \cdot min^{-1}$, 2°slope; 30 min at 25 $m \cdot min^{-1}$, 2°slope; 30 min at 28 $m \cdot min^{-1}$, 2°slope) and 5 min deceleration. Mice were run during the dark cycle (Omni-Max metabolic treadmill, Omni Tech Electronics, Columbus, OH) and were motivated to run by occasional gentle prodding using a soft nylon brush.

Intestinal Lymphocyte (IL) Preparation. IL were isolated according to Hoffman-Goetz and Quadrilatero (2003), modified from Lefrancois (1993). Following sacrifice, intestinal compartments (small and large intestines) were immediately removed, Peyer's patches and visible fat dissected out, and IL prepared as single cell suspensions by isolation over a column containing 0.3 g of pre-washed nylon wool, washing and layering over a density gradient medium (Lympholyte-M; Cedarlane Laboratories, Hornby, Ont.) to exclude epithelial cells and remove cellular debris. A sample of IL, which contained both intraepithelial and lamina propria lymphocytes, was stained with Turk's solution and counted manually by light microscopy.

Western Blot Analysis of Cytochrome C. Cytoplasmic protein samples were prepared from whole IL samples, separated by electrophoresis on a 12% SDS PAGE gel, and transferred onto a PVDF membrane (Sigma Chemical, St. Louis, MO, USA). Quality of transfer and equal protein loading was confirmed with by Ponceau S staining (Sigma

Chemical). Membranes containing the separated proteins were incubated in primary antibody for 1 h (1:200 in 10% milk-TBST): cytochrome c (Clone: 6H2; mouse-anti-rat IgG_1, mol wt=11 kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by 1 h incubation with secondary antibody (1:2000): horseradish peroxidase-conjugated anti-mouse in 10% milk-TBST. Protein was determined using ECL Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging System (Cambridge, UK). Each gel contained samples from all treatment groups as well as a biotinylated protein ladder to identify the molecular weight of the immunoblotted protein.

Histology. Within each experimental group, 1 cm sections of distal colon/large intestine (n=4) and terminal ileum/small intestine (n=4) were excised, cleaned of fecal matter and rinsed with PBS. Samples were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin (Harris) and eosin (H & E) and viewed under light microscopy at 40X magnification. Inflammatory scores were assessed based on the grading system of Melgar et al. (2005): (0) normal, (1) slight inflammation, (2) moderate inflammation and/or edema and (3) heavy inflammation and/or ulceration and/or edema. Crypt scores were evaluated based on Cooper et al. (1993): (0) intact crypt, (1) loss of basal one-third of crypt, (2) loss of basal two-thirds, (3) loss of entire crypt with no alteration to the epithelium and (4) loss of entire crypt and epithelium. Samples were masked as to group allocation during visualization. Tissue samples were scored independently by two veterinary pathologists as well as the researchers.

8-iso Prostaglandin $F_{2\alpha}$. Following sacrifice (Study II) cardiac blood was immediately collected into a 1ml syringe containing 0.08ml of heparin, centrifuged (6 min, 1500*g*), plasma collected, and stored at -20˚C. Plasma concentrations of 8-iso prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) were determined by direct enzyme immunoassay (EIA) using a commercially available kit (Assay Designs, Ann Arbor, Michigan, USA). Briefly, at room temperature samples were hydrolyzed (25µL part 10N NaOH: 100µL sample) for 2 h and neutralized with 12N HCL to pH 6-8. Following centrifugation (5 min, 14000*g*), the supernatant was collected and incubated with 8-iso-$PGF_{2\alpha}$ antibody at 4˚C for 24 h. Absorbance was determined at room temperature at 405 nm.

Statistical Analysis. For Study I (DSS study) there were two independent factors: diet (two levels: no bLf, bLf) and DSS (two levels: no DSS and DSS. For Study II (AE study) there were two independent factors: diet (two levels: no bLf, bLf) and exercise (three levels: SED, IMM, 24 h POST). Data were analyzed using SPSS (Version 15; Chicago, IL, USA) and $p<0.05$ was accepted as being different from chance alone. Values are expressed as group means ± SEM.

6.7. Results

Study I: DSS and bLf on Cytochrome c Levels in Mouse IL: Figure 6.2A shows that there was a significant main effect of DSS on cytochrome c levels in mouse IL ($F_{1,45}$ = 6.92, $p<0.05$). This effect was due to increased levels of cytochrome c in DSS treated compared to non DSS mice. There was also an effect of diet (bLf exposure) on mouse IL levels of cytochrome c ($F_{1,45}$ = 8.09, $p<0.01$) with the bLf fed groups having greater

cytochrome c levels compared with the no bLf controls (Figure 6.2B). There was no significant interaction between lactoferrin and DSS exposure.

Study I: DSS and bLf on Mouse Intestinal Histology: There were significant changes in small and large intestine histology due to DSS treatment. Table 6.2 summarizes the inflammatory and crypt scores as markers of tissue changes. Large intestine samples displayed pathological inflammation evidenced by complete crypt obliteration and epithelial erosion following DSS treatment (Figure 6.3 shows a representative tissue section). Additionally, there were large amounts of mononuclear (lymphocytes, macrophages) and polymorphonuclear (neutrophils) cellular infiltrates. There was no evidence of attenuation of DSS induced inflammatory damage in animals fed bLf (Figure 6.3D).

DSS induced alterations, albeit more subtle, within the small intestine. Inflammatory scores were slightly increased (Table 6.2) and, as shown in Figure 6.4, DSS altered crypt structure and resulted in mild increases in cellular infiltrates. As observed in the large intestine, provision of bLf in the diet did not reduce DSS induced damage to the small intestine (Figure 6.4D).

Table 6.2: Inflammatory and Crypt Scores

Treatment Group	**Inflammatory Score[1]**		**Crypt Score[2]**	
	Small Intestine	Large Intestine	Small Intestine	Large Intestine
Acute Exercise Study				
no bLf/SED (n=4)	0	0	0	0
bLf/SED (n=4)	0	0	0	0
no bLf/IMM (n=4)	0	0	0	0
bLf/IMM (n=4)	0	0	0	0
no bLf/24h POST (n=4)	0	0	0	0
bLf/24h POST (n=4)	0	0	0	0
DSS Study				
no bLf/no DSS (n=4)	0	0	0	0
bLf/no DSS (n=4)	0	0	0	0
no bLf/DSS (n=4)	1.3 ± 0.8	3.5 ± 0.3	0	2.3 ± 0.3
bLf/DSS (n=4)	1.0 ± 0.5	3.3 ± 0.3	0	2.5 ±0.3

[1] Inflammatory and [2] Crypt Scores were based on the scoring systems outlined by Melgar et al. (2005) and Cooper et al. (1993), respectively. See text for details of scoring.

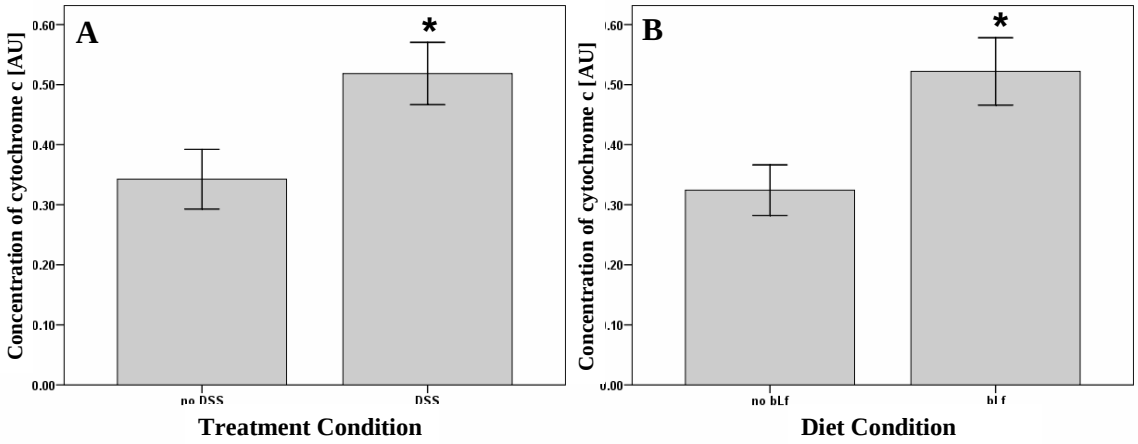


Figure 6.2: Protein levels of cytochrome c, expressed in arbitrary densitometric units, in mouse IL as a function of [A] DSS condition (*$p<0.05$) and [B] diet (bLf) treatment (*$p<0.01$). Values are means ± SEM. Refer to the text for details of statistical analysis.

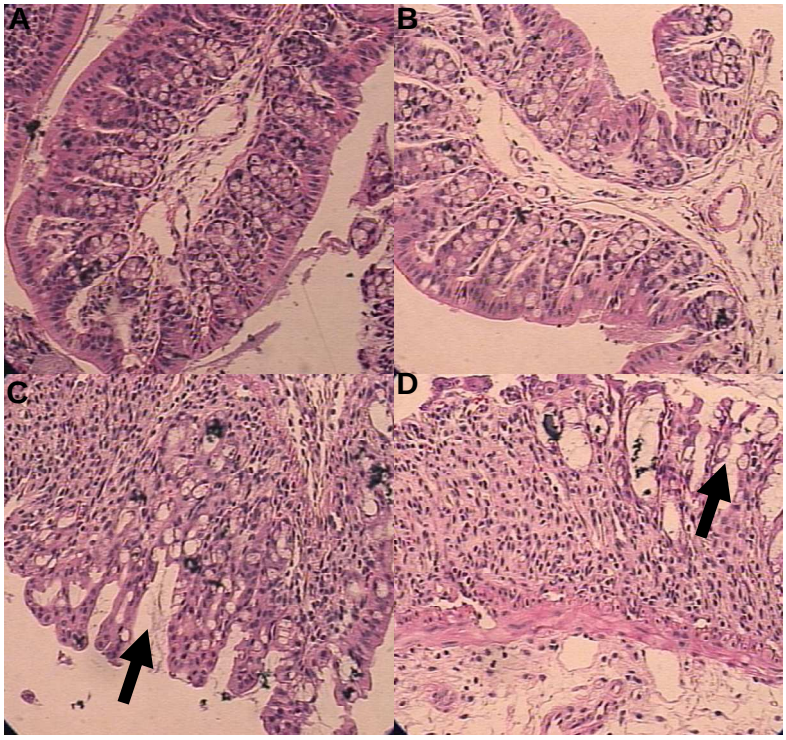


Figure 6.3: Representative hematoxylin and eosin (H & E) stained sections of mouse large intestine viewed by light microscopy under 40x magnification. All tissue sections are from mice given DSS and bLf treatments. A: DSS for no bLf/ no DSS, B: bLf/ no DSS, C: no bLf/DSS, D: bLf/DSS. Arrows indicate crypt disruption and areas of inflammatory pathology.

Study II: AE and bLf on Cytochrome c Levels in Mouse IL: Neither exercise nor diet influenced the expression of cytochrome c in mouse IL (SED: 0.78 ± 0.1 AU, IMM: 0.57 ± 0.06 AU, 24h POST: 0.68 ± 0.1 AU; bLf: 0.64 ± 0.06, no bLf: 0.73 ± 0.1). There were no interactions between acute exercise and diet on mouse IL cytochrome c levels.

Study II: AE and bLf on Mouse Intestinal Morphology: As shown in Table 6.1 and in representative histology (Figure 6.5), there was no evidence of pathological changes in either the small or large intestine as measured by crypt disruption or histology scores due to acute exercise exposure. Dietary bLf had no effect on the inflammatory indicators either alone or in combination with AE (data for AE x bLf are shown in Table 6.2).

Study II: AE and bLf on 8-iso-Prostaglandin $F_{2\alpha}$. There was a significant main effect of exercise on plasma concentrations of 8-iso-$PGF_{2\alpha}$ ($F_{2,63} = 6.94$, $p<0.01$) (SED: 318.1 ± 97.6 pg/ml, IMM: 856.4 ± 23.8 pg/ml, 24 h POST: 1024.3 ± 146.8 pg/ml). This was due to elevated levels in both the IMM ($p<0.05$) and 24 h POST ($p<0.01$) group relative to the SED control. Neither a significant diet effect nor interaction effect were observed. There was, however, a non-significant 20% reduction in plasma levels of 8-iso-$PGF_{2\alpha}$ in mice fed bLf compared to those on bLf free diets (no bLf: 831.9 ± 125.0 pg/ml, bLf: 663.8 ± 105.4 pg/ml).

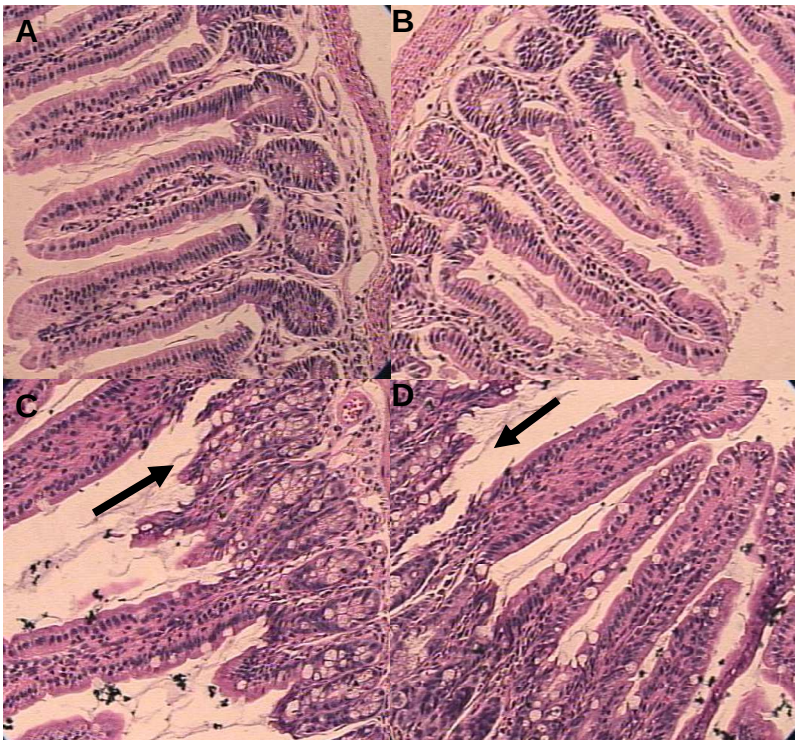


Figure 6.4: Representative hematoxylin and eosin (H & E) stained tissue sections of mouse small intestine viewed by light microscopy under 40x magnification. All tissue sections are from mice given DSS and bLf treatments. A: no bLf/ no DSS, B: bLf/ no DSS, C: no bLf/DSS, D: bLf/DSS. Arrows indicate crypt disruption and areas of inflammatory pathology.

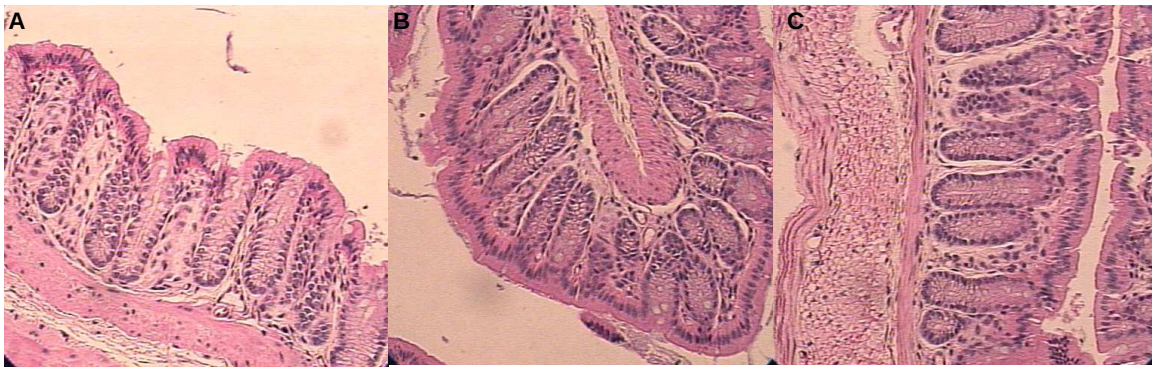


Figure 6.5: Representative hematoxylin and eosin (H & E) stained tissue sections of mouse large intestine viewed by light microscopy under 40x magnification. All tissue sections are from mice given repeated bouts of acute exercise. A: SED, B: IMM, C: 24 h POST. Only the no bLf treatment groups are shown.

6.8. Discussion

The primary objective of this research was to examine the effects of AE on mouse small and large intestinal inflammation as evidenced by histological changes and if these alterations were similar to those caused by DSS. We also determined if bLf attenuates any of these potential alterations. A second aim was to determine if DSS or AE, coupled with bLf, influenced the levels of the cell death associated protein cytochrome c, as a marker for lymphocyte mitochondrial disruption. A novel finding was that both DSS and bLf treatments increased the expression of cytochrome c in mouse IL. DSS treatment resulted in gross histological changes within the mouse small and large intestines and these effects were not observed in animals subjected to repeated bouts of treadmill exercise.

Administration of 5% DSS for 4 consecutive days in the drinking water was associated with marked structural alterations and increases in inflammatory and crypt scores within the large intestine. We observed an increased presence of inflammatory cells as well as mucosal ulcerations and edema following DSS treatment. These pathophysiological changes in the colon have been reported by others. For example, Takizawa et al. (1995a; 1995b) showed increased inflammatory cells coupled with the development of mucosal erosions 4 days following 3% DSS in the colon of rats. Body weights of female C57BL/6 mice were reduced at day 4 and evidence of infiltration of inflammatory cells occurred as early as day 3, with crypt loss, edema and focal ulcerations present at day 5 following 3% DSS treatment (Melgar et al., 2005). Our study confirms the inflammatory effects of DSS in the colon and extends these observations to

the mouse small intestine but to a lesser degree. Ohtsuka and Sanderson (2003) observed increases in neutrophils, confirmed by increased myeloperoxidase activity, in the small intestine of DSS treated mice. Administration of DSS increased intestinal disease indicators in mice with shortening of intestinal length, visual rectal bleeding, and decreased body weight (Spagnuolo and Hoffman-Goetz, 2008b). We found no evidence of an attenuation of DSS-induced structural alterations in mice receiving bLf. This lack of bLf effect was not entirely unexpected as the potentially protective anti-inflammatory benefits (i.e., cytokine alteration) may not have been sufficient to counter the numerous pathways through which DSS induces disease (disruption of intestinal barrier, recruitment of immune cells, changes in microflora, cytokine alterations). These results provide evidence of small and large intestinal alterations, indicative of inflammatory tissue damage, in mice subjected to DSS treatment and, further, of no effect of 2.0% dietary bLf supplementation on these tissue alterations.

Repeated bouts of intense exercise in mice were associated with increased oxidative stress as evidenced by the elevated plasma concentrations in 8-iso prostaglandin $F_{2\alpha}$. This lipid metabolite is a marker of exercise induced oxidative stress (Hoffman-Goetz and Quadrilatero, 2003; Mastaloudis et al., 2004; Steensberg et al., 2002). Although oxidative stress was induced by the AE protocol used in this study, there were no gross histological alterations indicative of inflammation within the small and large intestine. This finding is in contrast to that of Rosa et al. (2008) who reported that following 4 d of exhaustive treadmill running morphological alterations within the terminal ileum of mice were observed; these alterations included reduced muscular layer thickness and mild increases of cellular infiltrates. The reasons for this difference in

outcomes between the two studies are not known but may reflect a less strenuous exercise regimen reported here compared to that of Rosa and colleagues. That the AE in the current study was less strenuous is suggested by: 1) fewer number of exercise bouts (3 vs. 4), 2) differences in the durations of each bout (90 min. continuous exercise vs. durations based on times to exhaustion), and 3) lower intensity of the physical work (68%, 74% and 80% VO_2 peak at 22 $m \cdot min^{-1}$, 25 $m \cdot min^{-1}$, and 28 $m \cdot min^{-1}$ in the present study [Quadrilatero and Hoffman-Goetz, 2005] vs. 85% of maximal running velocity). These results suggest that aerobic exercise, at the intensity, duration and frequency used in this study, does not cause alterations indicative of intestinal inflammation.

DSS treatment resulted in increased cytochrome c levels in mouse IL and implies that DSS exposure results in cell death of lymphocytes within the intestine. This result is consistent with previous findings that DSS increases cell death, characteristic of apoptosis and necrosis, as evidenced by the increases in the % of PI^+/ANN^- (necrotic), % of PI^+/ANN^+ (late apoptotic), and protein levels of the pro-apoptotic caspase 3 in mouse IL (Spagnuolo and Hoffman-Goetz, 2008b). Apoptotic and necrotic death of mouse intestinal epithelial cells (IEC) by DSS has been demonstrated (Renes et al., 2002; Vetuschi et al., 2002) and given the anatomic proximity of IL to IEC, it is not surprising that similar mechanisms of death in IL also occur. Thus, the published evidence indicates that DSS treatment results in IL death and this current study extends the observation to demonstrate that cytochrome c levels (and presumably mitochondrial dysfunction) increase in mouse IL with DSS exposure.

Conversely, three bouts of intense treadmill exercise, each separated by a 24 h rest period, did not increase cytochrome c levels in mouse IL and is further support that

this exercise protocol does not impart similar inflammatory alterations to that of DSS. This exercise modality, however; does result in greater Annexin V expression and fewer IL numbers indicative of apoptotic cell death (Spagnuolo and Hoffman-Goetz, 2008a, Hoffman-Goetz and Spagnuolo 2007a). Expression of phosphatidylserine (as indicated by Annexin V) on the outer leaflet of the cell membrane is an early marker of apoptosis which precedes both the release of cytochrome c and the decrease in mitochondrial transmembrane potential (Denecker et al., 2000). Thus, a finding of no increase in cytochrome c levels in mouse IL following AE does not exclude apoptotic pathways independent of mitochondrial disruption (e.g., lysosomal, ER and TNF receptor mediated) (Jaatela and Tschopp, 2003; Hoffman-Goetz et al., 2005) or the kinetics of cytochrome c release relative to phosphatidylserine expression on IL with AE challenge. It is also possible that cytochrome c was complexed within the apoptosome and not readily detected by Western blot analysis.

Dietary bLf was associated with elevated expression of cytochrome c in mouse IL indicative of increased cell death. We have shown that bLf increases apoptotic indicators in mouse IL including decreased concentrations of Bcl-2 (Spagnuolo and Hoffman-Goetz, 2008a) and increased levels of Annexin V (Spagnuolo et al., 2007). Others (Sakai et al., 2005; Yoo et al, 1997) suggest bLf or its metabolites favour cell death. Mader et al. (2005; 2007) demonstrated that bLfcin induces apoptosis of Jurkat T cells through alterations in cell membrane integrity and increased mitochondrial permeability which was associated with increased cytochrome c release. Thus, the findings suggest that bLf may promote cytochrome c release and subsequent cell death through modulation of

mitochondrial membrane permeability. The physiological relevance of bLf as a potential inducer of apoptosis of IL (or IEC) has not been determined.

6.9. Conclusion

In summary, three bouts of intense treadmill exercise in mice did not result in inflammatory damage in small or large intestine as indicated by tissue histology. AE was also not associated with differential expression of cytochrome c in mouse IL. In contrast, 4 days of exposure to 5% DSS in the drinking water of mice produced gross histological alterations within the large intestine and minor changes within the small intestine. Cytochrome c levels in IL were higher following DSS treatment and support our previous observations that DSS treatment results in IL death. Finally, although bLf did not attenuate the DSS-induced histological alterations in mouse intestine, there is a possibility that this protein can promote IL death since cytochrome c levels were elevated following dietary exposure. The clinical relevance of dietary bLf in modulating cell death of lymphocytes in the intestine during normal and pathophysiological states remains to be determined. Nonetheless, given that lactoferrin did not protect against intestinal pathology following exposure to DSS, dietary bLf should probably not be recommended as a supplement at this time for athletes experiencing intestinal distress.

Chapter 7: Dietary Bovine Lactoferrin Does Not Prevent Aberrant Crypt Formation in Mice After Dextran Sulfate Sodium and Acute Exercise Challenge

7.1. Objectives

To determine the effects of dietary bovine lactoferrin (bLf) on aberrant crypt formation and on levels of the apoptotic proteins caspase 3 and Bcl-2 in intestinal lymphocytes of female C57BL/6 mice injected with the carcinogen azoxymethane (AOM) and following DSS or AE challenge.

7.2. Hypotheses

Hypothesis 1: bLf administration will decrease the number of aberrant crypts in AOM injected mice subjected to DSS or AE relative to the number of aberrant crypts in mice not receiving bLf supplementation.

Hypothesis 2: bLf administration will increase levels of caspase 3 and decrease levels of Bcl-2 in mice given DSS or AE in conjunction with the carcinogen AOM relative to the levels of caspase 3 and Bcl-2 in mice not receiving bLf supplementation.

7.3. Study Design

Inflammation has been demonstrated to be a promoter of colon carcinogenesis (Tanaka et al., 2000; Onose et al., 2003). Given the ability of dietary bLf to influence both apoptotic and cytokine measures following DSS and AE challenge (Chapters 4, 5, 6), it was the aim of this study to determine if this anti-inflammatory benefit was associated with direct mucosal protection against colon carcinogenesis. This study was thus designed to determine the possible protective benefit of dietary bLf on the formation of pre-cancerous lesions (i.e., aberrant crypts) in the colon following both inflammatory insult and tumour initiation events (i.e., AOM injection). The study design for experiment 5 is outlined in Figure 7.1.

Study duration: Two week acclimation, two week injection period, two week rest period, plus 12 d of dietary bLf administration.

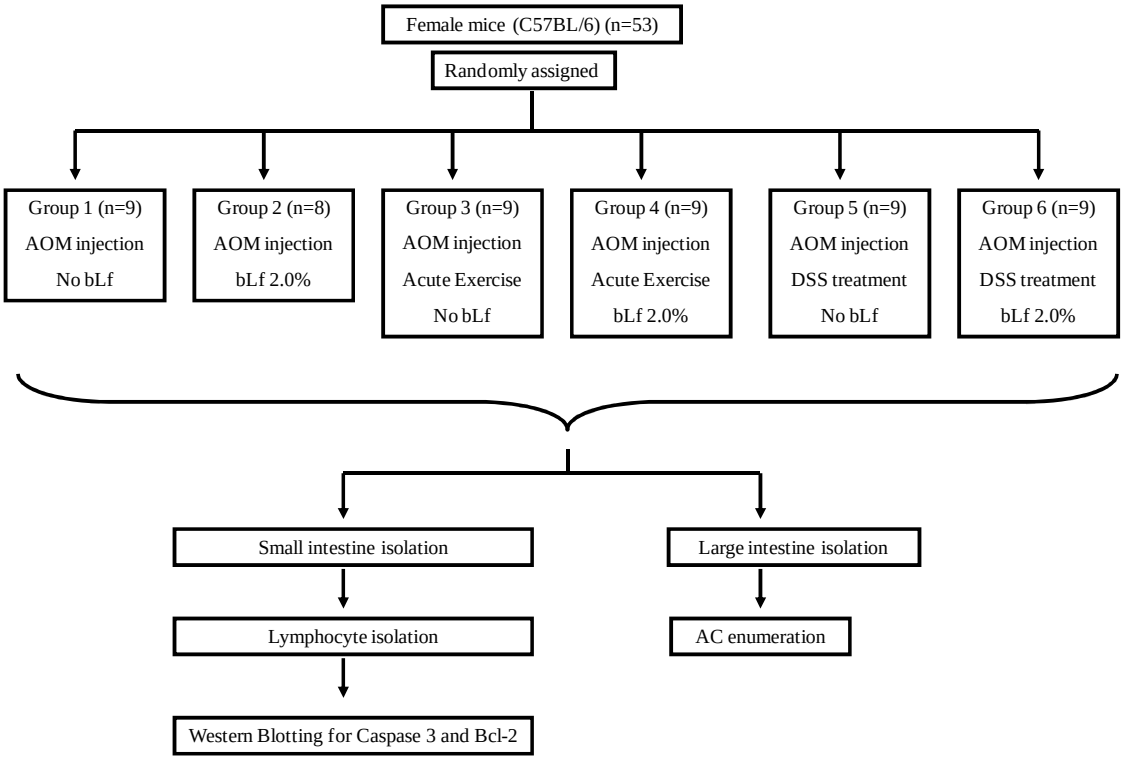


Figure 7.1: Schematic outline of the study design for experiment 5.

A two way ANOVA (2x3 factorial design) and post-hoc Tukey tests were used to analyze the study data. In this study the independent variables were diet condition (0% bLf or 2.0% bLf) and treatment condition (control, DSS, AE). Dependent variables include number of aberrant crypts, spleen weight, intestinal length, and animal weight prior to sacrifice. Pre-planned comparisons were run on IL apoptotic protein levels of caspase 3 and Bcl-2 as well as on IL counts, given the direction of effects on those outcome measures demonstrated in earlier experiments with DSS and/or AE.

7.4. Overview

Colonic inflammation increases aberrant crypt (AC) formation, pre-neoplastic lesions which may progress into tumours. We have shown that bovine lactoferrin (bLf) has small anti-inflammatory properties following dextran sulfate sodium (DSS) or acute exercise (AE) challenge in mice. The purpose of this study was to examine the effects of bLf in azoxymethane (AOM) injected mice given DSS or AE on AC formation and intestinal lymphocyte (IL) death. Female (n=53) C57BL/6 mice injected with AOM were randomized into two dietary conditions (no bLf, bLf) followed by allocation to three treatment conditions (Control, AE or DSS). AC were determined by light microscopy in colon samples and IL isolated, enumerated and apoptotic protein levels determined by Western blotting. DSS, but not AE, increased AC formation ($p<0.001$) and bLf had no effect on AC numbers. bLf did not affect the loss of IL which occurred in DSS ($p<0.01$) and AE ($p<0.05$) mice nor did it prevent the increase in IL caspase 3 levels following DSS or AE ($p<0.05$). The results suggest that dietary bLf, at the dose and exposure duration used in this study, does not protect the mucosa from AC formation or influence IL loss following AOM injection coupled with DSS or AE challenge.

7.5. Introduction

Colon carcinogenesis is a complex process characterized as three distinct stages: initiation, promotion and progression (Trosko, 2006). Experimentally, initiation can be induced by injection with colon specific carcinogens, such as 1,2-dimethylhydrazine (DMH) or azoxymethane (AOM). The earliest detectable measure of colon carcinogenesis is the formation of aberrant crypts (AC) (Bird, 1987). AC differ from normal crypts by exhibiting altered width and height, increased thickness of epithelial lining, and irregular luminal openings (Bird, 1995). The enumeration of AC has been used as a biomarker of early colonic tumour development (Mori et al., 2005).

Inflammation is believed to be a promoter of tumourigenesis. Animal studies show that following initiation by a carcinogen, inflammation promotes the formation of AC and tumour development. Onose et al. (2003) described increased tumour incidence in rats given DMH (initiation) followed by an inflammatory agent, dextran sulfate sodium (DSS) (promotion), compared to non-DSS rats. Increased AC in rat colon following AOM and DSS treatment was observed compared to animals given AOM without DSS (Tanaka et al., 2003). To further emphasize inflammation as a promoter of carcinogenesis, anti-inflammatory agents were able to reduce AC and tumour growth. For example, fewer aberrant crypt foci (ACF), which are clusters of AC, were observed following AOM and DSS treatment in mice given three different peroxisome proliferator-activated receptor ligands compared to mice receiving AOM and DSS alone (Tanaka et al., 2001).

Intestinal inflammation can be induced in rodents by adding DSS in the drinking water. This causes death of intestinal lymphocytes (IL) (Spagnuolo and Hoffman-Goetz, 2008b), activation of immune cells within the colon (Takizawa et al., 1995a; 1995b), increased concentrations of pro-inflammatory cytokines (i.e., TNF-α) (Egger et al., 2000) and increased free radicals (Naito et al., 2003). Another modality which may produce inflammation in the bowel is intense exercise. Acute exhaustive exercise (AE) results in some physiological changes similar to DSS-induced inflammation; these include increased oxidative stress (Hoffman-Goetz and Spagnuolo, 2007b), increased pro-inflammatory cytokine levels in the intestine (Hoffman-Goetz et al., 2008), IL death (Spagnuolo and Hoffman-Goetz, 2008a) and increased inflammatory cells within the intestine (Rosa et al., 2008). Whereas DSS promotes AC and tumour formation, it is not known whether intense AE promotes colon carcinogenesis.

IL function as effector and regulatory cells and control over their apoptosis and proliferation is critical for homeostasis. We have shown earlier that mouse IL undergo cell death following DSS (Spagnuolo and Hoffman-Goetz, 2008b) and AE challenge (Spagnuolo and Hoffman-Goetz, 2008a) that is characterized by an increased percentage of apoptotic IL, increased levels of the pro-apoptotic protein caspase 3 and decreased expression of the anti-apoptotic protein Bcl-2. Both DSS and AE may contribute to increased cell death in the intestine, but the impact on IL when administered as a tumour "promoter" (i.e., following carcinogen injection) has not been characterized.

Bovine lactoferrin (bLf), an 80 kDa dietary protein with anti-inflammatory properties, is found in mammalian milk, colostrums and dairy products. Dietary administration of bLf reduces the expression of the pro-inflammatory cytokine tumour

necrosis factor-α (TNF-α) in mouse intestine (Spagnuolo et al., 2007). Dietary bLf also has a small but significant effect on apoptotic protein expression (e.g., decreased Bcl-2) and a larger effect on pro-inflammatory markers (e.g., reduced TNF-α and NFκB) in mouse IL following DSS (Spagnuolo and Hoffman-Goetz, 2008a) and AE (Spagnuolo and Hoffman-Goetz, 2008b) challenge. There is also evidence of a potential anti-carcinogenic role of bLf, as Sekine et al. (1997a) reported fewer numbers of AC in rat colon following AOM injection and 4 weeks of bLf supplementation. The role of bLf in influencing inflammatory and carcinogenic events when both are applied independently (i.e., DSS alone; AOM alone) is clear from the literature. However the effects of bLf on AC formation when promotional events (DSS or AE) are used in conjunction with tumour initiation (AOM) have not been evaluated experimentally.

The purpose of this study was to examine the effect of bLf on the formation of AC and IL apoptotic protein levels in mice following both tumour initiation (AOM injection) and promotion (DSS and AE) events. We hypothesized that DSS and AE would increase the number of AC and alter IL apoptotic proteins to favour cell death (increase caspase 3 and decrease Bcl-2); we further hypothesized that dietary bLf administration in mice would reduce AC numbers and influence (reduce or increased) IL death and apoptotic protein levels.

7.6. Materials and Methods

Animals: Female C57BL/6 mice (n=53) 3-4 weeks old (Harlan Sprague Dawley, Indianapolis, IN, USA) were individually housed at 21±1 ºC and on a 12-12h reversed light-dark cycle. Mice were acclimated to the vivarium for 2 weeks with *ad libitum*

access to tap water and maintenance diet (Laboratory Rodent Chow, PMI feeds Richmond, IN, USA). Following acclimation, mice had free access to specialized diets for 12 d prior to sacrifice.

Diets: Semi-purified AIN 76A standard diets were prepared 2 weeks prior to the start of the experiment and were formulated to contain 20% total protein including either 0% or 2.0% bLf (Erie Foods International Inc, Erie IL). Concentrations were based on previous studies showing that 2.0% bLf was sufficient to influence apoptosis as well as cytokine production (Spagnuolo et al., 2007; Spagnuolo and Hoffman-Goetz, 2008a; Spagnuolo and Hoffman-Goetz, 2008b). All protocols with live animals were approved by the University Animal Ethics Committee according to the principles of the Canadian Council on Animal Care.

Azoxymethane Injections: Mice were injected subcutaneously with AOM at 10mg/kg body weight, once a week for 2 weeks, followed by a 2 week rest period. Mice were then given free access to the specialized prepared diets with or without bLf (12 d); the induction period was 4 weeks in duration and this protocol was based on Sekine et al. (1997a).

DSS and Acute Exercise Protocol: Within diet conditions, mice were randomized to one of three treatment conditions: three sessions of acute treadmill running (each bout separated by a 24 h rest interval) with sacrifice 24 h after completion of the final exercise session (AE, n=18), or 5.0% DSS in the drinking water (n=17), or a sedentary/no DSS

control (CTL) (n=18). Thus, there were a total of 6 groups: no bLf/CTL (n=9); bLf/CTL (n=8); no bLf/AE (n=9); bLf/AE (n=9); no bLf/DSS (n=9); bLf/DSS (n=9). DSS (MP Biomedicals, Solon, OH; mol wt=36-50 kDa) was solubilized in tap water at room temperature and mice had *ad libitum* access to DSS containing drinking water for 4 d prior to sacrifice. The treadmill protocol consisted of 10 min warm-up, 90 min continuous exercise (30 min at 22 $m \cdot min^{-1}$, 2°slope; 30 min at 25 $m \cdot min^{-1}$, 2°slope; 30 min at 28 $m \cdot min^{-1}$, 2°slope) and 5 min deceleration. The work intensity is approximately 68%, 74% and 80% of VO_2 peak at 22 $m \cdot min^{-1}$, 25 $m \cdot min^{-1}$ and 28 $m \cdot min^{-1}$ (Quadrilatero and Hoffman-Goetz, 2005). Mice ran during the dark cycle (Omni-Max metabolic treadmill, Omni Tech Electronics, Columbus, OH) and were motivated by occasional gentle prodding using a soft nylon brush.

Aberrant Crypt Determination: Mice were sacrificed by sodium pentobarbital (0.06-0.08 ml) overdose, the colon immediately removed, cleaned from fecal matter and visible fat, opened longitudinally, fixed in formalin and stained with 0.2% methylene blue (Bird, 1987). Samples were masked to the microscopist during AC enumeration (Nikon Eclipse 50i digital imaging system).

Intestinal Lymphocyte Preparation: IL from mouse small intestine were isolated as previously described by Spagnuolo et al. (2007). Briefly, following sacrifice, the small intestine was immediately removed, Peyer's patches and visible fat dissected out, IL prepared as single cell suspensions by isolation over a column containing 0.3 g of pre-washed nylon wool, washed and layered over a density gradient medium (Lympholyte-

M; Cedarlane Laboratories, Hornby, Ont.). IL, containing both intraepithelial and lamina propria lymphocytes were enumerated manually by light microscopy following staining with Turk's solution.

Western Blot Analysis of Caspase 3 and Bcl-2: Cytoplasmic protein samples were prepared and separated by electrophoresis on a 12% SDS PAGE gel and transferred onto a PVDF membrane (Sigma Chemical). Following transfer, membranes were stained with Ponceau S to confirm the quality of transfer and equal protein loading. Membranes containing the separated proteins were incubated for 1 h with primary antibody (1:200 in 10% milk-TBST): Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG1, mol wt=28kDa) or caspase 3 (clone: H-227; rabbit anti-human clone IgG, mol wt=35kDa) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation for 1 h with secondary antibody: horseradish peroxidase-conjugated anti-mouse (Bcl-2) or anti-rabbit (caspase 3) IgG at a concentration of 1:2000 in 10% milk-TBST. Protein was determined using ECL Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging System (Cambridge, UK). Each gel contained samples from all 6 treatment groups along with a biotinylated protein ladder to identify the molecular weight of the immunoblotted protein.

Statistical Analysis: Cell counts and IL protein levels of caspase 3 and Bcl-2 were analyzed by pre-planned comparisons because earlier studies showed that these variables were altered by AE or DSS. The remaining outcome measures (inflammatory indicators, food intake, aberrant crypts) were analyzed using a 2x3 analysis of variance design

(ANOVA) with diet (two levels: no bLf, bLf) and treatment condition (three levels: CTL, AE, DSS) as the independent factors using SPSS (Version 15; Chicago, IL, USA). Post hoc analysis was performed using Turkey's HSD test and $p \leq 0.05$ was accepted as being significantly different from chance alone. All values are expressed as group means ± SEM.

7.7. Results

Inflammatory Indicators, Food Intake and IL Counts: Animal weights at the start of the experiment (no bLf/CTL 21.0 ± 0.02g; bLf/CTL 20.9 ± 0.5g; no bLf/AE 21.6 ± 0.4g; bLf/AE 20.9 ± 0.4g; no bLf/DSS 20.5 ± 0.3g; bLf/DSS 20.8 ± 0.6g) did not differ significantly across groups. In contrast, there was a significant treatment effect prior to sacrifice ($F_{2,45} = 17.04$, $p<0.0001$) due to the reduced body weights of mice given DSS compared to CTL and AE mice (Table 7.1). There was no effect of diet nor was there an interaction between diet and treatment on mouse weight at sacrifice. Intestinal length was reduced ($F_{2,45} = 8.75$, $p<0.001$) and spleen weight was increased ($F_{2,42} = 10.36$, $p<0.0001$) in mice given DSS relative to CTL and AE mice (Table 7.1). Dietary bLf had no effect on the length of the intestinal compartment (small and large intestine) or spleen weights of mice. Neither diet nor interaction effects were observed on food intake but there was a significant main effect of DSS, AE or CTL treatment ($F_{2,47} = 5.71$, $p<0.01$). This was due to the reduced food intake in the DSS compared to the AE mice and not between the DSS vs. CTL or the AE vs. CTL mice (CTL 43.0 ± 2.8 g; AE 50.9 ± 1.96 g; DSS 39.4 ± 2.5 g, Tukey's test, $p<0.01$). IL counts were also significantly affected by treatment (Figure 7.2). Mice given DSS ($t_{22} = 2.57$, $p<0.01$) or AE ($t_{21} = 1.98$, $p<0.05$) had reduced cell

numbers compared to CTL animals. There was no significant effect of diet (bLf: 4.0 ± 0.43 x10^7 cells vs. no bLf: 3.8 ± 0.43 x 10^7 cells) nor was there any interaction effect.

Apoptotic Protein Levels in IL: Figure 7.3 Panel A shows the effect of treatment condition on Bcl-2 levels in mouse IL. There was a significant decrease in Bcl-2 protein levels (t_{15} = 2.43, $p<0.05$) in the DSS group relative to the CTL; no significant difference was observed between the CTL and AE groups. The effect of treatment condition on the levels of caspase 3 in mouse IL is shown in Figure 7.3 Panel B. There were significant increases in IL levels of caspase 3 in the DSS (t_{15} = 2.40, $p<0.05$) and AE (t_{14} = -2.01, $p<0.05$) groups relative to CTL. There were no significant effects of bLf on Bcl-2 (bLf: 2.1 ± 0.34 [AU] vs. no bLf: 2.2 ± 0.35 [AU]) or caspase 3 (bLf: 0.84 ± 0.05 [AU] vs. no bLf: 0.85 ± 0.028 [AU]) levels in IL; there were no significant interaction effects between treatment and diet on apoptotic proteins in mouse IL.

Table 7.1: Acute exercise (AE) and dextran sulfate sodium (DSS) effect on indictors of intestinal inflammation

Treatment Condition	Animal End Body Weight (g)	Small and Large Intestinal Length (cm)	Spleen Weight (g)	Intestinal Bleeding
Control	22.1 ± 0.4	45.0 ± 0.6	0.08 ± 0.005	No
AE	22.7 ± 0.3	44.6 ± 0.5	0.08 ± 0.002	No
DSS	19.1 ± 0.6**	41.6 ± 0.8*	0.11 ± 0.010$^{\#}$	Yes

AE = Acute exercise, DSS = dextran sulfate sodium. Tukey's post hoc analysis: ** $p<0.001$, *$p<0.005$, $^{\#}p<0.002$.

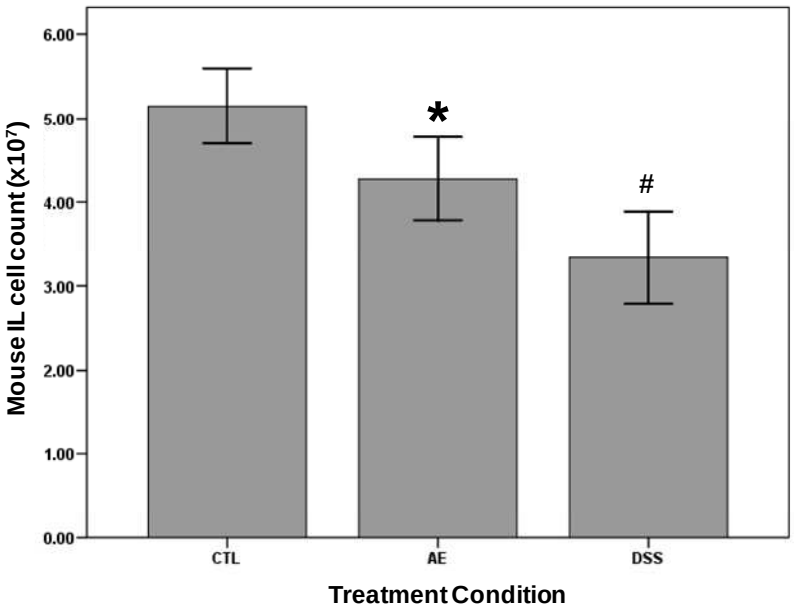


Figure 7.2: Mouse IL count as a function of treatment (CTL, AE, DSS) condition. All values are expressed as means ± SEM. *p <0.05, #p<0.01 vs. control. See text for details of statistical analysis.

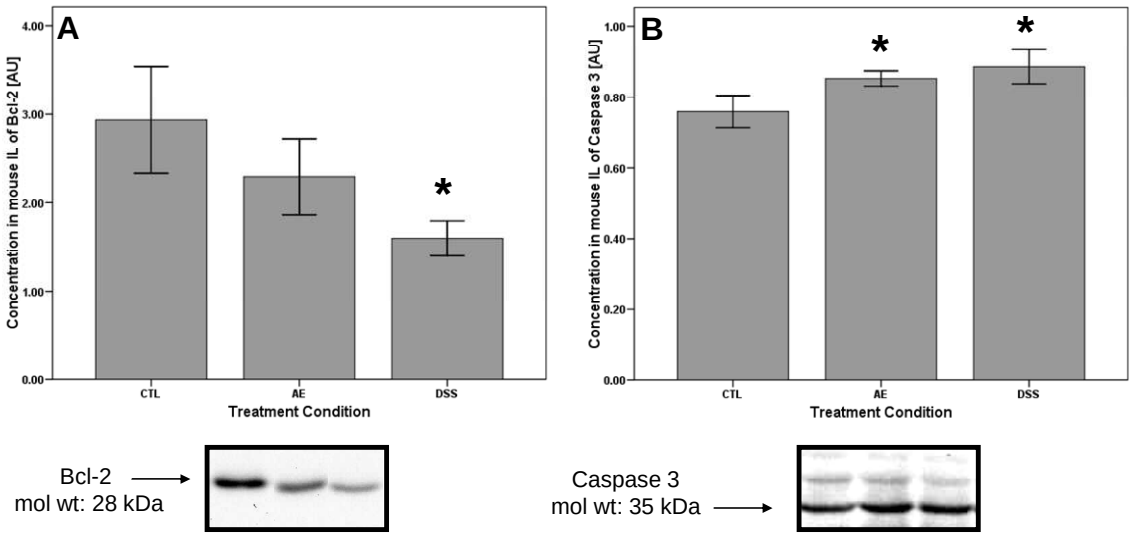


Figure 7.3: Concentrations of apoptotic proteins in mouse IL expressed as arbitrary densitometric units (AU). [A] anti-apoptotic Bcl-2 and [B] pro-apoptotic caspase 3. All values are expressed as means ± SEM. *p <0.05 vs. control. See text for details of statistical analysis.

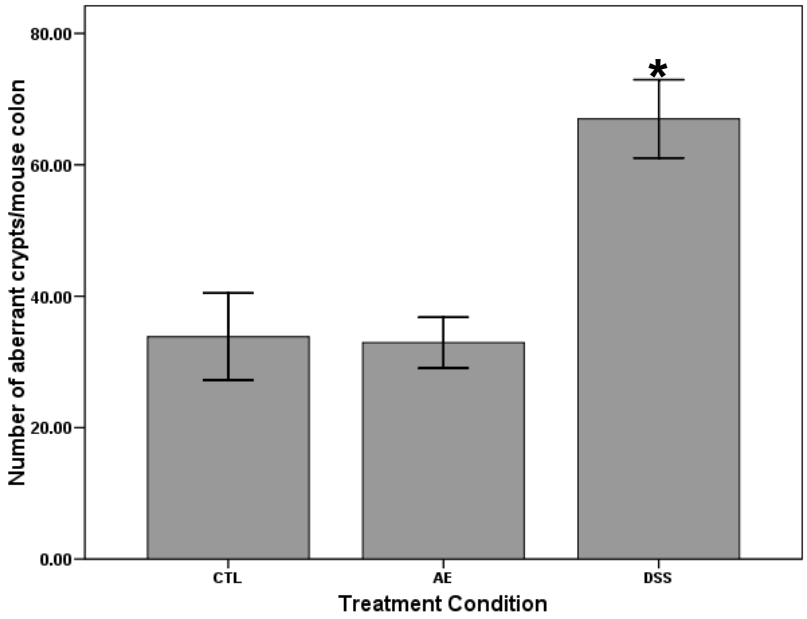


Figure 7.4: Number of aberrant crypts (AC) per mouse colon as a function of treatment (DSS, AE or CTL) condition. All values are expressed as means ± SEM. *p<0.005 vs. CTL and AE.

Aberrant Crypts: Figure 7.3 shows the number of AC in the CTL, AE and DSS treatment conditions. There was a significant main effect of treatment ($F_{2,31}$ = 9.21, $p<0.001$) with mice given DSS having a greater number of AC ($p<0.005$) relative to AE and CTL mice. There was no effect of bLf on AC numbers ($F_{1,31}$ = 0.37, p=0.55) (bLf: 43.9 ± 5.8 vs. no bLf: 42.7 ± 5.6) nor was there any interaction between diet and treatment conditions ($F_{2,31}$ = 0.75, p=0.48).

7.8. Discussion

The objectives of this study were to examine the effects of dietary bLf in mice following AOM injection in conjunction with either DSS or AE treatment on: 1) AC formation and 2) apoptotic protein levels in IL. Since we had previously demonstrated that bLf influences apoptotic and cytokine parameters in the IL of healthy mice and in mice exposed to agents (DSS) or protocols (AE) which may promote intestinal inflammation, we were interested in whether the anti-inflammatory properties translated into direct mucosal protection in a model of colon carcinogenesis (AOM + DSS or AOM + AE). The results of this study indicate that DSS, but not AE, increased the number of AC in mouse colon and that, contrary to our hypothesis, bLf did not prevent this increase nor did it reduce total AC numbers as a function of treatment. Dietary bLf did not affect IL loss or levels of pro-apoptotic protein, caspase 3, which accompanied DSS and AE challenge in mice.

Mice given DSS in drinking water and injected with AOM had increased numbers of AC, a finding consistent with other investigations (Tanaka et al., 2003; Onose et al., 2003). The increased numbers of AC with DSS exposure were likely due to

inflammation. Similar to the findings of others (Cooper et al., 1993; Melgar et al., 2005), we observed that DSS increased spleen weight and intestinal bleeding, decreased body weight at sacrifice, and reduced intestinal length of mice. Dietary bLf did not mitigate these disease indicators nor did bLf attenuate the DSS-induced increases in AC formation. Our results indicate that dietary bLf does not protect the intestinal mucosa from AC formation following tumour initiation (AOM injection) and promotion (DSS treatment) events. This finding was not entirely unexpected: at similar doses (2.0% for 12 d) and during only DSS treatment, dietary bLf reduced the expression in mouse IL of TNF-α and NFκB but did not protect the intestine from DSS-induced damage (Spagnuolo and Hoffman-Goetz, 2008b). Therefore, the inability of bLf to reduce AC formation may reflect the intensity of inflammation following DSS exposure. In contrast, Togawa et al. (2002a) reported that bLf administration reduced levels of pro-inflammatory cytokines (TNF-α, IL-1β) and provided intestinal mucosal protection following DSS treatment. Although the reasons for the difference between our results and those of Togawa et al. (2002a) are not known, factors such as dose of bLf (~800-1000mg vs. 1400mg), mode of administration (dietary exposure vs. gastric intubation), rodent model (female C57BL/6 mice vs. male Sprague-Dawley rats) and concentration and duration of exposure to DSS in the drinking water (5% DSS for 4d vs. 2.5% for 7d) may be involved. More importantly, the current study included tumour initiation events (AOM injection) as well as inflammation (DSS treatment) whereas Togawa et al. (2002a) used a protocol which did not involve tumour initiation. Indeed, the increased physiological stress provided by both tumour initiation and promotion may account for the inability of bLf to protect the intestinal mucosa. There is also a dose-dependency of bLf to affect cellular parameters

(Buccigrossi et al., 2007) with higher doses resulting in greater bLf effects. In addition, greater concentrations of DSS exposure produced larger inflammatory stress as evident by elevated pro-inflammatory cytokine production and increased intestinal damage (Egger et al., 2000). Together these findings imply that the greater the magnitude of the inflammation producing exposure, the higher the bLf dose that will be required. Future studies will be needed to determine if a dose response relationship occurs between bLf and DSS exposure in mice given a colonic carcinogen such as AOM.

Administration of dietary bLf did not reduce AC formation following AE challenge (no bLf/AE vs. bLf/AE). Similar to DSS treatment, the potential benefits provided by bLf may be insufficient to compensate for the multiple alterations associated with acute intense exercise (e.g., ischemia/reperfusion injury, increased pro-inflammatory cytokines, and increased free radicals). Although bLf administration favours the expression of anti-inflammatory cytokines following AE challenge, there was no decrease in plasma lipid metabolites or influence on IL loss (Spagnuolo and Hoffman-Goetz, 2008a). Thus, bLf administration may not be associated with direct mucosal protection at least at the concentration and exposure duration provided in this study. Nevertheless, we observed a non-significant 38% decrease in AC numbers in CTL mice given bLf compared with no bLf mice (no bLf/CTL: 40.1 ± 10.8 and bLf/CTL: 25.0 ± 3.2). Our finding is in contrast to Sekine and colleagues (1997a) who found that 2.0% bLf reduced AC formation in rats treated with AOM. A key difference between these studies is that one involved both tumour initiation and promotion events and the other involved only initiation events. The additional stress applied by the promotional event may have overwhelmed any small protection provided by bLf. Other factors include differences in

bLf exposure (28 d vs. 12 d), the animal model used (male F344 rats vs. female C57BL/6 mice) and the dose of carcinogen (15 vs. 10 mg AOM/kg body weight).

An important and novel finding was that mice given repeated AE stress did not have increased numbers of AC. Intense exercise stress is associated with several physiological events which could potentially increase AC formation such as increased muscle damage (Aoi et al., 2004), increased pro-inflammatory cytokines (Pedersen et al., 1998), ischemia-reperfusion injury (Moses, 2005), and greater oxidative stress (Quadrilatero and Hoffman-Goetz, 2005). That there were no increases in AC numbers in mouse colon suggests that AE, at least in the intensity, duration and frequency administered in this study, does not lead to alterations in the bowel which promote AC formation. The exercise protocol used in this study did result in IL loss and increased IL levels of pro-apoptotic caspase 3, and was identical to previous studies showing increased plasma levels of corticosterone and lipid metabolites as well as increased HSP 70, SOD1, caspase 3 and decreased Bcl-2 in mouse IL (Hoffman-Goetz and Spagnuolo, 2007a; Spagnuolo and Hoffman-Goetz, 2008a). However, these physiological alterations were not sufficient to increase AC formation and therefore, AE does not act as a promoter of colon carcinogenesis. In support of this notion are the results which indicate that AE does not induce intestinal inflammation at a magnitude comparable to that induced by DSS. In fact, AE had no significant impact on any indicators of disease activity (e.g., mouse body weight at sacrifice, intestinal length) despite the impact on IL numbers and apoptotic protein expression. However, that repeated bouts of AE did not promote AC formation does not exclude AE as potentially contributing to AC or tumour formation. For example, Demarzo and Garcia (2004) found increased ACF in male rats when

exhaustive swim exercise was provided prior to AOM injection. Hoffman-Goetz et al. (1994) found that timing of exercise training (before vs. after injection with the MMT 66 line) affected the number of lung tumour metastases in Balb/c mice. Thus, the timing of exercise challenge may be crucial to the onset of AC and/or tumour formation. In further support of this hypothesis are the following observations: 1) cholic acid administered during the initiation phase, but not post initiation, increased ACF formation and tumour incidence (Baijal et al., 1998) and 2) DSS treatment that proceeded AOM injection resulted in 100% tumour incidence compared to 0% when DSS preceded AOM injection (Tanaka et al., 2003). It is possible that AE challenge given prior to AOM injection may result in greater AC formation compared with AE given post AOM injection. Together these findings suggest that the timing of AE may be crucial in influencing the formation of AC although this remains to be tested empirically.

Following DSS or AE challenge and AOM injection in mice, there was evidence of IL death as levels of caspase 3 increased and overall IL numbers decreased. Levels in IL of the anti-apoptotic protein Bcl-2 were also reduced following DSS treatment. There was no observed effect on IL numbers or apoptotic proteins with bLf treatment in mice given AOM followed by DSS or AE treatment. Although dietary bLf was not associated with changes in these cell death measures, given the large physiological effects of DSS and AE, this lack of effect was not unexpected. In a non-AOM model, bLf did not influence IL death, or caspase 3 protein levels following DSS (Spagnuolo and Hoffman-Goetz, 2008b) and AE (Spagnuolo and Hoffman-Goetz, 2008a) exposures. However, there are also reports that bLf or its metabolites promotes both apoptosis (Mader et al., 2005; 2007) and proliferation (Buccigrossi et al., 2007). Nevertheless, the current study

shows that at a 2.0% dose for a duration of 12 d, dietary bLf did not affect IL death in mice given the carcinogen AOM followed by DSS or AE challenge.

7.9. Conclusion

In summary, although bLf has been previously shown to favour small anti-inflammatory responses following exposure to either DSS or AE, it was not able to attenuate DSS-induced increases in AC formation in mice. DSS but not AE challenge given after injection with AOM was associated with increased AC numbers in mouse colon. Dietary bLf did not affect IL death in mice subjected to either DSS or AE protocols following AOM injection nor did it alter protein levels of pro-apoptotic (caspase 3) and anti-apoptotic (Bcl-2) proteins. These findings indicate that the dose and duration of bLf provided in this study were not sufficient to protect against aberrant crypt formation in mouse colon. Further studies will be needed to determine if higher intakes or longer durations of bLf exposure affects AC formation in mouse colon following initiation with AOM and promotion with DSS.

Chapter 8: Discussion

8.1. Overall Findings

The purpose of the series of experiments described in this thesis was to determine the effects of dietary bovine lactoferrin (bLf) on cytokine levels and apoptosis in mouse intestinal lymphocytes (IL) in: 1) a normal, non-inflamed state, 2) following repeated bouts of acute exercise (AE), and 3) following dextran sulfate sodium (DSS) induced intestinal inflammation. An additional objective was to characterize the ability of bLf to protect the intestinal mucosa following DSS and AE challenge and on aberrant crypt formation when these two protocols were administered following carcinogen (AOM) injection. Overall, the results indicate that administration of dietary bLf in mice favours an anti-inflammatory cytokine environment mainly through reduced IL levels of TNF-α and NFκB. In addition, bLf decreased the levels of the anti-apoptotic protein Bcl-2, increased cytochrome c levels, and increased surface expression of phosphatidylserine in mouse IL suggesting a role for this dietary protein in the induction of apoptosis. Importantly, bLf did not protect the murine intestinal mucosa against DSS-induced structural alterations or the formation of aberrant crypts.

8.2. Induction of Intestinal Inflammation

The induction of intestinal inflammation was achieved by the addition of DSS to the drinking water of mice for 4 consecutive days. Evidence for the effectiveness of DSS exposure were alterations in several common indicators of disease activity that included

shortened intestinal length, increased spleen weight, intestinal bleeding and reduced body weights at time of sacrifice. Moreover, distinct characteristics of inflammation, such as the appearance of gross morphological alterations and the infiltration of leukocytes within the intestinal compartment were observed following DSS treatment. Administration of DSS to elicit intestinal inflammation is a widely utilized protocol (Strober et al., 2002) and was effective in the research described herein. Acute strenuous exercise has also been suggested as an inducer of intestinal inflammation (Marshall, 1998; Shek and Shephard, 1998) but the exact exercise regimen (i.e., speed, duration and frequency of administration) has not been experimentally determined. A protocol involving three repeated bouts of acute exercise was used, as others (e.g., Hoffman-Goetz and Spagnuolo, 2007a; Hoffman-Goetz et al., 2008; Heer et al., 1987; Concordet and Ferry, 1993; Nieman et al., 2005; Nieman et al., 2007a) have shown that repeated intense aerobic exercise in rodents or in humans is associated with increases in circulating leukocytes and inflammatory cytokines as well as apoptosis, cellular necrosis, and tissue pathologies ranging from thymus to intestine. However, the acute exercise regimen used in this research did not induce intestinal inflammation in mice. This conclusion is based on the findings that exercise did not result in tissue damage, immune cell infiltration, increases in aberrant crypts or cause any changes in the aforementioned disease indicators (i.e., shortened intestinal length, intestinal bleeding and reduced body weight at sacrifice).

Intestinal homeostasis is maintained by various regulatory mechanisms such as antioxidant defenses and anti-inflammatory cytokines (Papadakis and Targan, 2000). Exposure to stimuli that overcome either regulatory mechanism is one way by which intestinal inflammation may be induced. In support of this position are studies showing

that tissue damage caused by inflammation occurs concurrently with increases in either anti-inflammatory cytokines or antioxidants. For example, elevated concentrations of IL-10 mRNA coincided with increased disease activity and numbers of immune cells in the colon of mice treated with DSS (Egger et al., 2000). In athletes following a cycle race, lymphocyte concentrations of the antioxidant SOD were elevated along with increases in markers for oxidative stress and tissue damage (e.g., urate and malonaldehyde in plasma and carbonyl content in lymphocytes) (Tauler et al., 2006). The increases in cellular defenses are suggestive of a host adaptive mechanism whereby cellular responses to protect or limit against damage are initiated. When these adaptive responses are overwhelmed by external stimuli the result may be tissue damage. Conversely, an increase in either anti-inflammatory cytokines or antioxidants could limit the onset of inflammation (Niess et al., 1999). Findings from this thesis research indicate that the exercise regimen resulted in increased oxidative stress, as measured by increased plasma concentrations of 8-iso prostaglandin $F_{2\alpha}$, increased levels of IL-10 in mouse IL, and elevated IL levels of HSP 70, but exercise did not cause observable intestinal tissue damage or infiltration of immune cells. It is thus possible that the increases in IL-10 and HSP 70 that accompanied exercise were able to prevent any tissue damage that may have resulted from the increased oxidative stress.

The proposed hypothesis by which exercise induces inflammation includes intestinal tissue damage followed by bacterial translocation (Marshal, 1998; Shek and Shephard, 1998). Indeed, the presence of bacteria is suggested to be the stimulus necessary for the induction of inflammation (Strober et al., 2002). This is supported by observations that 1) under germ free conditions (i.e., without commensal flora) mice do

not develop intestinal inflammation (Elson et al., 2005), 2) antibiotic administration alleviates inflammation (Sartor, 2004) and 3) Crohn's disease in humans occurs in the colon and terminal ileum which has the highest bacterial content (Kelsall, 2008). Thus, the inflammation inducing protocol must be able to alter mucosal permeability in order to expose the commensal flora to the underlying immune network (Kelsall, 2008). The most direct method to increase intestinal permeability is to induce death of the intestinal epithelial cells (IEC) (Farhadi et al., 2003). DSS but not AE resulted in gross changes in intestinal histology and the recruitment of immune cells. This indicates that the exercise protocol used was not sufficient to cause any observable morphological damage to the intestinal barrier. This does not exclude the possibility that alterations in more subtle intestinal permeability function (e.g., altered antigen uptake or changes in junction zones) occurred following exercise. Future studies would be needed to explore this. However, because the exercise regimen did not cause observable physiological alterations in mouse intestine suggestive of inflammation (i.e., infiltration of immune cells) it is probably unlikely that damage to the epithelial barrier occurred. That the exercise regimen did not induce intestinal inflammation may be related to the inability of any exercise-associated stress to counter endogenous intestinal protective mechanisms. Future studies would be needed whereby the exercise regimen would be titrated to determine the duration, intensity and frequency of the work that are associated with IEC death and intestinal inflammation.

In further support of the conclusion that the exercise protocol used here did not result in intestinal inflammation is the observation that IL underwent cell death via apoptotic mechanisms. Following exercise, in mouse IL there was increases in pro-

apoptotic protein levels of caspase 3, decreases in anti-apoptotic protein levels of Bcl-2 in mouse IL, and reduced IL numbers, which together suggest apoptotic cell death. Indeed, apoptosis of lymphocytes following exhaustive or strenuous exercise is a common observation (Hoffman-Goetz and Quadrilatero, 2003; Mooren et al., 2002; Wang and Huang, 2005). The significance of the apoptotic process in lymphocyte removal is that it provides a controlled mechanism of cell death in which inflammation does not proceed (Verstege et al., 2006). If IL death following exercise were to occur via a necrotic pathway, as it was demonstrated to occur following DSS treatment [i.e., increased percent of Annexin V^-/PI^+ (necrotic)] then pathological inflammation would most likely ensue. Indeed, inflammation following necrotic death has been reported (Renes et al., 2002; Schulze-Bergkamen et al., 2006; Gibson, 2004). Thus, it was not surprising that the exercise regimen used in this study did not induce inflammation in the intestine as necrotic IL death with repeated treadmill exercise was not detected. This result is consistent with Mooren et al. (2002) who did not observed necrotic death in peripheral blood lymphocytes of volunteers subjected to exhaustive exercise. Since inflammation occurs following necrotic and not apoptotic death, DSS, and not AE induced intestinal inflammation would be expected.

Given the role of lymphocytes in intestinal mucosal defenses, such as tumour surveillance and protection against infection (Kunisawa et al., 2007) the loss of IL may have detrimental consequences for the individual. An alternative view is that IL loss (via apoptosis) following exercise is an adaptive mechanism by which the intestine protects against the development of inflammation. If, for example, exercise caused tissue damage that compromised the epithelial barrier, the result would be bacterial translocation and

subsequently the activation of the intestinal lymphocytes. Evidence for the involvement of lymphocytes in intestinal inflammation, is their presence in increased numbers in the inflamed mucosa of patients with IBD (Muller et al., 1998). Moreover, the mechanism of therapeutic action of sulfasalazine in IBD patients was by increasing apoptosis of intestinal lymphocytes (Doering et al., 2004; Mudter and Neurath, 2007). Therefore, it may be that by effectively removing lymphocytes through apoptotic processes prior to intestinal damage, any ensuing inflammatory response may be less damaging as fewer immune cells would be present to react to bacterial antigen.

8.3. Dietary bLf and Inflammatory Cytokines

The balance between pro- (T_H1) and anti- (T_H2) inflammatory cytokines is central to immunological homeostasis. During intestinal inflammation there is an increased production of pro-inflammatory cytokines that favours the activation and recruitment of immune cells. bLf has been shown to alter the cytokine milieu, including a decrease in TNF-α and an increase in IL-10, during inflammatory responses within the intestinal epithelium (Togawa et al., 2002a; 2002b). Similarly, decreased TNF-α and increased IL-10 were reported in the plasma of rats following bLf administration (Hayashida et al., 2004). Theoretically, the source of the cytokines could be from epithelial or immune cells located in muscle, the splanchnic bed, or lymphoid compartments (e.g., thymus, spleen, or intestine) (Bagby et al., 1996). However, whether the cytokine changes caused by bLf were as a result of effects directly on IL were not previously described in literature. Therefore, one objective of this thesis research was to examine the effects of dietary bLf on mouse IL levels of the inflammatory cytokines TNF-α and IL-10.

A novel finding of this research was that dietary bLf administration resulted in a decrease in TNF-α levels directly within mouse IL which may be related to the observed decrease in levels of NFκB. Since NFκB is a transcription factor partly responsible for the production of TNF-α, one mechanism to reduce TNF-α levels is by inhibiting the ability of NFκB to bind to DNA. Indeed, evidence from the literature suggests the inhibition of NFκB leads to reduced TNF-α production and that this may be due to altered DNA binding. For example; Haversen et al. (2002) reported that Lf binds to the TNF-α promoter region in monocytes, thus preventing NFκB-DNA interactions and reducing TNF-α production. A similar effect was found with gliotoxin, a potent NFκB inhibitor. Intraperitoneal injection of gliotoxin in mice treated with DSS decreased the expression of TNF-α and improved disease activity scores (Herfarth et al., 2000); the action of gliotoxin was attributed to its ability to decrease the DNA binding activity of NFκB (Li and Bever, 2001). Therefore, the bLf induced reductions in TNF-α may be related to decreased activity of NFκB potentially through modifying NFκB-DNA interactions.

Another potential mechanism by which TNF-α may be reduced is by directly interfering with NFκB activation. NFκB is bound to the IκB inhibitory protein in the cytoplasm and requires an external signal to liberate it from its inhibitor. The binding of TNF-α to the TNF-receptor (TNFR) results in the recruitment of TRAF2, a specialized adaptor protein, which participates in IKKβ activation. The activation of IKKβ in turn phosphorylates the IκB inhibitory protein and releases active NFκB (Baud and Karin, 2001). Thus, decreasing the extent of NFκB activation could occur by preventing TNF-α binding to the TNFR. Although there is evidence that bLf binds to a number of cell surfaces (reviewed in Legrand et al., 2006) there is no empirical data showing that bLf

binds to the TNFR. Alternatively, increasing the concentration or preventing the degradation of the inhibitory protein could also decrease active NFκB levels. Although no studies have directly demonstrated the ability of bLf to impart such properties, Minekawa et al. (2004) found that human breast milk increased IκB concentrations in intestinal epithelial cells. This resulted in decreased NFκB signaling. Thus increased IκB concentrations may be a mechanism by which NFκB levels (and thus TNF-α) are reduced by dietary bLf.

The effects of dietary bLf on IL-10 concentration in mouse intestinal lymphocytes were less clear. Although bLf administration was not associated with increased IL levels in healthy mice (i.e., experiment 1), IL-10 levels during DSS induced intestinal inflammation were reduced when mice were supplemented with bLf. Egger and colleagues (2000) suggested that elevated IL-10 mRNA with DSS treatment is a physiological compensation mechanism whereby the cell attempts to protect itself from the external stress. Thus, a reduction of IL-10 (as observed in this research with bLf administration during DSS treatment) may imply that bLf lessens the cellular requirement for a compensatory response. Indeed, similar effects were reported by Haversen et al. (2003) in which the numbers of $CD4^+$/IL-10 producing cells were decreased in the colon of DSS treated mice given human Lf. It is possible that the requirement of less IL-10 is related to the "anti-inflammatory" environment imparted by bLf. This is partially supported by the findings of this research that bLf decreased TNF-α levels in mouse IL. Lower levels of TNF-α may be indicative of a lessening of the inflammatory response. Therefore, less TNF-α may reduce the requirement for the T_H2 (anti-inflammatory) cytokine IL-10; IL-10 has been shown to decrease TNF-α levels (Papadakis and Targan,

2000). Although further work is needed to address the mechanism of this response, the results presented are, at least suggestive of, possible anti-inflammatory properties of bLf.

In summary, dietary bLf reduces TNF-α levels in IL and may lessen the compensatory (IL-10) host response to inflammation. The pathway by which bLf reduces TNF-α appears to be associated with decreased NFκB concentrations; whether the decreased levels of NFκB is by altering the ability of NFκB to bind to DNA or interfering with signals that activate NFκB remain to be elucidated. The finding that bLf biases toward anti-inflammatory responses make it an attractive therapy in the potential treatment of inflammatory disorders.

8.4. Dietary bLf, Intestinal Lymphocyte Apoptosis, and Inflammation

An objective of this research was to determine the effect of dietary bLf on apoptosis of mouse IL. Given the observations that bLf altered apoptotic proteins to favour cell death within the intestinal epithelium during carcinogenesis (Fujita et al., 2004a; 2004b) it was hypothesized that dietary bLf would induce similar results in mouse IL during inflammation. The importance of the apoptotic process in the pathogenesis of IBD is highlighted by the observations that patients with IBD exhibit increased numbers of activated $CD4^+$ and $CD8^+$ IL within their inflamed colonic tissue (Muller et al., 1998) and that IL isolated from inflamed mucosa exhibit apoptotic resistance (Sturm et al., 2004; Boirivant et al., 1999; Levine and Fiocchi, 2001).

The defective response of IL to apoptosis may be related, in part, to altered levels of apoptotic proteins. In support of this are the following observations in T cells isolated from IBD patients: 1) increased Bcl-2/Bax ratios (Ina et al., 1999) and 2) decreased

concentrations of the pro-apoptotic protein Bax (Itoh et al., 2001). The manipulation of these proteins may lead to increased apoptosis and possible alleviation of (or reduction in) the extent of inflammation. In the present research, bLf fed mice had decreased levels of the anti-apoptotic protein Bcl-2 with no change in levels of the pro-apoptotic protein Bax. This finding suggests that the Bcl-2/Bax ratio decreases with bLf exposure and, further, that this dietary protein potentially leads to increased mitochondrial outer membrane permeability (MOMP) and activation of the apoptotic machinery. In support of the observation that bLf may alter MOMP is the finding that dietary bLf increased cytoplasmic protein levels of cytochrome c. This protein is located on the mitochondrial inner membrane and once in the cytoplasm is vital to the formation of the apoptosome which activates caspase 3 (Adams and Cory, 2002). These observations coupled with the observed increase in Annexin V expression (which binds to phosphatidylserine) with bLf administration indicate that apoptotic death of IL may occur and that this is through mitochondrial associated pathways. A similar effect was noted by Mader et al. (2005; 2007) who demonstrated that Jurkat T cells incubated with bLfcin had reduced levels of Bcl-2 and increased concentrations of cytochrome c and caspase 3. Collectively, these results imply that bLf modification of the mitochondrial anti-apoptotic protein Bcl-2 may be one mechanism by which mouse IL death is promoted.

Given the numerous proteins capable of regulating mitochondrial outer membrane permeability (e.g., Bcl-X_L, Bak, Bad…etc) (Youle and Strasser, 2008) a decreased level of Bcl-2 does not represent the only mechanism by which mitochondrial membrane permeability will be altered. Nevertheless, several investigators have demonstrated the important and central role of Bcl-2 in the regulation of apoptosis. In mice with over-

expression of Bcl-2, and following cecal ligation and puncture, the following findings were noted: 1) decreased intestinal epithelial cell apoptosis and reduced caspase 3 concentrations that correlated with enhanced survival (Coopersmith et al., 2002) and 2) enhanced lymphocyte survival in the spleen and thymus that increased survival rates of the mice (Hotchkiss et al., 1999). Conversely, reduced Bcl-2 concentrations induced by missense RNA resulted in increased apoptosis of gastric cancer cells (Hao et al., 2007). Therefore, although anti-apoptotic Bcl-2 is not the only protein involved in the regulation of apoptosis, increased levels of Bcl-2 are associated with less cell death and decreased concentrations are associated with greater cell death. Thus, the ability of bLf to decrease Bcl-2 may be an important mechanism of regulating IL apoptosis.

The finding that bLf affected apoptotic proteins to favour apoptotic cell death was not, however, associated with reductions in mouse IL numbers. This is contrary to the original hypothesis that bLf would induce apoptosis of activated IL and thus offer intestinal protection during inflammation. It is tempting to speculate, that the inability of bLf to reduce IL numbers during inflammation was associated with the inability of bLf to protect the intestinal mucosa. However, that reduced IL numbers were not observed with bLf treatment may be explained by the findings that bLf did not increase IL levels of caspase 3, the primary executioner caspase. Given decreased protein levels of Bcl-2 and increased cytochrome c protein levels with bLf supplementation, the inability of bLf to reduce IL numbers may reflect the time of tissue sampling; alterations in mitochondrial membrane permeability may have not yet resulted in the activation of caspase 3. In fact, the kinetics of apoptosis is such that alterations in mitochondrial permeability occur upstream and prior to caspase activation (Huppertz et al, 1999). Thus, the kinetics of

apoptosis and the cross-sectional nature of this experiment may explain the lack of both increased caspase 3 protein levels and reduced IL numbers.

8.5. Dietary bLf, Mucosal Protection, and Clinical Implications

During or following periods of intestinal inflammation, therapeutic interventions should result in alleviation of disease symptoms and the restoration of homeostasis. Whether in patients with IBD, or in athletes suffering from gastrointestinal distress, successful recovery from tissue damage would include repair of the intestinal epithelium and the restoration of the epithelial barrier function. To evaluate whether the alterations in IL apoptosis or inflammatory cytokine levels following bLf treatment resulted in direct mucosal protection intestinal histology was examined. Treatment with DSS was associated with severe intestinal inflammation characterized by the occurrence of several disease indicators (shortened intestinal length, intestinal bleeding and decreased body weight at sacrifice). Mice supplemented with bLf enriched diets showed no alleviation of these disease symptoms; moreover, bLf did not prevent DSS-induced mucosal tissue damage or the recruitment of immune cells to the mouse intestinal mucosa. The inability of bLf to protect the mucosa may be related to the overwhelming stress induced by DSS. Indeed, DSS has been associated with several alterations following treatment which include direct cell cytotoxicity (Cooper et al., 1993), alteration in bacterial microflora (Okayasu et al., 1990) and upregulation of adhesion molecules that favour lymphocyte recruitment (Ni et al., 1996). It is also possible that the lack of effects of bLf in this DSS model is related to the mode of DSS induced IL death. Although apoptotic death of IL following DSS treatment was observed, death was predominately characterized as

necrotic. If bLf affects IL death through the mitochondrial pathway of apoptosis, as suggested by the findings of this research and by those of Mader et al. (2005; 2007), than the modifying effects of bLf on apoptosis would have little impact on DSS induced necrotic death.

Numerous studies have shown that inflammation promotes and accelerates the formation of aberrant crypts (AC) and colonic tumours (Tanaka et al., 2001; Onose et al., 2003). Mice were subjected to tumour initiation events (i.e., AOM injection) and then exposed to DSS in an attempt to mimic inflammation as a promoter of tumourigenesis. The findings of this research demonstrate that bLf did not prevent or reduce the formation of AC nor did it attenuate the DSS-induced increase in AC numbers. The lack of bLf effect was most likely related to the inability of this dietary protein to significantly influence DSS-induced alterations and thus not attenuate the detrimental physiological consequences of inflammation (i.e., tissue damage).

Dietary bLf, which has been shown to reduce TNF-α, NFκB and Bcl-2 and increase cytochrome c, may thus have limited ability to significantly influence the intestinal immune system during periods of severe inflammatory stress. The clinical importance of these findings are that dietary bLf, at least in the concentration and duration provided in this thesis research, may not be useful in the treatment of IBD or colon carcinogenesis. Dietary bLf may also not be sufficient as a supplement to prevent the gastrointestinal distress associated with strenuous exercise. The administration of 5% DSS for 4 days in drinking water resulted in severe intestinal damage and may represent an extreme sequelae of inflammation. The inability of dietary bLf to impart mucosal protection during this process is suggestive that it would offer little protection in either

exercise induced gastrointestinal distress or in the relapsing and remitting bouts of intestinal inflammation associated with IBD. Therefore, given the results of this study it would not be recommended that bLf be used as a supplement for athletes experiencing bowel distress or in those suffering from IBD. However, clearly these suggestions would need to be tested empirically both in a clinical population and experimentally.

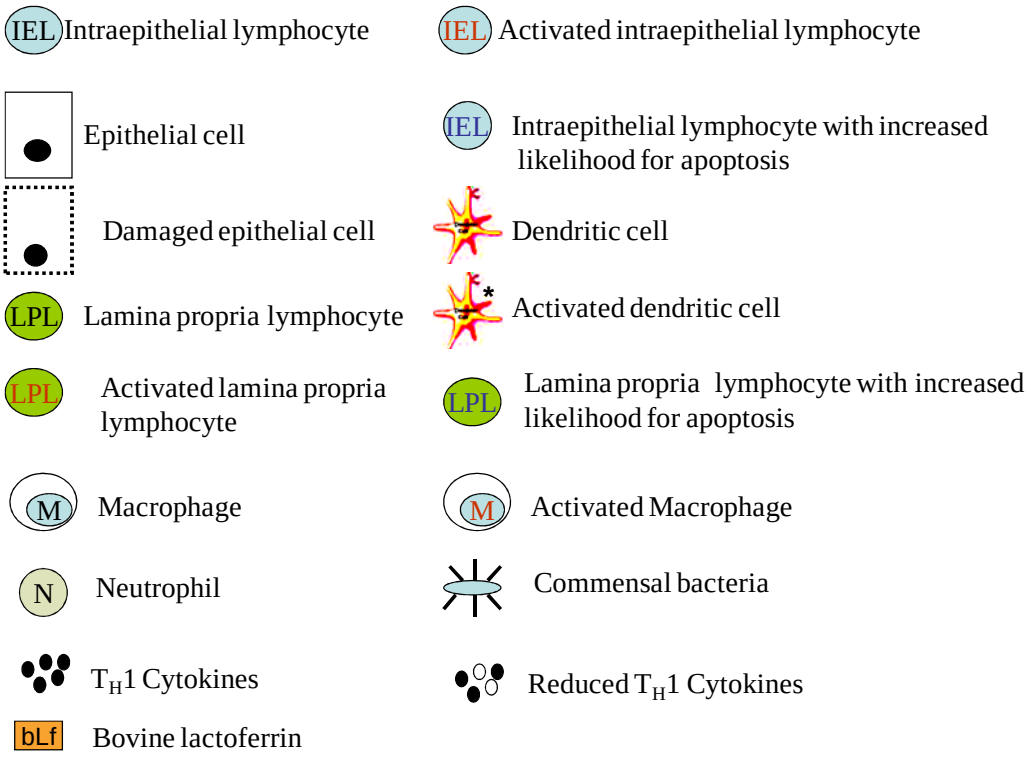
Athletes are known to supplement their diets to improve performance and potentially prevent against the onset of the gastrointestinal distress that accompanies exercise. Froiland et al. (2004) reported 89% of Division 1 athletes used supplements of which 12.6% used whey protein. Whey proteins have been found to increase lean muscle mass and strength compared to placebo controls in humans (Candow et al., 2006) and elevated lymphocyte glutathione concentrations and increased peak power during a 30s isokinetic cycling sprint (Lands et al., 1999). Although the benefits of whey protein ingestion are noted there is a paucity of data on the direct effects of bovine lactoferrin on the bowel distress associated with exercise. There is evidence, however; that exogenous administration of bovine colostrum (BC), which is the first milk produced after parturition, can impart beneficial immune modulatory effects in athletes. Crooks et al. (2005) showed increased IgA concentrations in the saliva of distance runners following supplementation with BC. In trained cyclists completing a high intensity exercise regimen, the exercise associated reductions in serum levels of IgG_2 were attenuated with BC treatment; decreased incidence of upper respiratory tract illness were also reported (Shing et al., 2007). Supplementation with other dietary components has also been shown to influence immune function in athletes. Administration of quercetin, a dietary flavanoid, did not influence pro-inflammatory measures directly in muscle but did reduce

post exercise plasma levels of IL-8 (pro-inflammatory) and IL-10 (anti-inflammatory) in trained cyclists following 3 consecutive days of exhaustive exercise (57% of maximal work rate) (Nieman et al., 2007a); decreased incidence of upper respiratory tract infections with quercetin supplementation have also been observed (Nieman et al., 2007b). Collectively, these studies demonstrate a potential for nutritional supplementation in improving the health of athletes through the modulation of immune function. Whether the immune effects of dietary bLf observed in this research are associated with improved health in athletes, however, requires further clinical examination.

An overall effect of bLf in providing intestinal mucosal protection during DSS treatment, or the carcinogenesis process was not observed, nonetheless bLf had modest activity on cytokines and apoptotic proteins and DSS as originally hypothesized. As presented in Figure 2.3, the original hypothesis was that addition of bLf during an inflammatory state would shift the cytokine milieu from a T_H1 to a T_H2 environment and would induce apoptosis of activated IL. Although, levels of IL-10 were not affected, the observed decrease in TNF-α does support a role for dietary bLf in promoting an anti-inflammatory cytokine environment. In addition, dietary bLf was shown to significantly decrease levels of the anti-apoptotic protein Bcl-2 and increase cytochrome c levels in mouse IL. These changes in apoptotic protein levels suggest an increased potential for bLf to cause IL apoptosis. Thus, Figure 2.3 has been modified to reflect the findings of this research and is presented below as Figure 8.1. The important changes are highlighted in the figure and figure legend. The legend has been adjusted as follows: apoptotic IEL has been changed to IEL with increased likelihood for apoptosis and apoptotic LPL has

been changed to LPL with increased likelihood for apoptosis. In addition, since dietary bLf reduced TNF-α and had no effect on IL-10 the legend label T_H2 cytokines has been replaced with reduced T_H1 cytokines.

Legend for Figure 8.1.



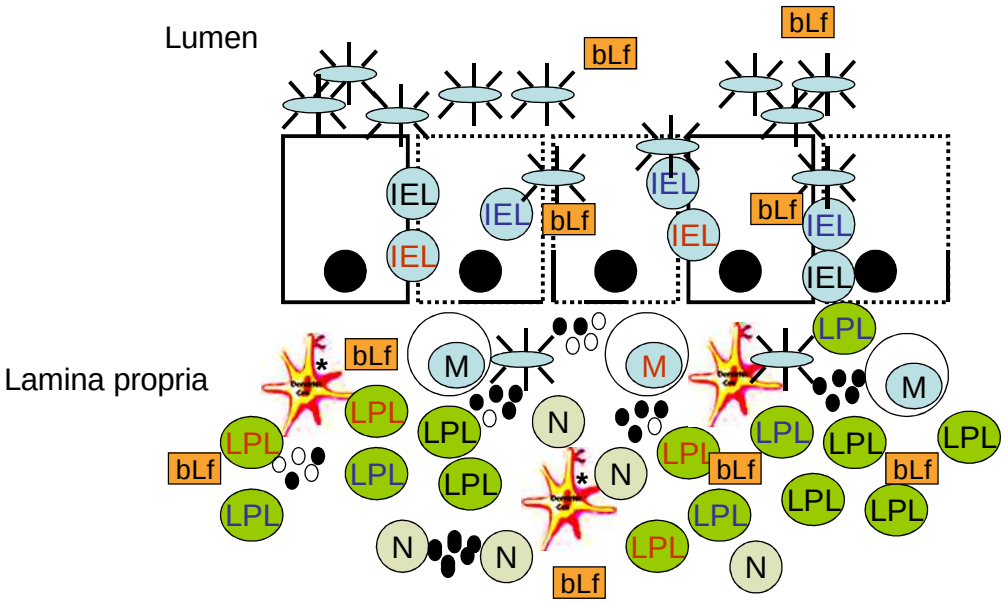


Figure 8.1: Schematic outlining the effects of dietary bLf administration during inflammatory stress.

8.6. Limitations

There are limitations to the research described in this thesis. These include, the nature of the data obtained from Western blotting techniques, the quantification of few apoptotic proteins and cytokines, the small number of time points analyzed, the use of mice, the small variations in dose and duration of bLf treatments tested, the small sample size and the limited number of inflammatory markers quantified.

Firstly, the technique of Western blotting allows for protein levels to be determined relative to experimental controls. Thus, it is not a definitive method of quantifying actual cellular protein concentrations. Furthermore, Western blotting does not quantify levels of messenger RNA (mRNA); as such, this technique does not provide information regarding gene expression. Nevertheless, proteins and not mRNA participate

directly in cellular functions and thus proteins are more relevant in the assessment of biological activity.

Second, apoptosis is controlled and regulated by several proteins. The experiments of this thesis quantified only certain indicators of apoptosis (caspase 3, Bcl-2, Bax, cytochrome c, externalization of phosphatidylserine via Annexin V^+) in mouse IL. Inclusion of other apoptotic proteins could have provided additional information. For example, measuring caspase 8, caspase 9 or caspase 12 may have provided insight as to whether apoptosis was carried out by the extrinsic, intrinsic or the endoplasmic reticulum pathway, respectively (Hoffman-Goetz et al. 2005). As such, better inferences would be made about the mode of apoptotic death. However, given the central role of caspase 3 in the execution of many apoptotic pathways (i.e., intrinsic and extrinsic pathways), the importance of Bcl-2 and cytochrome c in mitochondrial associated apoptosis (van Loo et al., 2002) and the importance of phosphatidylserine externalization in the signaling of apoptotic death (Vermes et al., 1995), the markers used in this research are valuable in the overall assessment of apoptosis.

Third, there are many cytokines involved in intestinal inflammation, such as IL-7 and IL-4, which are T_H2 cytokines and IFN-γ, IL-1β and IL-8, which are T_H1 cytokines (Papadakis and Targan, 2000). Furthermore, the redundant nature of the cytokine network implies that more than one cytokine can perform a particular function (Goldsby et al., 2003). For example, IL-1β is similar to TNF-α in that it imparts comparable cellular functions such as activation of macrophages, up-regulation of adhesion molecules and increasing vascular permeability and is also important in IBD pathogenesis (Neuman, 2007). The quantification of additional cytokines could have provided further information

into the potential immunomodulatory nature of dietary bLf. However; IL-10 and TNF-α are important cytokines that are central to events that occur in intestinal inflammation (MacDermott, 1996; Williams, 2001). The importance of IL-10 was highlighted by Lindsay et al. (2004) who demonstrated that DSS induced intestinal damage was reduced upon IL-10 addition. The importance of TNF-α has been demonstrated by several investigators (Baert et al., 1999; van Dullemen et al. 1995) who reported reduced inflammation upon the addition of anti-TNF-α antibodies. Therefore, the information gained by examining the effects of bLf on IL-10 and TNF-α, not only provides insight into important immunomodulatory mechanisms but, also builds a solid framework for future investigations.

Another potential limitation of this research was that only a limited number of time points were analyzed during each experiment. For example, IL were isolated and analyzed prior to and post DSS administration and three time points were analyzed in the exercise study (prior to, immediately following, and 24 h post the acute exercise protocol). Apoptosis is a kinetic process that proceeds over several minutes or several hours (Huppertz et al., 1999). The results presented here provide only cross-sectional data of what may potentially be occurring. To overcome this limitation and obtain an overall picture of any apoptotic pathway would involve sampling at many time points (i.e., 2h, 4h, 8h…etc post exercise or DSS). However, the data presented in this research provide a starting point for later experiments on the kinetics of apoptosis and bLf.

Given that this was a diet study, dose and duration of exposure play a large role in determining the physiological response. Any modifications of these dietary parameters could thus affect the degree of this response. Varying the concentration (i.e., higher or

lower doses) of bLf or increasing the exposure time could have resulted in more drastic (or more subtle) alterations in apoptotic and/or inflammatory cytokine levels. Nevertheless, the results build upon previous work which demonstrated that 2.0% bLf protected against AOM induced aberrant crypt formation (Sekine et al., 1997a; 1997b) and also provide a solid framework for future studies involving dietary bLf and intestinal inflammation.

Another potential limitation of this research was the small number of inflammatory measures. A common method of measuring inflammation is the quantification of myeloperoxidase (MPO), an enzyme located within neutrophils. Since neutrophils are the first cell to the site of injury they are frequently measured as indicators of inflammation (Lu et al., 2006). Limited amount of intestinal tissue prevented the use of MPO analysis in determining inflammation. Another common method of measuring inflammation is quantifying plasma levels of C-reactive protein. However, since this would not have measured inflammation directly within the intestine this method was not performed. Therefore, the use of an H&E stain (a common histological stain) allowed for measures of inflammation directly within the intestine using small amounts of tissue and was also able to analyze, to some degree, the extent of neutrophil infiltration. Although, this was the sole measure of inflammation it provides important and relevant information.

Given the low sample size (i.e., between 9-16 mice per group in these experiments) there is a potential limitation regarding statistical power. Power is defined as the probability that the statistical test will reject a false null hypothesis (Blair and Taylor, 2008). Two factors that largely determine statistical power are sample size and

level of significance (Blair and Taylor, 2008). Given the constant level of significance established at the onset of the experiments (α=0.05) the main factor influencing statistical power in these experiments is sample size. Thus in the statistical analysis performed in this thesis it is possible that the values which approached significance (e.g., bLf effects onTNF-α in experiment 3: p=0.10 or bLf effects on cell count in experiment 2: p=0.06) could have been considered significant if the sample size, and thus power, were increased. However, the sample size was calculated based on several factors including: power (at 80%), level of significance (α=0.05) and estimated mean difference (based on previous experimental data). In addition, time and budgetary constraints limit the ability of increasing sample size to values much greater than 16 mice per group. Therefore, the statistical analysis performed on the experimental data provides both relevant and reliable interpretations.

Finally, the use of mice as an experimental model has its own limitations since it is not a given that similar treatment effects would occur in humans or other animal species. A major advantage of using a single inbred mouse strain (C57BL/6) is the small genetic variability between animals (Festing, 1999). However, the results obtained in this thesis research are limited in generalizability to even other inbred mouse strains. Melgar et al. (2005) noted that when subjected to similar concentrations and durations of DSS exposure C57BL/6 mice had more severe intestinal damage and increased disease activity scores compared to Balb/c mice. Although the reason for this difference is not known, it was suggested that the C57 strain was more susceptible to DSS-induced cellular damage and thus resulted in greater inflammation. Therefore, whether the effects of dietary bLf

would occur similarly in other mouse strains is not known; generalizations to humans are even more problematic.

8.7. Future Research

Apart from the quantification of additional apoptotic and cytokine proteins future studies could involve modifications of both the exercise regimen and the DSS protocol. The altered exercise regimen might involve higher work intensities and longer durations in an attempt to induce observable intestinal structural alterations. This may allow for greater inferences to be made into the potential use of bLf as a supplement for athletes who experience gastrointestinal distress following strenuous exercise. Conversely, alterations in the DSS protocol would include decreasing the concentration of DSS (For example, from 5% to 3%) or reducing the exposure time (For example, from 4 to 3 days) in an attempt to lessen the degree of inflammatory damage. Additional studies would include increasing the dose and duration of dietary bLf administration to determine if varying exposures would influence apoptotic and inflammatory cytokine levels sufficiently to provide intestinal protection. Finally, future research could focus on determining the mechanism by which bLf reduces TNF-α and NFκB. This may be achieved by examining if bLf affects: 1) the DNA binding activity of NFκB, 2) the binding of TNF-α to the TNFR, or 3) levels of the inhibitory IκB protein.

8.8. Conclusions

In conclusion, the results presented in this thesis research show that 2.0% bovine lactoferrin provided in the diet to mice reduces the levels of TNF-α and NFκB in mouse IL following DSS and AE challenge. The repeated bouts of acute exercise protocol resulted in oxidative stress, increased cellular adaptive responses (HSP 70), and increased host compensatory mechanisms (IL-10), but did not induce intestinal inflammation; DSS treatment in contrast, was effective at causing severe intestinal inflammation. Overall, dietary bLf treatment was associated with mouse IL apoptosis evident through increased expression of phosphatidylserine on the external leaflet of the IL membrane (as measured by Annexin V^+-FITC binding), increased levels of cytochrome c and decreased levels of the anti-apoptotic protein Bcl-2. Bovine lactoferrin induced modifications in both cytokine and apoptotic protein levels in mouse IL but did not protect the intestinal mucosa from DSS induced alterations. Moreover, when AE or DSS were applied as potential promoters of colon carcinogenesis (i.e., post AOM injection), dietary bLf did not prevent the formation of aberrant crypts nor did it prevent the DSS-induced increase in aberrant crypts. Therefore, dietary bovine lactoferrin results in only limited changes in cytokines to favour anti-inflammatory responses, and apoptotic proteins to favour pro-apoptotic events. Moreover, these modifications do not appear to contribute to the direct mucosal protection against inflammation or inflammation as a promoter of colon carcinogenesis. However, the experimental design may not have been powerful enough to detect a dietary effect given the small sample size tested. Thus further research, with greater mice per group, would be required to fully determine whether bLf can protect the intestine during inflammation. Nevertheless, at this time the use of dietary bLf in the

treatment of inflammatory bowel diseases, or as a supplement to athletes is not recommended. Yet the results from these experiments are promising and offer some optimism that the anti-inflammatory effects may be considered in the array of clinical and experimental strategies to reduce the detrimental effects of intestinal inflammation.

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APPENDICIES

Appendix I - List of Abbreviations

8-iso-$PGF_{2\alpha}$ – 8-iso Prostaglandin $F_{2\alpha}$

24 h POST – sacrifice 24 hours following cessation of the final exercise bout

AC – aberrant crypts

ACF – aberrant crypt foci

ACTH - adrenocorticotropic hormone

AE – acute exercise

AIF – apoptosis inducing factor

ANN – annexin

ANOVA – analysis of variance

AOM – azoxymethane

APAF-1 – apoptotic protease activating factor-1

ATP – adenosine triphosphate

AU – arbitrary densitometric units

BC – bovine colostrum

bLf – bovine lactoferrin

CAR – carrageenan

CD – Crohn's Disease

CDC – chenodeoxycholic acid

CORT – corticosterone

CTL – control

d – days

DC – dendritic cells

DFF – DNA fragmentation factor

DMH – 1,2-dimethylhydrazine

DNA – deoxyribonucleic acid

DSS – dextran sulfate sodium

endo G – endonuclease G

EC – epithelial cell

EIA – enzyme immunoassay

ER – endoplasmic reticulum

Fas L – Fas ligand

FITC – fluorescein isothiocyanate

GALT – gut associated lymphoid tissue

GC – glucocorticoids

GSH – glutathione

GSSG – oxidized glutathione

h – hours

hLf – human lactoferrin

H & E – hematoxylin and eosin

HSP – heat shock protein

Ig – immunoglobulin

IBD – inflammatory bowel disease

IEC – intestinal epithelial cells

IEL – intraepithelial lymphocytes

IFN-γ – interferon-γ

IKKβ – I kappa β kinase

IL- – interleukin

IL – intestinal lymphocytes

IMM – sacrifice immediately after the final exercise bout

IR – ischemia reperfusion

Lf – lactoferrin

Lfcin – lactoferricin

LP – lamina propria

LPL – lamina propria lymphocytes

LPS – lipopolysaccharide

mRNA - messenger RNA

mbTNF-α – membrane bound protein

MLN – mesenteric lymph nodes

MMP – mitochondrial membrane permeability

MOMP – mitochondrial outer membrane permeability

MPO – myeloperoxidase

NFκB – nuclear factor κ B

NK – natural killer cells

NSAID – non-steroidal anti-inflammatory drug

PBL – peripheral blood lymphocytes

PE – phycoerythrin

PI – propidium iodide

PMSF – phenylmethylsulfonyl fluoride

PP – Peyer’s patches

PVDF – polyvinylidene fluoride

RNA – ribonucleic acid

ROS – reactive oxygen species

SDS – sodium dodecyl sulfate

SED – sedentary mice

SEM – standard error of the mean

SMA – superior mesenteric artery

SOD – superoxide dismutase

tBid – truncated Bid protein

TBST – tris-buffered saline tween

TCR – T-cell receptor

TF – transcription factor

TNBS - trinitrobenzenesulfonic acid

TNF-α – soluble tumor necrosis factor-α

TNFR – tumor necrosis factor receptor

TRADD – TNFR associated death domain

TRAF – TNF receptor associated factor

UC – ulcerative colitis

Appendix II - Strenuous Exercise Assessment

Mice were run on a treadmill according to a protocol outlined by Hoffman-Goetz and Quadrilatero (2003) which demonstrated increased oxidative stress and elevated plasma levels of the stress hormone corticosterone. This published protocol consisted of a 10 min warm-up followed by a single session of 90 min of continuous exercise (30 min at 22 $m \cdot min^{-1}$, 2°slope; 30 min at 25 $m \cdot min^{-1}$, 2°slope; 30 min at 28 $m \cdot min^{-1}$, 2°slope) and a 5 min deceleration period. Oxygen consumption values (VO_2 max) when performed at a 0° slope were 68%, 74% and 80% at 22 $m \cdot min^{-1}$; 25 $m \cdot min^{-1}$; 28 $m \cdot min^{-1}$, respectively (Quadrilatero and Hoffman-Goetz, 2005). The increased slope (2° slope) and the repeated administration of this protocol (3 bouts separated by 24 h rest periods) would further add to the work intensity required by the mice to complete the exercise regimen. Furthermore, toward the completion of the exercise bouts, the mice required encouragement to run and this was achieved by gentle prodding with a nylon brush. In no instance was a mouse able to complete the entire exercise regimen (i.e., all three bouts) without this encouragement. Therefore, it is suggested that the exercise protocol used in this research was at a strenuous level for the mice.

Appendix III - Lactoferrin Absorption

Methods

Mice were sacrificed by sodium pentobarbital (0.06-0.08 ml) overdose. Blood was collected immediately by cardiac puncture into a 1 ml syringe containing 0.08ml of heparin. Plasma was obtained following centrifugation (6 min, 1500*g*) and frozen at -20 °C for Lf analysis. Bovine Lf was analyzed in plasma samples using a commercially available ELISA kit (Bethyl Laboratories, Montgomery Texas, USA) according to the manufacturer's protocol. The reported sensitivity of the kit was from 7.8ng/ml to 500ng/ml. Blood hemoglobin was analyzed by a clinical veterinary laboratory (University of Guelph) with an Advia 120 Hematology Analyzer.

Results

Blood hemoglobin (Hg) and red blood cell counts were not significantly affected by the dietary lactoferrin condition (Table A1). Bovine lactoferrin was not detected in plasma samples.

Table A1: Analysis of blood from mice receiving 0%, 0.2%, or 2.0% bovine lactoferrin.

Group	RBC (10^{12})/L	Hemoglobin (Hg) (g/L)
0 % bLf	8.4 ± 0.1	136.4 ± 1.2
0.2 % bLf	8.7 ± 0.5	134.5 ± 7.9
2.0% bLf	8.1 ± 0.2	133.0 ± 2.5

Discussion

To exclude the possibility that lactoferrin, an iron binding protein, induces anemia (i.e., iron deficiency) the blood concentration of hemoglobin (Hg) was measured. Hg concentrations are commonly used to detect anemia and values less than 110 g/L indicate iron deficiency (Hernell and Lonnerdal, 2002). Dietary bLf did not alter Hg concentrations in the blood compared to controls nor did it reduce Hg values below the lower limit. That bLf did not induce anemia is similar to other observations involving bLf. For example, Paesano et al. (2006) reported increased Hg concentrations in pregnant women following bLf supplementation. Hernell and Lonnerdal (2002) showed that formula fortified with bLf did not influence Hg levels in infants. Therefore, the findings of this study suggest that dietary bLf does not result in a state of iron deficiency.

In agreement with other investigators (Kuhara et al., 2000; Wang et al., 2000; Wakabayshi et al., 2004), plasma bLf was not detected using an ELISA technique. It is possible that the commercially available ELISA used lacked the sensitivity to detect bLf at very low concentrations (i.e., the floor for detection using the ELISA kit was 7.8 ng/ml). However, Wakabayashi et al. (2004) using a more sensitive SELDI affinity mass spectroscopy technique (which is sensitive at pmol/g concentrations) were also unable to detect Lf or its fragments in the portal blood of rats following oral administration through drinking water. Digestion of bLf results in functional fragments such as bovine Lfcin and these are detectable in the feces of rats at pmol/g levels (Kuwata et al., 2001). Bovine Lf survives gastric transit within humans and is excreted in the feces (Troost et al., 2001). Given equipment and time constraints, fecal bLf was not quantified. Collectively, these results imply that orally administered bLf may exert its function on cells directly in the

gastrointestinal tract and may not be taken up into the circulation in detectable concentrations. In support of this suggestion is the following evidence: First, Lf was absorbed by villous columnar epithelial cells, macrophages, intraepithelial lymphocytes and lamina propria lymphocytes throughout the pig small intestine; bLf was detected within the cytoplasm of exfoliating, apoptotic epithelial cells (Kitagawa et al., 2003). Second, CaCo-2 intestinal epithelial cells took up bLf in vitro with highest concentration near the nucleus (Ashida et al., 2004); receptors for lactoferrin have been identified on numerous mammalian immune cells including neutrophils (Deriy et al., 2000) and lymphocytes (Mazurier et al., 1989). Taken together, bLf may not enter into the circulation but may exert its functionality directly within the gastrointestinal tract.

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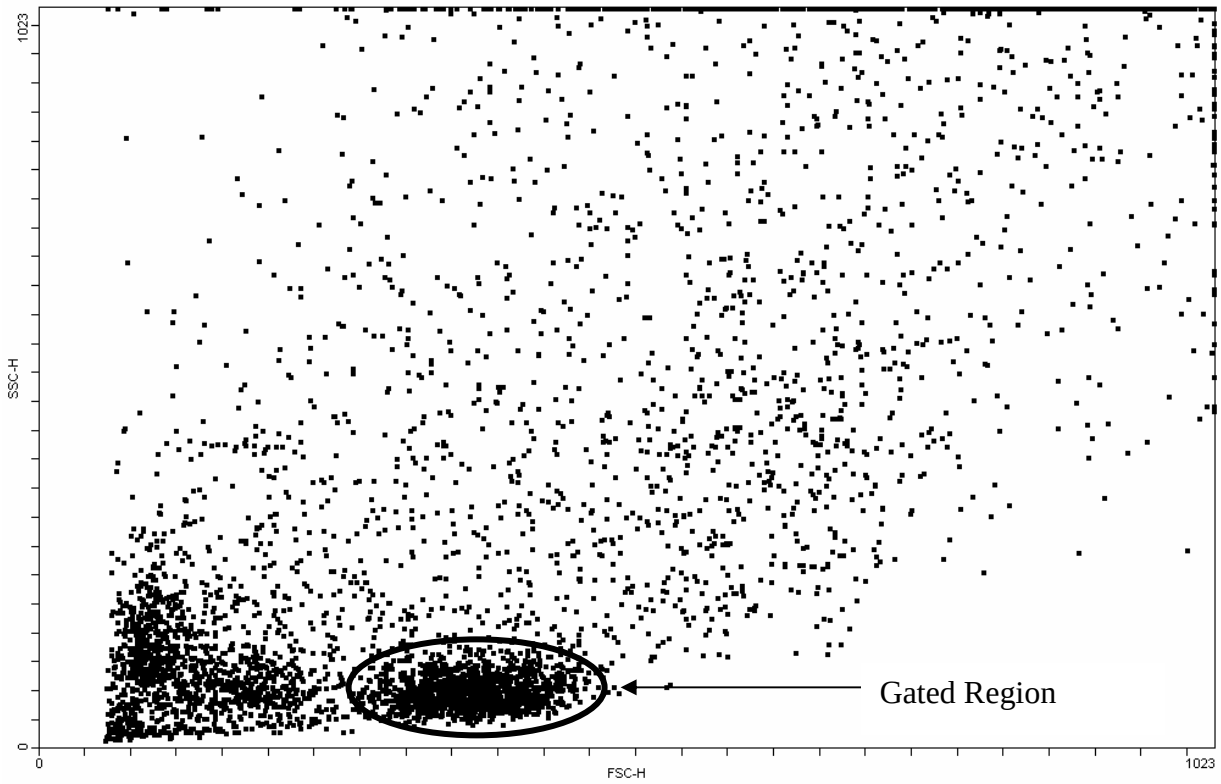
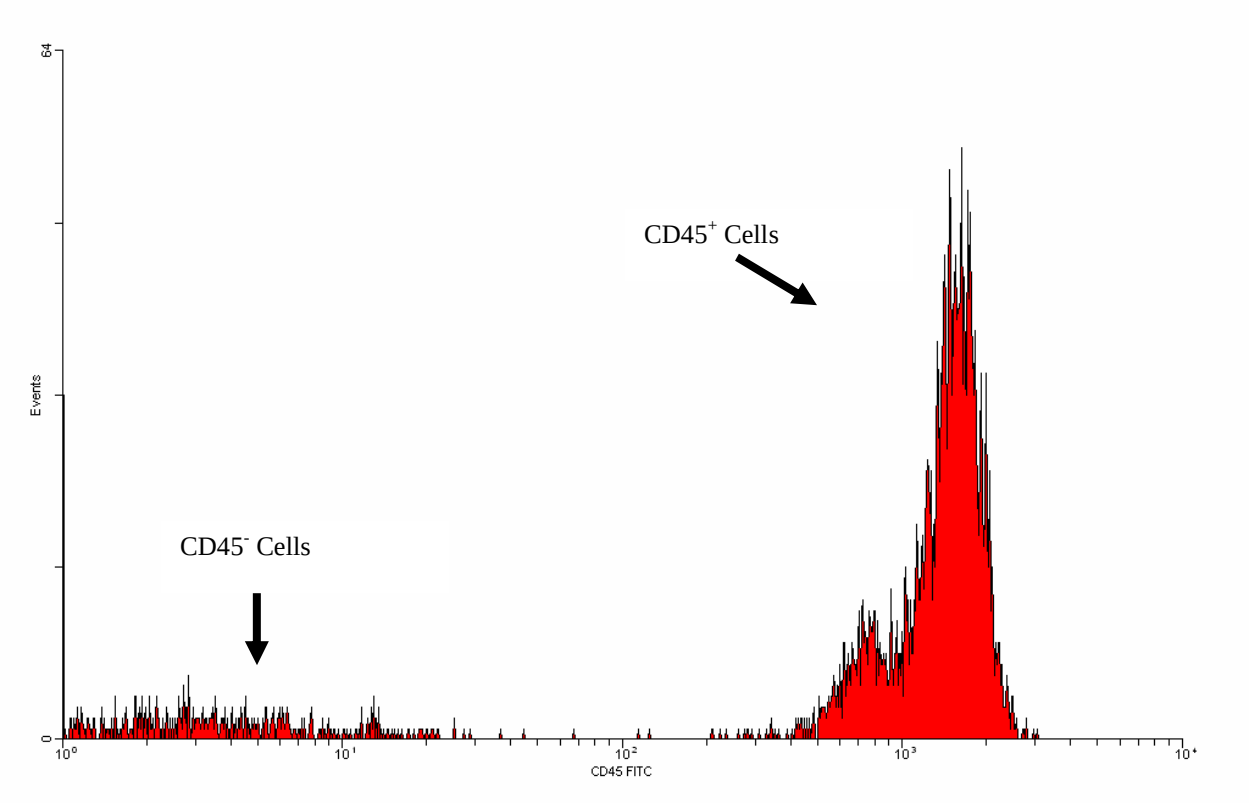
Appendix IV - Representative Flow Diagrams

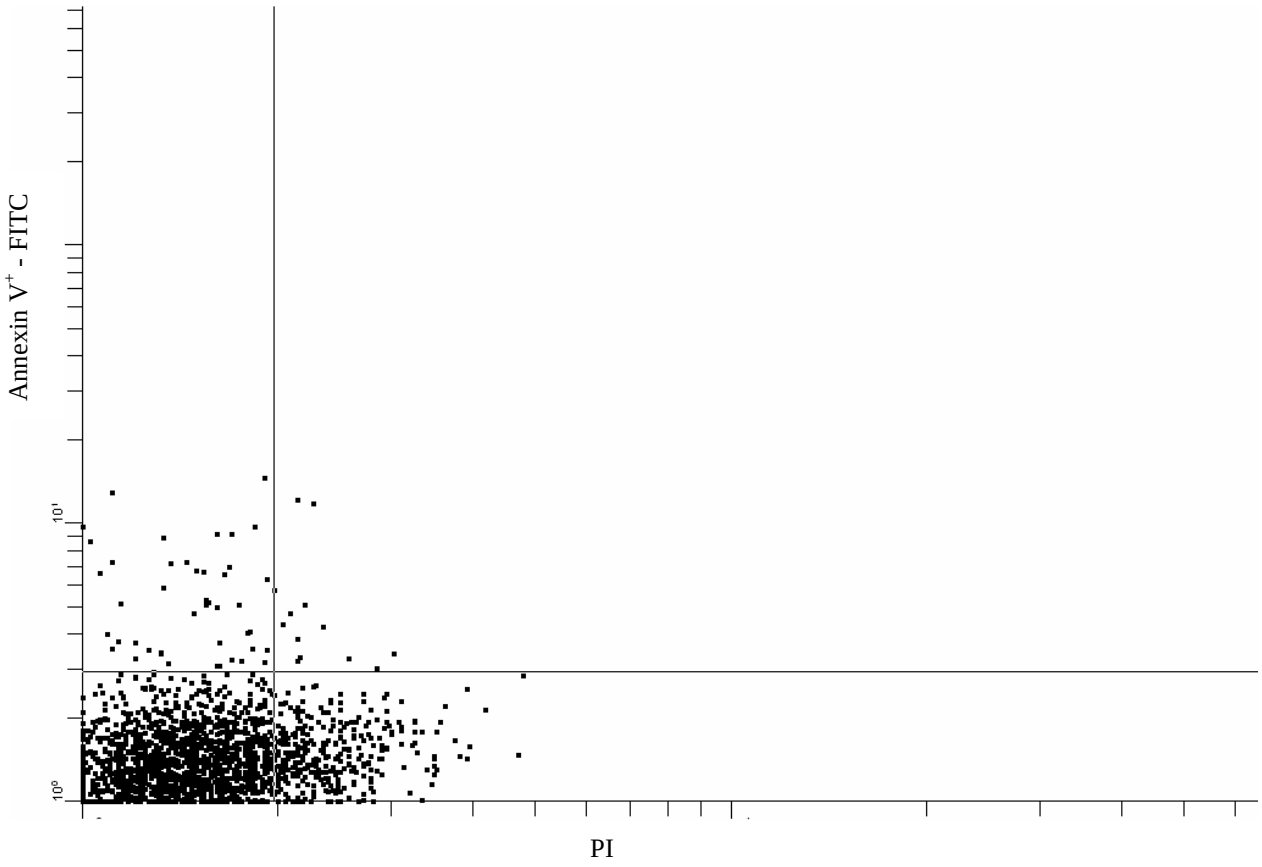
Intestinal lymphocytes incubated with CD45-FITC antibody



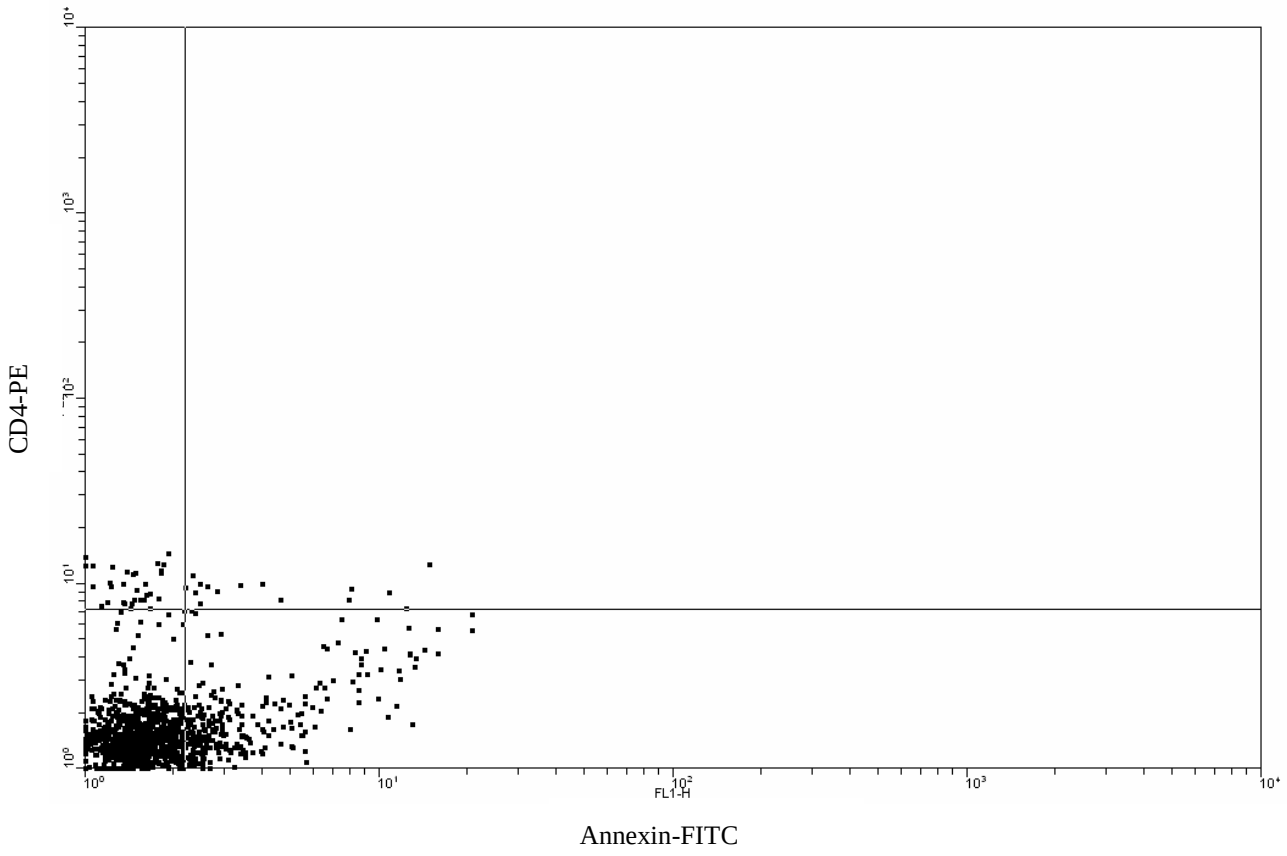
Intestinal lymphocytes incubated with CD45-FITC antibody (gated for $CD45^+$ cells only)



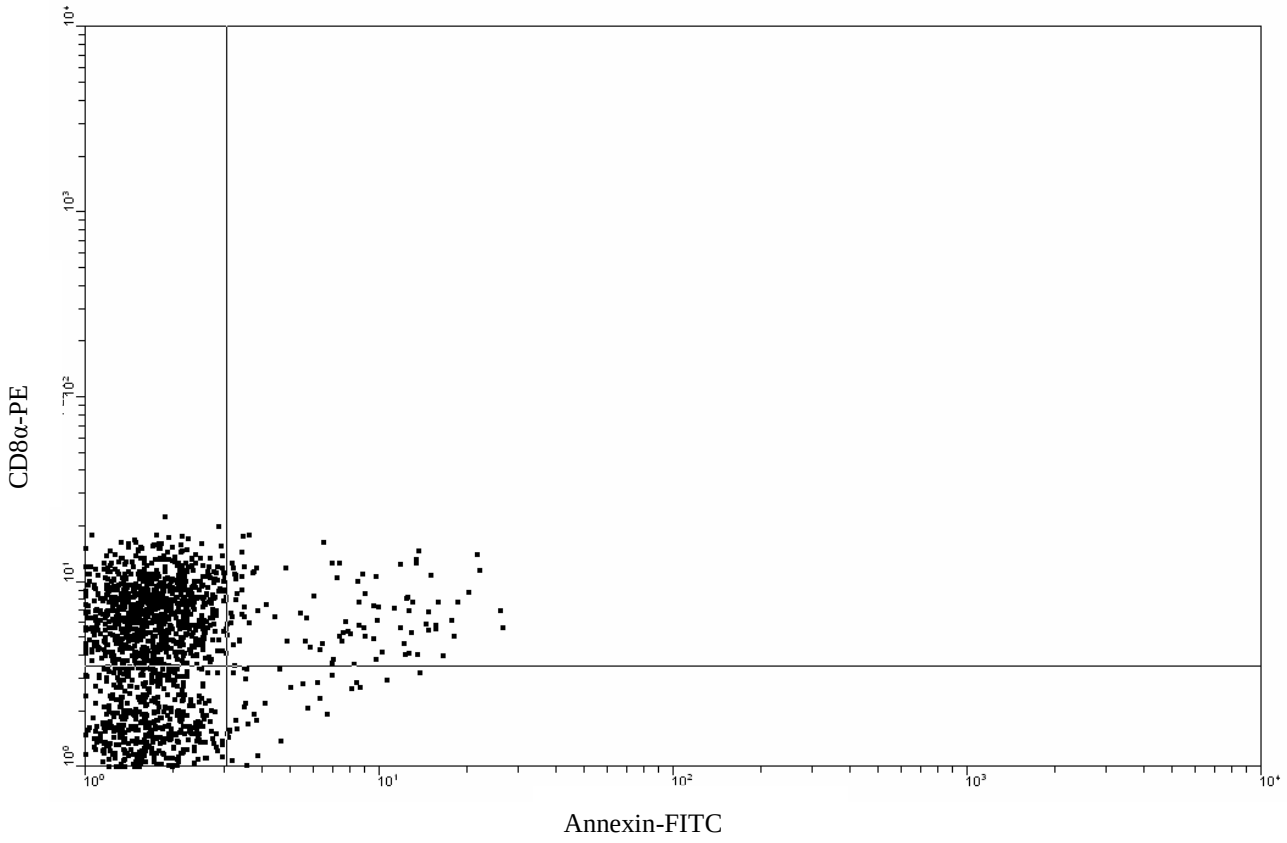
Intestinal lymphocytes incubated with Annexin-FITC and Propidium Iodide (PI) (gated for $CD45^+$ cells only)



Intestinal lymphocytes incubated with Annexin-FITC and CD4-PE (gated for $CD45^+$ cells only)



Intestinal lymphocytes incubated with Annexin-FITC and CD8-PE (gated for $CD45^+$ cells only)



Appendix V - Representative Statistics

Chapter 3:

Descriptives

CD4TOTAL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control (0%bLf)	16	8.9350	2.34441	.58610	7.6858	10.1842	3.82	13.62
0.2% bLf	16	12.2756	3.33230	.83308	10.5000	14.0513	4.69	17.60
2.0%bLf	15	12.0093	4.62612	1.19446	9.4475	14.5712	6.46	21.70
Total	47	11.0534	3.78220	.55169	9.9429	12.1639	3.82	21.70

ANOVA

CD4TOTAL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	109.410	2	54.705	4.387	.018
Within Groups	548.621	44	12.469		
Total	658.031	46			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: CD4TOTAL

Tukey HSD

(I) TreamentGrop	(J) TreamentGrop	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (0%bLf)	0.2% bLf	-3.34063*	1.24843	.028	-6.3687	-.3126
	2.0%bLf	-3.07433*	1.26907	.050	-6.1524	.0038
0.2% bLf	Control (0%bLf)	3.34063*	1.24843	.028	.3126	6.3687
	2.0%bLf	.26629	1.26907	.976	-2.8118	3.3444
2.0%bLf	Control (0%bLf)	3.07433*	1.26907	.050	-.0038	6.1524
	0.2% bLf	-.26629	1.26907	.976	-3.3444	2.8118

*. The mean difference is significant at the .05 level.

Chapter 4:

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Exercise	1.00	SED	20
	2.00	IMM	30
	3.00	24 h POST	19
Diet	1.00	nobLf	34
	2.00	bLf	35

Descriptive Statistics

Dependent Variable: Concentration_Prostanes

Exercise	Diet	Mean	Std. Deviation	N
SED	nobLf	276.1992	219.70437	9
	bLf	352.4027	565.97211	11
	Total	318.1111	436.38159	20
IMM	nobLf	989.6181	809.34655	16
	bLf	704.2003	576.66223	14
	Total	856.4231	713.34323	30
24 h POST	nobLf	1107.2790	657.46070	9
	bLf	949.6043	649.35771	10
	Total	1024.2923	639.91274	19
Total	nobLf	831.9174	728.83379	34
	bLf	663.7508	623.73607	35
	Total	746.6155	677.85206	69

Tests of Between-Subjects Effects

Dependent Variable: Concentration_Prostanes

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6253803.656[a]	5	1250760.731	3.153	.013
Intercept	35057039.1	1	35057039.12	88.375	.000
Exercise	5505140.667	2	2752570.334	6.939	.002
Diet	246056.335	1	246056.335	.620	.434
Exercise * Diet	390180.104	2	195090.052	.492	.614
Error	24991068.6	63	396683.628		
Total	69707867.3	69			
Corrected Total	31244872.2	68			

a. R Squared = .200 (Adjusted R Squared = .137)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Concentration_Prostanes

Tukey HSD

(I) Exercise	(J) Exercise	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SED	IMM	-538.3120*	181.81576	.012	-974.7290	-101.8950
	24 h POST	-706.1812*	201.77281	.002	-1190.5017	-221.8608
IMM	SED	538.3120*	181.81576	.012	101.8950	974.7290
	24 h POST	-167.8692	184.66422	.637	-611.1235	275.3850
24 h POST	SED	706.1812*	201.77281	.002	221.8608	1190.5017
	IMM	167.8692	184.66422	.637	-275.3850	611.1235

Based on observed means.

*. The mean difference is significant at the .05 level.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Diet	1.00	nobLf	42
	2.00	bLf	37
Exercise	1.00	SED	29
	2.00	IMM	30
	3.00	24 h POST	20

Descriptive Statistics

Dependent Variable: Concentration_CORT

Diet	Exercise	Mean	Std. Deviation	N
nobLf	SED	416.2117	125.10021	14
	IMM	582.1376	151.18207	16
	24 h POST	394.6887	95.31805	12
	Total	473.2721	152.66300	42
bLf	SED	346.7116	110.64841	15
	IMM	456.6523	58.41417	14
	24 h POST	394.4464	114.59654	8
	Total	398.6318	104.80052	37
Total	SED	380.2634	120.98282	29
	IMM	523.5778	131.93172	30
	24 h POST	394.5918	100.49030	20
	Total	438.3140	136.83834	79

Tests of Between-Subjects Effects

Dependent Variable: Concentration_CORT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	506608.707[a]	5	101321.741	7.754	.000
Intercept	13973966.1	1	13973966.07	1069.376	.000
Diet	79344.814	1	79344.814	6.072	.016
Exercise	326658.781	2	163329.391	12.499	.000
Diet * Exercise	46051.486	2	23025.743	1.762	.179
Error	953920.282	73	13067.401		
Total	16637942.8	79			
Corrected Total	1460528.989	78			

a. R Squared = .347 (Adjusted R Squared = .302)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Concentration_CORT

Tukey HSD

(I) Exercise	(J) Exercise	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SED	IMM	-143.3144*	29.76878	.000	-214.5343	-72.0945
	24 h POST	-14.3284	33.22605	.903	-93.8195	65.1628
IMM	SED	143.3144*	29.76878	.000	72.0945	214.5343
	24 h POST	128.9860*	32.99924	.001	50.0375	207.9346
24 h POST	SED	14.3284	33.22605	.903	-65.1628	93.8195
	IMM	-128.9860*	32.99924	.001	-207.9346	-50.0375

Based on observed means.

*. The mean difference is significant at the .05 level.

Chapter 5

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
bLf	1.00	no bLf	14
	2.00	bLf	17
DSS	1.00	no DSS	16
	2.00	DSS	15

Descriptive Statistics

Dependent Variable: PIposANNneg

bLf	DSS	Mean	Std. Deviation	N
no bLf	no DSS	1.0700	.79676	8
	DSS	7.8083	7.38459	6
	Total	3.9579	5.76981	14
bLf	no DSS	4.5588	6.01833	8
	DSS	9.7478	7.25474	9
	Total	7.3059	7.02065	17
Total	no DSS	2.8144	4.52159	16
	DSS	8.9720	7.10760	15
	Total	5.7939	6.60169	31

Tests of Between-Subjects Effects

Dependent Variable: PIposANNneg

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	355.773[a]	3	118.591	3.364	.033
Intercept	1018.493	1	1018.493	28.895	.000
bLf	55.829	1	55.829	1.584	.219
DSS	269.549	1	269.549	7.647	.010
bLf * DSS	4.548	1	4.548	.129	.722
Error	951.698	27	35.248		
Total	2348.108	31			
Corrected Total	1307.470	30			

a. R Squared = .272 (Adjusted R Squared = .191)

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
bLf	1.00	no bLf	14
	2.00	bLf	17
DSS	1.00	no DSS	16
	2.00	DSS	15

Descriptive Statistics

Dependent Variable: PIposANNpos

bLf	DSS	Mean	Std. Deviation	N
no bLf	no DSS	6.4113	2.77754	8
	DSS	10.8700	6.31493	6
	Total	8.3221	4.97344	14
bLf	no DSS	9.0975	3.24910	8
	DSS	14.2122	9.68934	9
	Total	11.8053	7.64755	17
Total	no DSS	7.7544	3.23279	16
	DSS	12.8753	8.41203	15
	Total	10.2323	6.70935	31

Tests of Between-Subjects Effects

Dependent Variable: PIposANNpos

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	272.104[a]	3	90.701	2.271	.103
Intercept	3121.820	1	3121.820	78.164	.000
bLf	68.859	1	68.859	1.724	.200
DSS	173.655	1	173.655	4.348	.047
bLf * DSS	.815	1	.815	.020	.887
Error	1078.358	27	39.939		
Total	4596.133	31			
Corrected Total	1350.461	30			

a. R Squared = .201 (Adjusted R Squared = .113)

Chapter 6

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Diet	1.00	no bLf	32
	2.00	bLf	31
Exercise	1.00	SED	20
	2.00	IMM	18
	3.00	24 h Post	25

Descriptive Statistics

Dependent Variable: CytC

Diet	Exercise	Mean	Std. Deviation	N
no bLf	SED	.6750	.44335	10
	IMM	.6451	.25265	9
	24 h Post	.5995	.40551	13
	Total	.6359	.37184	32
bLf	SED	.8902	.65189	10
	IMM	.4910	.30059	9
	24 h Post	.7641	.57735	12
	Total	.7255	.54766	31
Total	SED	.7826	.55370	20
	IMM	.5681	.28079	18
	24 h Post	.6785	.49198	25
	Total	.6800	.46508	63

Tests of Between-Subjects Effects

Dependent Variable: CytC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.943[a]	5	.189	.863	.512
Intercept	28.368	1	28.368	129.700	.000
Diet	.087	1	.087	.400	.530
Exercise	.436	2	.218	.997	.375
Diet * Exercise	.381	2	.191	.871	.424
Error	12.467	57	.219		
Total	42.542	63			
Corrected Total	13.410	62			

a. R Squared = .070 (Adjusted R Squared = -.011)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: CytC

Tukey HSD

(I) Exercise	(J) Exercise	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SED	IMM	.2145	.15195	.342	-.1511	.5802
	24 h Post	.1040	.14030	.740	-.2336	.4417
IMM	SED	-.2145	.15195	.342	-.5802	.1511
	24 h Post	-.1105	.14457	.726	-.4584	.2374
24 h Post	SED	-.1040	.14030	.740	-.4417	.2336
	IMM	.1105	.14457	.726	-.2374	.4584

Based on observed means.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
Diet	1.00	no bLf	24
	2.00	bLf	25
DSS	1.00	no DSS	26
	2.00	DSS	23

Descriptive Statistics

Dependent Variable: CytC

Diet	DSS	Mean	Std. Deviation	N
no bLf	no DSS	.2004	.13343	13
	DSS	.4707	.18183	11
	Total	.3243	.20638	24
bLf	no DSS	.4848	.26887	13
	DSS	.5623	.29919	12
	Total	.5220	.28060	25
Total	no DSS	.3426	.25353	26
	DSS	.5185	.24895	23
	Total	.4252	.26410	49

Tests of Between-Subjects Effects

Dependent Variable: CytC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.951[a]	3	.317	5.955	.002
Intercept	8.998	1	8.998	168.958	.000
Diet	.431	1	.431	8.091	.007
DSS	.369	1	.369	6.923	.012
Diet * DSS	.113	1	.113	2.128	.152
Error	2.396	45	.053		
Total	12.205	49			
Corrected Total	3.348	48			

a. R Squared = .284 (Adjusted R Squared = .236)

Chapter 7:

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
bLF	1.00	no bLf	18
	2.00	bLf	19
Treat	1.00	SED	12
	2.00	AE	14
	3.00	DSS	11

Descriptive Statistics

Dependent Variable: AC

bLF	Treat	Mean	Std. Deviation	N
no bLf	SED	40.1429	28.69627	7
	AE	31.5714	15.02062	7
	DSS	66.7500	3.40343	4
	Total	42.7222	23.70854	18
bLf	SED	25.0000	7.10634	5
	AE	34.2857	14.98571	7
	DSS	67.1429	25.39966	7
	Total	43.9474	25.42412	19
Total	SED	33.8333	22.98550	12
	AE	32.9286	14.48322	14
	DSS	67.0000	19.76360	11
	Total	43.3514	24.26957	37

Tests of Between-Subjects Effects

Dependent Variable: AC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	9454.825[a]	5	1890.965	4.989	.002
Intercept	68696.165	1	68696.165	181.247	.000
bLF	141.819	1	141.819	.374	.545
Treat	8517.545	2	4258.773	11.236	.000
bLF * Treat	568.518	2	284.259	.750	.481
Error	11749.607	31	379.020		
Total	90740.000	37			
Corrected Total	21204.432	36			

a. R Squared = .446 (Adjusted R Squared = .357)

Post Hoc Tests

Multiple Comparisons

Dependent Variable: AC

Tukey HSD

(I) Treat	(J) Treat	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SED	AE	.9048	7.65884	.992	-17.9451	19.7546
	DSS	-33.1667*	8.12658	.001	-53.1677	-13.1656
AE	SED	-.9048	7.65884	.992	-19.7546	17.9451
	DSS	-34.0714*	7.84405	.000	-53.3771	-14.7657
DSS	SED	33.1667*	8.12658	.001	13.1656	53.1677
	AE	34.0714*	7.84405	.000	14.7657	53.3771

Based on observed means.

*. The mean difference is significant at the .05 level.