Effect of acute treadmill exercise and voluntary freewheel running on cytokine and apoptotic protein expression in intestinal lymphocytes of older female C57BL/6 mice

by

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Author's Declaration

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

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Nicholas Packer

August 17th, 2011

Abstract

Background: Colorectal cancer (CRC) is the second leading cause of Canadian cancer mortality. Inflammation is a fundamental risk factor in the aetiology of sporadic intestinal carcinoma. Reducing the frequency or duration of gastrointestinal inflammation may decrease CRC risk. Over 200 population studies demonstrate reduced odds of developing CRC among physically active persons. Preliminary data suggests that regular exercise may slow CRC pathogenesis by decreasing and increasing intestinal expression of pro- and anti-inflammatory cytokines, respectively. This research was designed to further our understanding of how exercise influences the colonic cytokine milieu, even in the presence of immunoscenescent changes.

Objectives: The objective of the first experiment (*Study #1*) was to compare cytokine and apoptotic protein expression in intestinal lymphocytes (IL) at baseline and in response to acute exercise-induced oxidant stress in both young and older C57BL/6 female mice. A second objective (*Study #2*) was to examine the effect of exercise training on the expression of pro- and anti-inflammatory cytokines and pro- and anti-apoptotic proteins in IL of older C57BL/6 female mice under 'resting' conditions. The final objective (*Study #3*) was to compare the effect of acute exercise-induced stress on IL cytokine and apoptotic protein expression in trained versus untrained older C57BL/6 mice.

Methods: Immediately following sacrifice, plasma was collected from the mice and stored (-80ºC) until corticosterone and 8-iso-$PGF_{2\alpha}$ assessment by enzyme immunoassay. Soleus and plantaris skeletal muscles were excised and frozen in liquid nitrogen (-80ºC) until spectrophotometric assessment of cytochrome *c* oxidase (CO) activity. Finally, the entire mouse intestinal compartment was removed and IL lysates were prepared for flow cytometric analysis of percent apoptosis (% Annexin V^+ IL) and for western blot analysis of pro-inflammatory (TNF-α, IL-1β), pleiotropic (IL-6) and anti-inflammatory (IL-10) cytokine, and pro-(caspase-3, -7) and anti-(Bcl-2) apoptotic protein expression.

Results: Findings from *Study #1* indicate that, in mice, acute exercise increases caspase-3 (IMM and 2Hr groups vs. SED; $p<0.05$) and TNF-α (IMM vs. SED and 2Hr groups; $p<0.001$), and decreases Bcl-2 (IMM and 2Hr groups vs. SED; $p<0.01$) expression in intestinal lymphocytes. Furthermore, IL expression of Bcl-2 was lower ($p<0.001$) and % Annexin V^+ IL was higher ($p<0.05$) in the older vs. young mice. The results from *Study #2* indicate that trained older mice had lower ($p<0.05$) expression of TNF-α and caspase-7 in IL, and lower ($p<0.05$) concentration of 8-iso-$PGF_{2\alpha}$ in plasma compared to sedentary untrained controls. Finally, *Study #3* shows that older trained mice display increased expression of pro-(TNF-α) and anti-(IL-10) inflammatory cytokines and pro-apoptotic (caspase-3, caspase-7) proteins, and decreased expression of anti-apoptotic (Bcl-2) protein in IL after acute exercise challenge compared to older untrained controls. In both *Study #1 & #3*, the treadmill protocol induced stress: plasma corticosterone and 8-iso-$PGF_{2\alpha}$ were higher in mice sampled immediately after acute exercise relative to the no acute exercise (sedentary) condition. This exercise effect did not differ by age (*Study #1*) or by training (*Study #3*) condition. In addition, *Study #2* & *Study #3* showed elevations in cytochrome *c* oxidase activity following long-term training.

Conclusion: Collectively, these results suggest that, in C57BL/6 female mice, IL expression of pro-apoptotic proteins and pro-inflammatory cytokines does not differ by age (young vs. older animals) in response to a single intense exercise bout. However, older mice display lower expression of 'protective' anti-apoptotic proteins and a higher percentage of early apoptotic IL compared to young mice. Additionally, long-term exercise may protect the bowel from inflammation by reducing inflammatory cytokine and apoptotic protein expression under 'resting' (no stress) conditions. Finally, long-term training preserves the IL cytokine and apoptotic protein responses in older mice to a magnitude similar to that previously described in young mice. Alternatively, older untrained mice display reduced responsiveness to acute treadmill exercise, suggestive of immunosenescence.

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List of Abbreviations

ACF - aberrant crypt foci

ANOVA - analysis of variance

Bcl-xl - B cell lymphoma extra-large transmembrane protein

CD - chromosome of differentiation markers, indicates T cell phenotype

COX-2 - cyclooxygenase enzyme 2

CTL - cytotoxic T cell lymphocyte

DSS - dextran sodium sulfate

FAS - FasR 'death receptor'

IGF-1 - insulin growth factor 1

LT - leukotrienes

$NF_k\beta$ - nuclear transcription factor involved in cytokine production

NIDDM - non-insulin-dependent diabetes mellitus or type II diabetes

NOS2 - nitric oxide synthase isoform 2

NWR - No Wheel Running

NSAID - nonsteroidal anti-inflammatory drugs

PG - prostaglandin

TNF-α - tumor necrosis factor-alpha

TNFR1/TNFR2 - TNF-alpha receptor 1 and 2

IFN-γ - interferon gamma

IKK - IkB kinase

IMM - immediately post-acute exercise

iNOS calcium insensitive inducible nitric oxide synthase

RTE - run-to-exhaustion

SED - sedentary

SOCS1 - suppressor of cytokine signaling 1

STAT-1/JAK - signal transducer activator of transcription 1 and janus kinase

STAT-3 - signal transducer and activator of transcription 3

STAT-3/$NF_k\beta$ - signal transducer activator of transcription and nuclear factor kB

TCR - T cell receptor

Th - T helper cells

TLR - toll-like receptor

VEGF - vascular endothelial growth factor

WR - Wheel Running

2Hr - assessment at 2Hr post-RTE

INTRODUCTION

'Immunosenescence' is an umbrella term that describes declines in immune function with age. Even in the absence of disease, aging is accompanied by impaired T cell function, decreased antigen responsiveness and chronic dysregulation of the cytokine milieu (Fulop et al., 2010). These declines in function contribute to high morbidity and mortality among the elderly. Chronic inflammation characterizes the aging process; this 'inflame-aging' is a key risk factor for the development of chronic diseases including cancer (Franceschi et al., 2000).

Colorectal carcinogenesis (CRC) is the second leading cause of Canadian cancer mortality (Statistics Canada, 2009). As the population ages, overall disease burden is expected to increase. The aetiology of sporadic colorectal cancer is varied, but chronic inflammation of the gastrointestinal (GI) mucosa is a fundamental risk factor. The bowel is an immunologically important organ densely populated with lamina propria (LP), intraepithelial (iIEL) and intestinal (IL) lymphocytes. In the GI tract, these cells have important immunoregulatory roles. Unremitting inflammation induces excessive lymphocyte recruitment and cytokine production. Cellular dysplasia and carcinoma are induced by this inflammatory milieu. Accordingly, reducing the frequency or duration of intestinal inflammation may lower GI cancer risk.

Regular exercise is a promising approach to decreasing colorectal cancer burden (Varo et al., 2003). Over 200 population studies demonstrate reduced odds of developing colorectal cancer (CRC) among physically active persons. Potential mechanisms for this effect include decreased intestinal transit time and altered concentration of intestinal and pancreatic hormones (Quadrilatero and Hoffman-Goetz, 2003). Preliminary data (almost exclusively from studies in young animals) suggests that training reduces intestinal inflammation by decreasing pro- and

increasing anti-inflammatory cytokine expression (Hoffman-Goetz et al., 2009). The purpose of this thesis research was to consider if the same 'anti-inflammatory' effects of training are observed experimentally in an older population. These findings further our understanding of how exercise influences the colonic cytokine milieu, even in the presence of immunoscenescent changes.

The structure of this thesis is as follows. Chapter 1 is a literature review providing a brief summary of relevant concepts including a discussion on intestinal immune function, colorectal cancer aetiology, cytokines and inflammation, cellular apoptotic pathways, immunosenescence, and exercise and immune function. Chapter 2 provides an overview of the study rationale and design of the thesis experiments**.** Chapters 3-5 are the hypothesis, experimental design, methods, results and conclusions for each study. These chapters are presented in the same format as the published work. Chapter 6 is an integrated discussion of the overall findings including a section on limitations with guidelines for future research.

CHAPTER 1: Literature Review

The purpose of this literature review is to provide a brief overview of intestinal immune function, immunosenescence, and the effects of acute treadmill and voluntary freewheel exercise. This discussion is intended to provide context for the studies constituting this thesis.

1.1 THE GASTROINTESTINAL TRACT

1.1.1 Intestinal Immune Function

The small and large intestines are primarily involved in chemical (e.g., cholecystokinin, secretin) and mechanical (e.g., peristalsis) digestion and in the absorption of food micro-particles across the lumen (Cunningham-Rundles and Lin, 1998). The gastrointestinal (GI) tract also plays a major role in protection against exogenous pathogens. The immunologic capacity of healthy intestinal tissue is of crucial importance given its continuous exposure to a variety of bacterial and viral antigens (MacDermott, 1996). Immune dysregulation, or the inability to maintain the integrity of the enteric barrier, can result in severe and varied pathology (Ma, 1997). Early in development, intestinal epithelial and intra-epithelial cells 'fuse' or anastomose through the formation of tight junctions (Ma, 1997). These structures are important in maintaining GI health and prevent uncontrolled translocation of nutrients across the intestinal lining (Kelly and Coutts, 2000). Another marker of healthy GI function is the presence of diverse colonies of microorganisms (Stagg et al., 2004). This gut flora enhances intestinal motility, chemical secretion, nutrient absorption, villous length and crypt depth (Hart et al., 2002).

Independent of physical barriers (e.g., tight junctions, epithelial cells) or immune-enhancing symbiosis (e.g., gut flora), gastrointestinal defence is almost exclusively mediated by

the gut-associated lymphoid tissues (GALT) (Luongo et al., 2009). This immunologically active tissue includes Peyer's patches and mesenteric lymph nodes and is an important contributor to mucosal immunity due to its distribution throughout the intestinal tract (Czerkinsky et al., 1999). The GALT is important to humoral immune defence and is a key site of B-cell mediated IgA production and mucosal secretory IgA (sIgA) secretion (Brandtzaeg, 2010). Secretory IgA has an important role in neutralizing exogenous pathogens, initiating antibody-dependent cell-mediated cytotoxicity and inducing respiratory burst activity in polymorphonuclear leukocytes (Fagarasan and Honjo, 2003). This front line defence is supported by the functions of antigen-presenting cells (i.e., dendritic and $CD4^+$ T 'helper' cells) as the GALT also represents the primary site of lymphocyte proliferation, differentiation and activation (Luongo et al., 2009).

1.1.2 Intestinal Lymphocytes

The lamina propria (LP) is basolateral to the intestinal lumen and is extensively populated by T-lymphocytes, major effector cells in gastrointestinal homeostasis and in the adaptive arm of immunity (Lefrancois and Lycke, 2001; Köhne et al., 1996). Lamina propria T cells protect against intestinal inflammatory dysregulation (Iliev et al., 2009). Constitutively found in the mucosa, intestinal intraepithelial lymphocytes (iIEL) are involved in homeostatic regulation and perform neoplastic surveillance functions (Henderson et al., 2010). Antigenic exposure of the mucosa initiates lymphocyte activation, proliferation and phenotypic selection, actions which determine downstream effector functions (Levine and Fiocchi, 2001; Ivanov et al., 2006). There is speculation that chronic 'anti-inflammatory' immune bias in the bowel of healthy individuals is the result of selective differentiation of lymphocytes in response to continuous exposure to gut

flora (Hart et al., 2002). Collectively, intestinal immune function reflects a balance between T cell responses (Bailey et al., 2005) and lymphocyte-derived inflammation (Powrie, 2004).

1.1.3 Maintaining Intestinal Homeostasis

Selective apoptosis (programmed cell death) of lymphocyte subsets typically proceeds through one of two mechanisms of controlled cell death: the intrinsic and extrinsic apoptotic cascades. The intrinsic apoptotic pathway is mediated by mitochondrial cleavage of inactive zymogens (Wyllie, 2010). Subsequent cytoplasmic translocation and activation of cleaved isoforms leads to caspase-mediated cell lysis (Wyllie, 2010). This pathway is initiated by extracellular/intracellular signals (e.g., cytochrome *c*, granzyme B) (Ow et al., 2008; Rousalova and Krepela, 2010) and follows a 'cascade' whereby different isoforms undergo sequential cleavage. The cleaved proteins each exert specific biological effects (Kuwana and Newmeyer, 2003). In contrast, the extrinsic pathway proceeds through cytokine-mediated activation of surface 'death receptors' or though a receptor/ligand binding mechanism (Levine and Fiocchi, 2001; Chen et al., 1998). These effects are antagonized by anti-apoptotic proteins which prevent the release, activation, and translocation of mitochondrial caspases (Kuwana and Newmeyer, 2003). Apoptosis regulates cell phenotypes within the lymphocyte population and is critical for intestinal homeostasis and in protecting against intestinal pathology (Bruner et al., 2002; Atkins and Furuta, 2010; Acheson et al., 2004).

1.2 COLORECTAL CANCER

1.2.1 Canadian Relevance

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide (WHO, 2004) with a lifetime risk of over 7% in the general Canadian population (Canadian Cancer Society, 2011). In 2011, the Canadian Cancer Society (2011) estimates that approximately 22,200 Canadians will be newly diagnosed with colorectal cancer. Among this newly diagnosed Canadian cohort, the annual mortality estimate is 8,900 (Canadian Cancer Society, 2011). This represents a lifetime incidence and mortality risk of 1 in 13.3 and 1 in 27.7, respectively (Canadian Cancer Society, 2011).

Sixty-three is the average age of initial sporadic colorectal cancer diagnosis (Lynch, 1999). Given an aging Canadian demographic, CRC is a disease of increasing concern. In 2009, an estimated 15.2% of the Canadian population was over 65 years; this percentage is projected to increase to 24.5% by 2036 (CIA, 2010). In other words, in less than 2 decades, a quarter of Canadians will be over 65 years old. When this statistic is coupled with the average cost of managing CRC, the implications to the national healthcare system are dire. Indeed, the total yearly cost of CRC treatment in Canada as of 2003 was over $333 million dollars (Maroun et al., 2004). This represents an average yearly expenditure of $20,319 – 39,182 (CDN) per patient (Maroun et al., 2004; Luo et al., 2010). These costs are only expected to increase, leading to a significant challenge for the Canadian healthcare system from an economic, societal and individual perspective (Wong et al., 2009).

1.2.2 Disease Aetiology and Pathogenesis

There are numerous theories regarding the aetiology of colorectal cancer. These include, but are not limited to: (1) exposure to the *bacteroides fragilis* enterotoxin (Toprak et al., 2006); (2) various genetic polymorphisms (Antonacopoulou et al., 2010); and (3) active inflammatory bowel disease (Westbrook et al., 2010). This brief review will focus on the 'inflammation hypothesis of colorectal cancer' which is evidenced by increased CRC risk among people with ulcerative colitis, inflammatory bowel disease and other inflammation-promoting disorders (e.g., sclerosing cholangitis) (Soetikno et al., 2002). This hypothesized relationship is further strengthened by increases in colorectal cancer risk proportional to the extent and duration of colitis (Kulaylat and Dayton, 2010).

Colitis-induced carcinogenesis is characterized by the spread of epithelial dysplasia and the occurrence of p53 abnormalities (Burmer et al., 1992). p53 plays a critical role in DNA repair, mitotic regulation and cellular apoptosis (Zambetti and Levine, 1993). Loss of heterozygosity (LOH) for p53 predicts the extent and severity of colonic dysplasia (Burmer et al, 1992). p53 LOH was observed in 85% of colorectal carcinoma patients, in 63% of human pathology specimens with high-grade dysplasia, and in 33% of specimens with low-grade dysplasia (Burmer et al., 1992). In addition, some sporadic colorectal cancer cases are characterized by chromosomal instability and mutagenesis in tumour suppressor (TS), adenomatous polyposis coli (APC), mutated in colon cancer (MCC) and deleted in colon cancer (DCC) genes; activation of the k-*ras* oncogene and loss of function of 18q also indicate elevated CRC risk (Wong et al., 2010). Chronic inflammation contributes to microsatellite and chromosomal instability, inducing intestinal dysplasia through p50 and $NF_k\beta$-dependent mechanisms (Huang et al., 2008).

Though there are multiple mechanisms by which inflammation induces dysplasia, 'typical' pathogenesis is characterized by chronic intestinal irritation leading to carcinoma (Demarzo et al., 2008). This is supported by research showing that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) prevents the development of colorectal cancer (Harris et al., 2009; van Staa et al., 2005). Epplein et al. (2009) found that regular use of anti-inflammatory drugs (e.g., aspirin and non-aspirin NSAIDs) was associated with a relative risk for colon cancer of 0.73 (95% CI: 0.61–0.89; p=0.009). A meta-analysis of 72 studies indicated that regular intake of over-the-counter anti-inflammatory drugs reduced CRC risk by 43% (case-control and cohort studies from 1980 onwards) (Harris et al., 2009). Moreover, these cancer-preventative effects were heightened for COX-2 specific NSAIDs – likely because COX-2 over-expression simulates a prostaglandin cascade that leads to the 'inflame-o-genesis of cancer' (Harris et al., 2009; Harris et al., 2007). Downstream products of COX-2 enzyme activity include arachidonic acid-derived lipid messengers such as prostaglandins (PG) and leukotrienes (LT) (Wang et al., 2005). PG and LT signalling molecules promote cellular and nuclear damage by forming lipid peroxy radicals which then up-regulate inflammatory cytokine translation. These inflammatory mediators promote: (1) DNA mutagenesis and oxidative stress; (2) inappropriate cell survival in dysplastic epithelium; and (3) rapid tumour cell growth (O'Connor et al., 2010).

1.2.3 Physical Activity

Physical activity, particularly long-term moderate intensity aerobic exercise, is a promising lifestyle intervention that reduces colorectal cancer incidence (Lee, 2003; Colditz et al., 1997). Many epidemiological studies demonstrate reduced odds of developing colorectal cancer among physically active persons.

A meta-analysis by Wolin et al. (2009) recently reported a strong inverse association between regular physical activity and colon cancer risk in both genders and in case-control and cohort studies, with an overall relative risk (RR) of 0.76 (95% CI: 0.72–0.81) compared to inactive individuals. Samad et al. (2005) in a meta-analytical review of 19 studies found the same inverse relationship between regular physical activity and colorectal cancer in men, with a RR of 0.79 (95% CI: 0.72–0.87). In a large population-based study, Thune and Lund (1996) describe how males and females differed in the protective effects of exercise. Physical activity (defined as walking or bicycling for ≥4 hours per week) reduced the risk of CRC in women (RR = 0.62; 95% CI: 0.40–0.97) with this risk reduction particularly evident in the proximal colon (RR = 0.51; 95% CI: 0.28–0.93). No effect of occupational activity and CRC risk was observed among women (Thune and Lund, 1996). For men, however, all forms of regular physical activity (including occupational activity) were associated with risk reduction (RR = 0.74). Sex differences in gastrointestinal transit time, stool bulk and bile acid production (Stephen et al., 1986; Lampe et al., 1993) may explain the reported differences in CRC risk reduction by gender. Thune and Lund (1996) also state that these differences may not have biological relevance as there were age (Males: $\bar{X} = 58.1$ years; Females: $\bar{X} = 54.6$ years) and power (due to a greater number of male cancer cases) differences between genders. Irrespective of gender differences, Thune and Furberg (2001) argue that the strong dose-response provides convincing evidence that physical activity is protective against CRC. In addition, it is possible that physical activity may even reduce risk of CRC recurrence: 4 hrs of walking per week significantly improved survival times among individuals already diagnosed with CRC (Halle and Schoneberg, 2009).

Given the role that inflammation plays in CRC pathogenesis, it is intriguing to consider whether regular exercise reduces the inflammatory processes that lead to mutation and dysplasia

(Quadrilatero and Hoffman-Goetz, 2003; Chan and Giovannucci, 2010). A recent study by Demarzo and colleagues (2008) showed that exercise training in rats had both anti-proliferative and anti-inflammatory effects in colonic mucosa. Moderate treadmill exercise reduced epithelial cell proliferation and COX-2 expression in colon tissues of male Wistar rats exposed to dimethyl-hydrazine, a potent carcinogen (Demarzo et al., 2008). Pharmaceutical and surgical treatments for CRC (and other inflammation related conditions) are expensive and can have profound negative side-effects on patient quality of life. Alternatively, physical activity is a low-cost and low-risk lifestyle practice with benefits across all facets of health (Belardineli et al., 2006; Brown et al., 2004; Keo et al., 2008). While there is likely no single mechanism that explains the protective effect of exercise, physical activity is suspected to reduce CRC risk by decreasing intestinal inflammation via multiple vascular, immune and epigenetic mechanisms (Rogers et al., 2008; Quadrilatero and Hoffman-Goetz, 2003).

1.3 INFLAMMATION

Inflammation refers to the aggregate response to physical stress; this can be induced by cellular shear forces (e.g., wounds, tissue injury), by exposure to cellular irritants (e.g., antigens, necrotic cells), or by exogenous pathogens (e.g., bacteria, viruses) (Li et al., 2007). Inflammation serves both to limit the extent of cellular damage and to initiate tissue repair. Healing involves the complex activation of and interaction between immune cells, including leukocytes, fibroblasts and endothelial cells (Adams et al., 1994). Cellular activation induces the production and release of inflammatory mediators (e.g., histamine, bradykinin and chemokines) which cause

vasodilation and increased vasculature permeability (Speyer et al., 2011; Proost et al., 1996). Many inflammatory processes are mediated by cytokines or other products of immune cells.

1.3.1 Cytokines

Cytokines, produced by a diverse number of immunologically active cells, are low molecular weight proteins that include interleukins (IL), tumour necrosis factors (TNF), lymphotoxins, interferons (IFN), colony-stimulating factors and chemokines (Curfs et al., 1997). Cytokines are classified by cellular origin. For heuristic purposes they are dichotomized into pro- and anti-inflammatory effectors or T_H1 (pro) and T_H2 (anti) lymphocytes and cytokines.

Elevated production of the pro-inflammatory cytokine, tumour-necrosis-factor alpha (TNF-α) has been shown to propagate the pathogenesis of atherosclerosis and rheumatoid arthritis (Montecucco and Mach, 2009). Similarly, the pro-inflammatory cytokine interleukin-1β (IL-1β) has been linked to the development of many chronic inflammatory diseases including: gout, type 2 diabetes, heart failure and myeloma (Dinarello, 2011). Normally, tightly regulated TNF-α and IL-1β production prevents infection and the replication of dysplasic cells. Genetic polymorphisms in pro-inflammatory (TNF-α, IL-1β) cytokine expression have been linked to inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) (Koss et al., 2000). Dysregulation of anti-inflammatory cytokine production has also been linked to disease pathology due to over-expression of IL-10 and IL-10R in atopic dermatitis and psoriatic epidermis, respectively (Mirnohammadsadegh et al., 1997).

Pro- and anti-inflammatory cytokines play an important role in maintaining 'normal' immune function in the intestine. Receptors for many cytokines, including IL-1β, TNF-α and IL-6 are expressed on surface epithelial cells and intra-epithelial lymphocytes (Panja et al., 1998). A

balance between pro- and anti- inflammatory processes is important in the maintenance of gastrointestinal health. Unremitting, chronic inflammation induces aberrant crypt foci (ACF) formation and an 'ACF-dysplasia-carcinoma' growth sequence in colitis models, as well as an 'ACF-adenoma-carcinoma' sequence in sporadic CRC (Kukitsu et al., 2008). Moreover, the inflammatory cytokines TNF-α, IL-1β and IFN-γ down-regulate intestinal expression of selenoprotein P (an extracellular anti-oxidant) (Speckmann et al., 2010) and thereby may 'prime' the intestinal tract towards dysplastic somatic mutations.

1.3.2 Pro-Inflammatory Cytokines

The 'classical' inflammatory cytokines are immunologically important for human health. These include TNF-α and IL-1β, cytokines which play a role in the pathogenesis of inflammatory diseases. In its active 'proteolytically cleaved' soluble form, TNF-α plays an important role in innate/cellular immune defence (Curfs et al., 1997). However, inflammatory dysregulation is often characterized by elevated TNF-α (Szkaradkiewicz et al., 2010). Many inflammatory intestinal conditions, including ulcerative colitis, Crohn's disease, inflammatory bowel disease and colorectal cancer, are typified by elevations in TNF-α concentration (Szkaradkiewicz et al., 2010). In CRC patients, elevated TNF-α in the bowel predicts both the extent of carcinogenesis and likelihood of tumour recurrence (Grimm et al., 2010). Moreover, therapeutic application of anti-TNF-α antibodies prevents CRC development in colitis-associated conditions (Popivanova et al., 2008). Given the strong positive association between IBD and colorectal cancer risk, TNF-α is suspected to play a role in the pathogenesis of cancer (Wilson, 2008).

In healthy individuals, the active isoform of IL-1β induces lymphocyte activation, endothelial growth and apoptosis of terminally differentiated T cells (Jung et al., 2003; Wang et

al., 2009). IL-1β production is elevated in response to physiologic stress. Andreozzi and colleagues (2007) report sharp increases in intestinal concentration of this pro-inflammatory cytokine in response to acute exercise challenge. These stress-induced increases in IL-1β can promote an inflammatory tissue environment by up-regulating intra-cellular $NF_k\beta$ and COX-2 pathways. In addition, IL-1β promotes tumorigenesis by enhancing vascularity of tumours through the expression of vascular endothelial growth factor (VEGF) (Jung et al., 2003). Over 95% of multiple myeloma patients had elevated IL-1β production (Lacy et al., 1999). IL-1β is activated by caspase-1; the cleaved substrate acts upon biological targets by binding to cell membrane surface receptors or to soluble intracellular receptors (Dinarello, 2010). Pharmacologic inhibition of IL-1β signalling in the form of anti-IL-β has been shown to reduce tumour metastasis and angiogenesis. Accordingly, IL-1β is an effective target of adjuvant therapy designed to decrease cancer mortality and morbidity (Dinarello, 2010).

1.3.3 Anti-Inflammatory Cytokines

Anti-inflammatory cytokines relevant to intestinal inflammation include IL-4 and IL-10. Interleukin-4 controls lymphocyte development. Given the 'anti-tumour' killing activity of cytotoxic T lymphocytes (CTL) (a type of T_H1 lymphocyte), it would seem that IL-4 should promote dysplasia by interfering with CTL function. However, Schuler et al. (2009) report that IL-4 deficient mice have severely impaired tumour immunity and develop mammary and colonic adenocarcinomas. Although the mechanism is uncertain, not only does IL-4 inhibit T_H1 lymphocytes but it also helps to prime CTL toward the generation of anti-tumour T_H2 effector cells.

Interleukin-10 is a potent cytokine that inhibits inflammatory cytokine production from T_H1 lymphocytes and thereby shifts the immune balance away from a cellular response towards an anti-inflammatory T_H2 'humoral' bias. In fact, IL-10 directly stimulates Ab production from B cells (Curfs et al., 1997). IL-10 (in combination with IL-12) has anti-tumour effects in colon and breast cancer tumour cell lines (Lopez et al., 2005). Activation of IL-10 was linked to tumour remission in ~70% of mice with colon, mammary or lung cancers (Lopez et al., 2005). IL-10 also enhances the activity of IL-4 (another anti-inflammatory cytokine) and decreases IFN-γ (a pro-inflammatory cytokine) production. Accordingly, IL-10 can limit the magnitude and duration of the inflammatory response. Due to its indirect (i.e., suppression of pro-inflammatory cytokines) and direct (i.e., induction of anti-inflammatory cytokines) immune functions, IL-10 is a key target for continued research.

1.3.4 Pleiotropic Cytokines

Interleukin-6 is a multi-functional cytokine that (in healthy individuals) plays an important immunologic role by: (1) enhancing adipose tissue metabolism (Pedersen and Fischer, 2007), (2) improving vascular health (Tafuto et al., 1994), and (3) suppressing allergic inflammation (Kishimoto, 1992). Alternatively, IL-6 has been implicated in the pathogenesis of a variety of chronic diseases including rheumatoid arthritis (Tafuto et al., 1994). IL-6 is involved in early tumour promotion and cellular proliferation in colitis-associated animal models of CRC (Grivennikov et al., 2009; Strassman et al., 1993). This may be due to the fact that IL-6 prevents apoptosis in lamina propria myeloid cells through initiation of a STAT-3 transcription cascade (Grivennikov et al., 2009).

IL-6 is an important cytokine to consider from an exercise-immunology perspective as it has been shown to increase in mouse intestinal lymphocytes (Hoffman-Goetz et al., 2010) and human skeletal muscle (Pedersen et al., 2003) as a result of exercise training and in response to acute exhaustive exercise (Pedersen et al., 1998). Typically, exercise training and acute exercise display opposing immune effects (Pedersen and Fischer, 2007). Indeed, several researchers note that IL-6 has pleiotropic effects – pro-inflammatory during acute exercise (Pedersen et al., 1998; Pedersen et al., 2003) and anti-inflammatory during training (Pedersen and Fischer, 2007). Interleukin-6 is regulated by the transcription factor $NF_k\beta$. In an inflammatory intestinal environment (e.g., elevated IL-1β or TNF-α) or early in the inflammatory response to physical stressors, IL-6 will induce inflammation. However, in the presence of anti-inflammatory cytokines (e.g., IL-10), IL-6 inhibits macrophage production of IL-1β and TNF-α (Pedersen and Fischer, 2007). Though IL-6 demonstrates both pro- and anti-inflammatory functions (Pedersen et al., 2003; Pedersen and Fischer, 2007) its effects are primarily inflammatory with increasing age (Dobbs et al., 1999) or in the presence of disease pathology (Ridker et al., 2000).

1.3.5 Transcription Factors

Transcription factors are a subclass of sequence-specific-binding factors that regulate protein expression at the level of the individual gene. Many of the damaging effects of inflammation, particularly those arising from cytokine exposure, occur due to altered transcriptional regulation (Fenton and Birmingham, 2010; Atreya and Neurath, 2008).

The transcription factor STAT-3 is activated by pro-inflammatory cytokines; this leads to cytokine translation and apoptotic inhibition in some cancer cell lines (Rose-John et al., 2009; Sahu and Srivastava, 2009). This combination of cytokine production and apoptotic inhibition

presents an ‘open window’ for dysplasia. IL-6/STAT-3 mechanisms may contribute to CRC development in inflammatory bowel disease patients (Albrecht et al., 2007; Mitsuyama et al., 2007). IL-6 binds to the soluble IL-6R and activates mucosal T cells by increasing STAT-3 activation (Mitsuyama et al., 2007). The resulting release of pro-inflammatory cytokines has been shown to activate TNF-R1 ‘death receptors’ and induce $I_K\beta$ kinase (IKK) cleavage; this leads to up-regulation of the $NF_k\beta$ pathway and production of more inflammatory mediators and DNA-damaging metabolites (Lin et al., 2010). A study by Greten et al (2004) indicated that blocking $NF_k\beta$ by inhibition of IKK_β reduced tumorigenesis by 80% in a dextran sodium sulfate (DSS) colitis mouse model. Inhibition of $NF_k\beta$ reduced the expression of the anti-apoptotic protein Bcl-xl and the pleiotropic cytokine IL-6. Hence, $NF_k\beta$ and STAT-3 control the expression of anti-apoptotic response genes. Interaction between STAT3 and $NF_k\beta$ cascades may play a role in the development and progression of GI cancers (Grivennikov and Karin, 2010).

1.4 APOPTOSIS

Apoptosis refers to a tightly regulated and highly conserved pattern of ‘pre-programmed’ cell death (Wyllie, 2010; Kerr et al., 1972; Hoffman-Goetz et al., 2005). This genetically controlled process involves the sequential activation of apoptotic pathways which induce specific changes to the cell plasma membrane and nucleus. Apoptosis-induced dysregulation is physically characterized by cell shrinkage, lysis of the plasma membrane (Parone et al., 2002) or by the formation of a membrane enclosed cell fragment (apoptotic body) (Cain et al., 2002). The appearance of surface markers such as phosphatidylserine expression on the outer leaflet of the plasma membrane indicates that cell lysis and death is imminent (Mignini et al., 2008). In

healthy persons, apoptotic cells are cleared from the body by phagocytes and apoptosis is considered important in homeostatic maintenance.

1.4.1 Intrinsic and Extrinsic Apoptotic Pathways

There are two broad apoptotic mechanisms: (1) mitochondria-dependent intrinsic and (2) cell-membrane receptor-mediated extrinsic pathways (Zhang et al., 2003). Both avenues of pre-programmed cell death are regulated by caspase proteins, a family of cysteinyl aspartate proteases that induce a broad cascade of cellular effects in response to sequential cleavage of targeted aspartate residues (Levine and Fiocchi, 2001; Chen et al., 1998). Caspases typically exist as inactive zymogens. Zymogen procaspase cleavage generates two large and two small biologically active components (Salvesen et al., 1999). The 'large' caspases are further divided into protein subclasses which differ in their biological activity. Initiator caspases act upstream to 'turn on' the apoptotic pathway and 'executioner' caspases act directly act on the plasma membrane.

The mitochondria-dependent intrinsic apoptotic pathway is induced by cellular exposure to a variety of stressors, including oxidant stress (Kile, 2009) and exercise (Hoffman-Goetz et al., 2005). Such stimuli trigger changes in mitochondria structure and/or permeability which induce the release of intermembrane proteins, including cytochrome *c* (Parone et al., 2002). In healthy mitochondria, membrane permeability is regulated by members of the Bcl-2 family. These regulatory molecules include pro-(Bax) and anti-(Bcl-2) apoptotic proteins. Bcl-2 protects against apoptosis by preventing both mitochondrial cytochrome *c* release and the activation of caspase-9, an 'initiator' caspase (Zhang et al., 2003). When the effects of oxidant stress 'overwhelm' the antagonistic capacity of Bcl-2, subsequent membrane pore formation allows for

the leakage of mitochondrial procaspases into the cytosol (Wyllie, 2009). After translocation, cytoplasmic procaspase-9, apaf-1 and cytochrome *c* combine in the cytoplasm to form an 'apoptosome' which activates caspase-9 and downstream 'executioner' caspases, including caspase-3 (Cain et al., 2002; Zhang et al., 2003). Activated 'executioner' caspases excerpt a broad array of damaging effects which lead to membrane dysregulation, morphological changes, and eventual cell death (Wyllie, 2010).

Many 'executioner' caspase functions are conserved in the extrinsic apoptotic pathway. The key difference, however, is that the extrinsic pathway is initiated by ligand binding to cell surface 'death receptors.' Most ligands belong to the tumour necrosis factor (TNF) pro-inflammatory cytokine family (Wallach and Kang, 2008). These ligands are comprised of three identical polypeptide sequences (Zhang et al., 2003) which bind tightly to membrane death receptors (Wallach and Kang, 2008). Ligand-receptor binding actively recruits intracellular molecules (e.g., FADD) which activate initiator caspases (e.g., procaspase-8) and downstream executioner caspases (e.g., caspase-3) (Peter and Krammer, 2003). Examples of key receptors that are activated by TNF-α ligation include TNF-R1, Fas, CD95 and TRAIL-R1/R2 (Zauli et al., 2003; Mignini et al., 2008). Activation of each receptor induces the extrinsic apoptotic pathway though subsequent pro-caspase cleavage and cell membrane dysregulation.

1.5 IMMUNOSENESCENCE

Immunosenescence is an 'umbrella' term used to describe the broad decline in immune functions observed with increasing age. Even in the absence of discernable disease pathology, these changes include impaired T cell receptor function, decreased number of naïve $CD4^+$ and $CD8^+$ T cells, decreased antigen responsiveness and chronic dysregulation of the cytokine milieu (Huang

et al., 2008). $CD4^+$ 'helper' T cells secrete cytokines in response to stimulation by antigen-presenting cells. These cytokines induce autocrine and paracrine effects important to the innate immune response. Alternatively, $CD8^+$ 'cytotoxic' T cells recognize, bind to and lyse pathogenically infected cells. Consistent trends towards inflammation characterize the aging process. This 'inflame-aging' is associated with many chronic diseases of the aged, including several types of dementia and some cancers (Singh and Newman, 2010). Collectively, these immune changes contribute to elevated morbidity and mortality among the elderly.

1.5.1 Lymphocyte Phenotypes and Cytokines

Intestinal lymphocytes are phenotypically distinguished by surface membrane proteins. Cluster of differentiation (CD) receptors determine, among other things, the cytokine-producing capacity of T cells in the gastrointestinal tract. With advancing age, memory ($CD45RO^+$) T cells increase and naïve ($CD45RO^-$/CD45RA) T cells decrease. Naïve T cells are needed to initiate an immune response to novel antigens whereas memory T cells are retained from earlier antigen exposure (Rothstein et al., 1990). In this manner the aging process 'crowds out' naïve T cells from the immune arsenal; this may partly explain the increased rate of infections and impaired immunity in elderly individuals.

Immunoscenescent changes and selective leukocytosis of T cell types contribute to persistent, low-grade inflammation in the bowel. For example, αβ T cells produce significantly greater levels of pro-inflammatory cytokines than do γδ T cells (Kohyama et al., 1997). In addition, γδ T cell number (compared to αβ T cells) decreases in the elderly (Colonna-Romano et al., 2004). This may bias the 'lymphocyte landscape' towards a cytokine-based αβ T cell response (Wack et al., 1998; Cossarizza et al., 1996; Picker et al., 1995). This 'inflame-aging' is

potentially driven by chronic antigenic load mediated by memory T cells, which results in the expansion of 'primed' pro-inflammatory cytokine-producing $CD28^-$ T cells in the colon (Zanni et al., 2003). Colonna-Romano and colleagues (2002) demonstrate that γδ T cells harvested from the old and very old are in a 'primed' state and show increased TNF-α in whole blood as well as increased TNF-α production by single T cells.

Moreover, T_H1 (e.g., IFN-γ, IL-2, and TNF-α) cytokine production from T cells increases significantly with age (Zanni et al., 2003). Even healthy elderly individuals demonstrate increased production of IL-6, TNF-α, and IL-1β in peripheral mononuclear cells (Fagiolo et al., 1993) and increased IL-6 and TNF-r1 in plasma (Stowe et al., 2010).

1.5.2 Apoptosis and Aging

Senescent changes in the immune system can promote the development of chronic diseases of aging, including cancer (Warner, 1999). It has been repeatedly shown that lymphocytopenia increases with age (Phelouzat et al., 1996**;** Aggarwal et al., 1999). Elevated splenic lymphocyte apoptosis was observed in old compared to young mice after exposure to the oxidant stressor 2-deoxy-D-ribose (which depletes intracellular glutathione) (Schindowski et al., 2001). Old compared to young rats had a three-fold increase in colonic lymphocyte apoptosis in response to exposure to the oxidant (and carcinogen) azoxymethane (AOM) (Kwon et al., 2007). Finally, in response to treatment with 2-deoxy-D-ribose, older adults (>60 years) had a greater percentage of apoptotic lymphocytes compared to younger adults (<35 years) (Schindowski et al., 2000).

1.6 PHYSICAL ACTIVTY

Regular physical activity significantly enhances health related quality of life by contributing dramatically to positive health outcomes and improved immune function (Chodzko-Zajko et al., 2009). Many effects of regular physical activity are recognized across the lifespan (Stessman et al., 2009). However, the effects of exercise on the immune system vary based on the intensity and duration of activity. Though regular moderate-intensity exercise conveys broad immune benefits, acute aerobic exercise induces oxidant stress, promotes downstream inflammation and compromises immunity (Quadrilatero and Hoffman-Goetz., 2005).

1.6.1 Acute Exercise

Acute episodes of physical activity induce the stress response by activating the hypothalamic-pituitary-adrenal (HPA) axis (Mastorakos and Pavlatou, 2005). This elevates plasma concentration of various stress hormones (e.g., catecholamine and cortisol) (Kjaer and Dela., 1996; Quadrilatero and Hoffman-Goetz., 2005). Catecholaminergic activation of (β-adrenergic) receptors on T lymphocytes induces pro-inflammatory cytokine production. In addition, T cell exposure to epinephrine and norepinephrine promotes inflammation through $NF_k\beta$ activation (Mastorakos and Pavlatou, 2005).

Thus, high-intensity exercise constitutively elicits an inflammatory response as indicated by elevated concentrations of TNF-α and IL-1β in plasma (Ostrowski et al., 1999). High-intensity acute exercise elicits a pro-inflammatory response both by skeletal muscle release of pro-inflammatory cytokines/myokines (such as IL-6) and increased cellular concentration of reactive oxygen (ROS) species. Uncontrolled production of ROS is less effectively buffered by endogenous antioxidants; the resulting consequences include cellular membrane damage, lipid

peroxidation, DNA damage, and the production of cell fragments (Mooren et al., 2002; Pedersen et al., 1998). In addition to ROS-mediated cellular damage, acute exercise increases expression of the transcription factor, $NF_k\beta$, critical for the production of the classical pro-inflammatory cytokine TNF-α (Vider et al., 2001). Rivier and colleagues (1994) reported that strenuous exercise (in the form of an incremental exercise test) resulted in spontaneous release of TNF-α and IL-6 from blood monocytes harvested from young master athletes. Given the role of inflammatory cytokines in initiating the extrinsic apoptotic pathway and in light of the tissue-damaging effects of ROS, the oxidant stress effects of acute exercise may contribute to pathologies of the colonic epithelium and eventually to dysplasia and colorectal cancer.

Intense exercise reduces immune function and may increase disease susceptibility (Mars et al., 1998; Hoffman-Goetz and Pedersen, 2010). Both animal and human studies indicate that acute exercise causes transient increases in leukocyte numbers (leukocytosis), followed by exercise-induced apoptosis (leukocytosis) and leukocyte death (lymphocytopenia) (Mooren et al., 2002; Hoffman-Goetz and Quadrilatero, 2003; Concordet and Ferry, 1993). Acute exercise-induced lymphocytopenia may be due to: (1) increased apoptotic protein expression (e.g., caspase-3) (Hoffman-Goetz and Spagnuolo, 2007); (2) reduction in toll-like receptors (e.g., TLR4 which plays a role in macrophage activation) (Li and Xu, 2010; Oliveria and Gleeson, 2010); or (3) leukocyte subset redistribution due to the effects of catecholaminergic and glucocorticoid hormones (Mignini et al., 2008).

Not all transient cytokine release is deleterious. IL-6 also increases lipolysis and inhibits the harmful effects of TNF-α (Pedersen and Fischer, 2007). Furthermore, the rapid increase in pro-inflammatory cytokine translation that accompanies acute exercise is typically followed by increases in anti-inflammatory cytokines (IL-4, IL-10) or receptors (IL-1ra, sTNF-α) or

antagonists for pro-inflammatory cytokines (IL-1ra) – which act to reverse the inflammatory state. Suzuki et al. (2002) report that acute exercise stress is initially characterized by inflammatory cytokine release; this is followed by plasma increases in the anti-inflammatory cytokines IL-1ra and IL-4 from 1-2 hrs after the acute exercise bout. Plasma IL-10 in men increases dramatically after a 42.2 km marathon race, presumably to counter the effects of TNF-α and IL-6 production (Suzuki et al., 2003). Similar plasma cytokine results have been reported by Ostrowski et al. (1999) for Copenhagen Marathon runners. It is possible that differential cytokine transcription and translation kinetics may buffer many of the marked inflammatory effects of acute exercise, and thereby prevent traumatic tissue damage.

1.6.2 Exercise Training

In contrast to the damaging effects of acute exercise, exercise training has many positive health effects (Stessman et al., 2009). These include increased intestinal lymphocyte concentration of endogenous antioxidants (Roshan et al., 2011), improved muscle mass and increased cellular respiratory capacity (Chodzko-Zajko et al., 2009). In fact, long-term voluntary freewheel running increases the concentration of citrate synthase, succinate dehydrogenase, and cytochrome *c* oxidase levels in skeletal muscle of young mice (Hoffman-Goetz et al, 2009; Hoffman-Goetz et al., 2010; Davidson and Hoffman-Goetz, 2006). These enzymes indicate cellular oxidative capacity and protect against the harmful effects of ROS.

Furthermore, regular physical activity alleviates oxidant stress by increasing anti-inflammatory and decreasing pro-inflammatory cytokine production in lymphocytes (Petersen and Pedersen, 2005; Hoffman-Goetz et al., 2009; Peters et al., 2006). In young C57BL/6 mice, intestinal lymphocyte expression of pro-inflammatory TNF-α decreases (Hoffman-Goetz et al.,

2009) and the expression of anti-inflammatory IL-10 increases (Hoffman-Goetz et al., 2010) in response to long-term freewheel running. This effect has been replicated in various tissues including the intestine (Hoffman-Goetz et al., 2009), whole blood (Bautmans et al., 2005), skeletal muscle (Gielen et al., 2003), hippocampus, cerebellum, pituitary (Chennaoui et al., 2008), liver and adipose tissue (Pedersen and Febbraio, 2005). Gomez-Merino and colleagues (2007) found that training reduced the expression of IL-1β and IL-12 in adipose tissue of male Wistar rats. Lira et al. (2009) showed that exercise training reduced IL-1β in rat skeletal muscle. Exercise training also increases IL-6 and IL-10 expression in intestinal lymphocytes of young C57BL/6 mice (Hoffman-Goetz et al., 2010). Finally, training increased expression of anti-inflammatory IL-4 in splenocytes from young BALB/c mice (Sugiura et al., 2002) and IL-1ra in smooth muscle from young Wistar rats (Gomez-Merino et al., 2007).

There is some support for the hypothesis that exercise training in older mice will prime the intestinal compartment towards an anti-inflammatory state (Gielen et al, 2003). This perspective is generated by findings from studies in young populations. Regular physical activity has been consistently shown to improve the immune profile across all ages (Chodzko-Zajko et al., 2009; Stessman et al., 2009) which may reduce (at least in part) immunoscenescent changes. Similar findings have been reported in humans as healthy sedentary males (45-64 years) demonstrated a significant reduction in serum IL-6 in response to 24-weeks of exercise training (Thompson et al., 2010). Moderate exercise training also decreased serum concentration of TNF-α in elderly (male) chronic heart failure patients (Smart et al., 2011).

Many of the changes in endogenous inflammatory cytokine production that accompany exercise training may be due to alterations in the number or ratio of memory to naïve T cells.

Woods and colleagues (1999) found that six months of exercise training increased the percentage and number of naïve T cells and decreased the number and percentage of memory T cells. Accordingly, exercise-induced changes in T lymphocyte subpopulations may account for some cytokine changes as exercise-induced reductions in memory T cells has been linked to decreased inflammatory cytokine production (Ibfelt et al., 2002).

CD28 (a membrane marker of naïve T cells) is responsible for antigen-mediated T cell activation, lymphocyte proliferation and T cell survival (Weng et al., 2009). $CD28^-$ T cells demonstrate significantly impaired immune functions including reduced antigen diversity, reduced responsiveness to new antigen stimulation and increased production of IFN-γ (Weng et al., 2009; Effros et al., 2005; Herndler-Brandstetter et al., 2008). Increased numbers of $CD28^-$ T cells are observed with increasing age. At birth essentially all T cells express the CD28 receptor. By age 80, however, up to 10-15% of $CD4^+$ ('helper' T cells) and 50-60% of $CD8^+$ ('cytotoxic' killer T cells) human T cells are $CD28^-$ (Fagnoni et al., 1996). This increase in $CD28^-$ T cells leads to impaired immune function, poor pathogen response and reduced vaccine effectiveness in the elderly (Saurwein-Teissl et al., 2002; Goronzy et al., 2004). Simpson and colleagues (2009) have shown that physically active middle-aged and elderly men have a lower percentage of circulating $CD28^-$ T cells compared with inactive individuals. Similar observations were made by Shimizu et al. (2008) who reported that exercise training in older adults (males and females, aged: 61-79 years) increased the number of $CD28^+$ T cells. Thus, exercise-induced increases in CD28 expression may explain some of the immune benefits of exercise in elderly populations.

To summarize, key findings from the literature are: (1) chronic inflammation of the gastrointestinal tract plays a major role in the aetiology of dysplasia and colorectal cancer

carcinoma; (2) acute exercise induces a transient inflammatory milieu within the gastrointestinal compartment; (3) immunoscenescent changes in cytokine and intestinal lymphocytes subpopulations bias towards an inflammatory intestinal environment; and (4) long-term exercise training decreases intestinal inflammation and induces anti-inflammatory cytokine expression. However, there are major gaps in the scope of current research. Primarily, most experimental studies have been conducted in young animal and human populations. To date, no research has investigated whether intestinal lymphocyte cytokine and apoptotic protein responses to acute exercise differ between young and old mice. In addition, it is unclear whether the 'anti-inflammatory' effects of exercise training would be seen in the gastrointestinal tract of older mice. It is also unclear how the significant immunological changes that occur with age might influence the acute exercise response in trained versus untrained mice. In light of these major research gaps, the main focus of this thesis was to describe the effect of long-term exercise training in older animals on intestinal lymphocyte cytokine and apoptotic protein expression at ‘rest’ and in response to acute treadmill exercise.

CHAPTER 2: Research Objectives, Components and Rationale

2.1 Research Objectives

Given the considerable Canadian burden of colorectal cancer and recent trends towards population aging, the effect of immunosenescence and inflammation on the risk of developing intestinal neoplasia is of particular concern. Moreover, numerous findings demonstrate the anti-inflammatory effect of regular physical activity in young populations. Given the lack of research examining this phenomenon in an older cohort, this thesis addressed the following.

The first objective was to compare intestinal lymphocyte cytokine and apoptotic protein expression in response to acute exercise-induced oxidant stress in young vs. older C57BL/6 mice. The second objective was to describe the effect of voluntary exercise training on the expression of pro- and anti-inflammatory cytokines and pro- and anti- apoptotic proteins in intestinal lymphocytes of older mice. The third objective was to compare the effect of acute exercise-induced oxidant stress on intestinal lymphocyte cytokine and apoptotic protein expression in trained vs. untrained older C57BL/6 mice. The overall goal of this research was to expand current knowledge on the effects of regular exercise training on (1) pro- and anti-inflammatory cytokine and (2) pro- and anti-apoptotic protein expression in intestinal lymphocytes of older individuals.

To accurately inform population-level public health interventions and cohort specific exercise recommendations it is crucial to understand the effects of acute exercise and exercise training on intestinal inflammation and apoptosis in animal models. Clarifying how training influences intestinal inflammation may provide new evidence linking physical activity and decreased cancer risk in the elderly.

2.2 Components and Rationale

Three experiments were conducted to address the research objectives.

2.2.1 Study #1

The inflammatory and apoptotic effects of exhaustive treadmill exercise were examined in the intestine of young and older C57BL/6 mice at multiple time points. The rationale for *Study #1* was based on previous research in young animals that indicates that inflammatory cytokine and apoptotic protein expression increases immediately (IMM) and 2 hours after (2Hrs) an acute treadmill exercise challenge compared to sedentary (SED) 'non-exercising' controls. Exhaustive treadmill running constitutes an oxidative challenge as confirmed by elevated plasma 8-iso-PGF_{2a}. There are no published accounts describing the effects of acute treadmill exercise on the intestinal cytokine and apoptotic response in older animals. Understanding how older mice respond to acute treadmill exercise informed the selection of appropriate time points for sacrifice and cytokine and apoptotic protein assessment in the main exercise training study.

2.2.2 Study #2

The effect of four months of voluntary (freewheel) running on inflammation and apoptosis in intestinal lymphocytes was examined under 'resting' conditions in older C57BL/6 female mice. The rationale was based on extensive research in young animals demonstrating that inflammatory cytokine and apoptotic protein expression is lower in intestinal lymphocytes of trained compared to untrained mice. There is considerable research describing the effect of exercise training on whole blood or plasma cytokine levels in elderly humans. However, there are few accounts detailing the effect of long-term exercise training on intestinal cytokine and apoptotic protein expression under resting conditions in elderly animals or humans. Baseline

measures are important to evaluate cumulative wheel running effects under normative conditions. Understanding how older mice respond to long-term freewheel running may elucidate potential mechanisms by which exercise reduces ‘inflame-aging’ or slows the progression of human colorectal carcinogenesis.

2.2.3 Study #3

The inflammatory and apoptotic effects of exhaustive treadmill exercise were compared in intestinal lymphocytes of trained versus untrained older C57BL/6 mice. The rationale was based on limited information about the effect of training on intestinal cytokine and apoptotic protein expression in response to acute exercise in the elderly. Some research suggests that the immune response to acute exercise is impaired with increasing age (Jozsi et al., 2000). Sacrifice and sample collection immediately post treadmill exercise is intended to increase knowledge about the time required for the transcription, translation and release of pro- and anti-inflammatory cytokines in colonic tissue of older animals (Hoffman-Goetz et al, 2010; Zhou et al, 2002; Zhou et al, 2003). To date, there are no published studies which have examined the effect of training on acute exercise-induced expression of cytokines and apoptotic proteins in intestinal lymphocytes of older mice.

CHAPTER 3: Apoptotic protein and inflammatory cytokine expression in intestinal lymphocytes after acute treadmill exercise in young and old mice

3.1 Objective

The objective of this experiment was to examine the effects of a single bout of exhaustive treadmill running on intestinal lymphocyte (IL) expression of the apoptosis-inducing cytokine TNF-α, the pro-apoptotic proteins caspase-3 and 7, the anti-apoptotic protein Bcl-2, and IL apoptotic status (% Annexin V^+) in young (3-4 months) and older (12-13 months) C57BL/6 female mice.

3.2 Hypothesis

Compared to young (3-4 months) animals, older (12-13 months) female C57BL/6 mice will have higher baseline (SED) and post-acute exercise (IMM and 2Hrs) expression of the apoptosis-inducing cytokine (TNF-α), pro-(caspases-3 and 7) apoptotic proteins and surface markers of early apoptosis (% Annexin V^+) in intestinal lymphocytes. They will also have lower expression of the anti-apoptotic protein Bcl-2 compared to young mice.

3.3 Experimental Design

This study was designed to evaluate the effect of a single episode of treadmill running on IL expression of the apoptosis-inducing cytokine (TNF-α), pro-(caspases-3 and 7) and anti-(Bcl-2) apoptotic proteins, and on surface markers of apoptosis (% Annexin V^+) in healthy young and older mice. The experimental design is shown in **Figure 3.1**.

Two-way analysis of variance (ANOVA) with age (two levels: young and older) and exercise challenge (three levels: SED, IMM and 2Hr) and Tukey's post-hoc test was used to

identify main effects and interactions between age and acute exercise groups (SPSS Version 19; Chicago, IL, USA). Homogeneity of variance was confirmed by Levene's test. Age and exercise challenge were the independent factors, and selected cytokines and apoptotic proteins were the dependent factors (SPSS for Windows Version 19; SPSS Inc, Chicago, IL, USA). Differences were accepted as significant from chance alone if $p<0.05$.

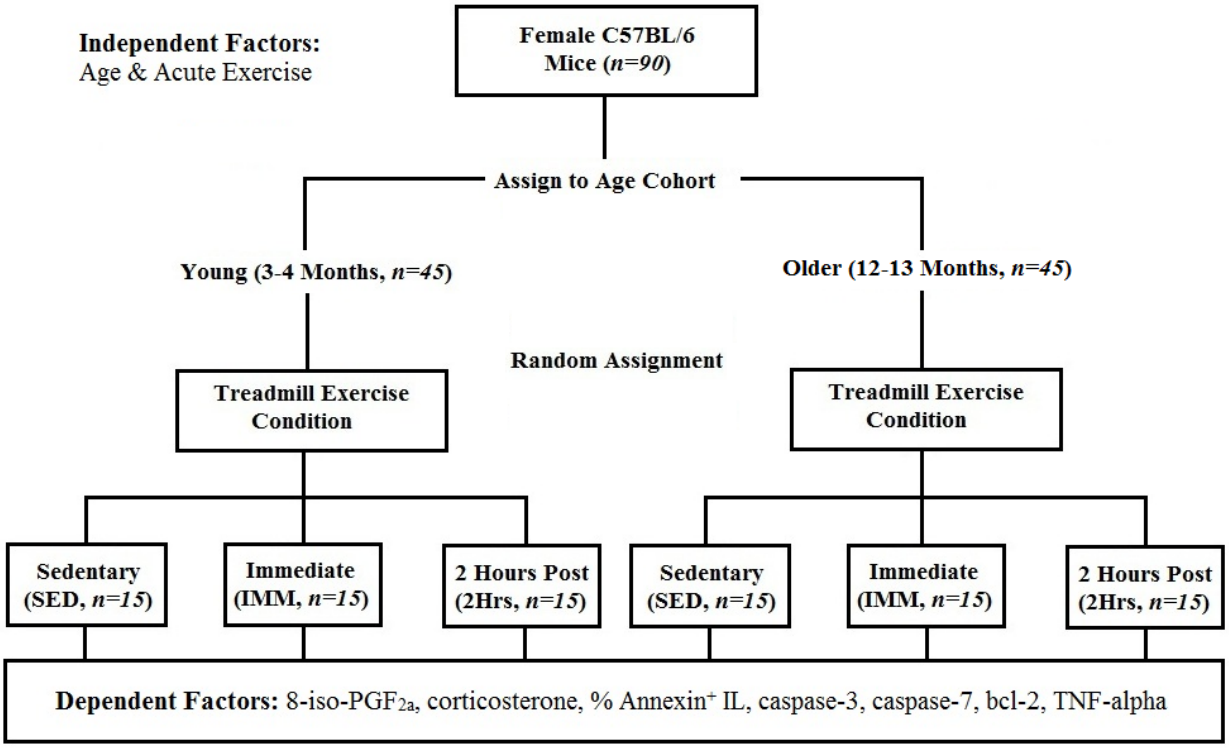

Figure 3.1 Study 1 - Outline of the experimental design.

The work presented in the remainder of this chapter has been accepted for publication as:

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3.4 Overview/Summary

Background: Gastrointestinal disturbances are common in athletes following intense exercise. Variations in apoptotic protein expression and cell death may contribute to acute exercise-induced intestinal inflammation. The effect of age on apoptotic protein response in the intestinal compartment in response to exercise is not known. **Aim:** Using a mouse model, we examined the effects of a single bout of treadmill running in young and old mice on (IL) expression of the apoptosis-inducing cytokine TNF-α, the pro-apoptotic proteins caspase-3 and 7, the anti-apoptotic protein Bcl-2, and IL apoptotic status (% Annexin V^+). **Methods:** young (3-4 months, n= 44) and old (12-13 months, n= 45) female C57Bl/6 mice were randomized to treadmill exercise (10 min warm-up, 20 min at 22 m min^{-1}, 30 min at 25 m min^{-1}, 30 min at 28 m min^{-1}, 2º slope) with sacrifice immediately (IMM) or 2hr after (2Hr), or to a non-exercised control (SED). IL were removed and prepared for analysis of % apoptosis (flow cytometry) and determination of apoptotic protein and cytokine expression (Western blotting). Plasma corticosterone and 8-iso-$PGF_{2\alpha}$ were measured by EIA. **Results:** Exercise was associated with a higher IL expression of caspase-3 in IMM and 2Hr groups vs. SED ($p<0.001$), a higher expression of TNF-α in the IMM group vs. SED ($p<0.001$), and a lower Bcl-2 expression in the IMM and 2Hr groups vs. SED ($p<0.01$). There was a trend ($p=0.07$) for increased caspase-7 expression after exercise. IL caspase-3 and 7 and TNF-α expression did not differ by age whereas Bcl-2 expression was lower

($p<0.001$) and % Annexin V^+ IL was higher ($p<0.05$) in old vs. young mice. Plasma corticosterone and 8-iso-$PGF_{2\alpha}$ were higher ($p<0.001$ and $p<0.05$) in IMM vs. SED mice but did not differ by age. **Conclusion:** The expression of the pro-apoptotic proteins, caspase-3 and caspase-7, and the apoptosis-inducing cytokine, TNF-α, in IL did not differ by age in this animal model in response to a single intense exercise challenge. However, old mice had lower expression of the 'protective' anti-apoptotic protein Bcl-2 and a higher percentage of early apoptotic IL. Whether repeated exercise results in less IL resiliency in elderly individuals remains to be determined.

3.5 Introduction

Athletes often experience pronounced gastrointestinal (GI) disturbances following strenuous exercise (Riddoch and Trinick, 1988). Choi et al. (2006) reported that well over 90% of long distance runners (22 out of 24) had upper GI mucosal lesions after exercise completion. Colonic lesions, bloody stools and diarrhoea were observed in men after long distance running (Sanchez et al., 2006). In response to an ultra-marathon (100 mile running race), approximately 85% of participants tested positive for gastrointestinal bleeding and lower GI symptoms (Baska et al., 1990). In a review of the clinical literature, Ho (2009) noted that lower GI disturbances were common among competitive endurance athletes and frequent recreational runners.

Although there are many proposed etiologic factors for intestinal problems observed in athletes, ischemia/reperfusion (I/R) injury is thought to be involved (Perko et al., 1998). During heavy exercise, splanchnic blood flow is reduced (Perko et al., 1998) and leads to I/R injury in human intestinal tissue. Oxidant stress arises during I/R and contributes to mucosal damage (Shah et al., 1999). Exercise-induced oxidant stress in the gastrointestinal tract also results in the

apoptotic loss of resident immune cells including intraepithelial and lamina propria lymphocytes (Quadrilatero and Hoffman-Goetz, 2004). This loss of lymphocytes is important because these cells serve an immunoregulatory and immunosurveillance function, protecting against dysplasia and intestinal carcinogenesis (Izcue et al., 2009).

Apoptosis is a form of programmed cell death (Wyllie, 2010). In the exercise context, there are two broad apoptotic pathways. The first is the intrinsic pathway whereby oxidant stressors trigger the leakage of mitochondrial caspases (e.g., caspase 3 and 7) into the cytosol with eventual membrane dysregulation, morphological changes and death (Wyllie, 2010). Cellular anti-apoptotic proteins, such as Bcl-2, help to prevent this caspase-induced cell damage (Kuwana and Newmeyer, 2003). The second is known as the extrinsic pathway and occurs through activation of transmembrane cell death receptors by certain pro-inflammatory cytokines (such as tumour necrosis factor-alpha [TNF-α]), ligation with TNF-R1, and Fas/FasL (CD95) activation (Mignini et al., 2008). Taun et al. (2008) reported an increase in plasma concentration of TNF-α and Fas ligand in response to 3 consecutive days of acute high-intensity exercise (85% $V0_2$ max, 30 min per day) in 12 healthy volunteers. Irrespective of which apoptotic pathway is activated by exercise, cell death occurs rapidly with the appearance of classical markers such as phosphatidylserine expression on the outer leaflet of the plasma membrane (Mignini et al., 2008). Intestinal lymphocyte (IL) apoptosis arising from intense exercise may contribute to gastrointestinal inflammation, associated symptomatology, and potentially the later development of bowel diseases (Packer et al., 2010; Ng et al., 2006).

Our knowledge about the effects of exercise on lymphocyte apoptosis and gastrointestinal inflammation has come largely from studies with young human subjects (Navalta et al., 2009).

However, there is some evidence of age-related differences in lymphocyte apoptotic response to acute exercise and oxidant stress. Goon et al. (2008) reported that older individuals have higher blood lymphocyte apoptosis both at rest and in response to Tai-Chi exercise. Higher apoptosis was observed in splenic lymphocytes of old compared to young mice sampled after exposure to the oxidant stressor 2-deoxy-D-Ribose (which depletes intracellular glutathione) (Schindowski et al., 2001). Elderly individuals (>60 years) had a greater percentage of apoptotic lymphocytes, compared to younger individuals (<35 years), in response to treatment with 2-deoxy-D-ribose and in response to exposure to anti-CD95 (Schindowski et al., 2000). This suggests that sensitivity to oxidant-induced reactive oxygen species and expression of Fas ligand (CD95) increases with age (Potestio et al., 1999). Old compared to young rats had a three-fold increase in colonic lymphocyte apoptosis in response to exposure to the oxidant azoxymethane (AOM) (Kwon et al., 2007). The apoptosis-inducing cytokine TNF-α was lower in older adults with higher levels of exercise compared to sedentary age-matched controls (Colbert et al., 2004). Gameiro et al. (2010) reported an increase in serum concentration of TNF-α in post-menopausal women. In contrast, lower production of TNF-α occurred in macrophages obtained from aged mice (Effros et al., 1991). Taken together, the direction of the effects – an increase or decrease in lymphocyte apoptosis and in TNF-α levels in response to intense exercise in older individuals - is unclear. Moreover, there is no research on how (or even if) age affects lymphocyte apoptosis and inflammatory cytokines in the gastrointestinal tract.

Understanding age-related apoptotic responses to exercise in the bowel is important for two reasons. First, with advancing age there is a decline in gastrointestinal-mucosal immune function and priming towards inflammation (Schiffrin et al., 2010). Second, although regular exercise conditioning is associated with many positive health effects and better functional

capacity in the elderly (Liao et al., 2011), little is known about acute exercise and lymphocyte function in the bowel. Given the increasing involvement of older adults in sport and physical activity (Petersen et al., 2010) and the higher incidence of bowel diseases with age (Schiffrin et al., 2010) it will be important to determine how exercise affects intestinal lymphocytes in older individuals.

Sampling tissue from the gastrointestinal tract of humans is problematic for several reasons. Tissue samples are normally limited to biopsies from individuals with pathologies such as inflammatory bowel diseases (IBD) (Pironti et al., 2010). Mucosal lymphocytes in patients with IBD show marked abnormalities in apoptotic responsiveness (Peppelenbosch et al., 2004) and TNF-α expression is increased in IBD (van Heel et al., 2002). In addition, tissue samples tend to be restricted anatomically to the large intestine (Pironti et al., 2010). Among the methodological advantages of using an animal model of exercise-induced lymphocyte apoptosis is the ability to directly obtain healthy gastrointestinal tissues for analysis. An animal model also allows for sufficient tissue collection for the creation of a single cell (intestinal lymphocyte) suspension.

Given the apoptotic effects of exercise-induced oxidative stress and the lack of data on lymphocyte apoptosis in the GI tract after exercise in the elderly, we examined the effects of a single episode of treadmill running in healthy young and old mice on the expression of the apoptosis-inducing cytokine (TNF-α), pro-(caspases-3 and 7) and anti-(Bcl-2) apoptotic proteins, and on surface markers of early apoptosis (% Annexin V^+) in intestinal lymphocytes. We hypothesized that old mice would have higher baseline and post-acute exercise expression of inflammatory and pro-apoptotic indices compared to young animals.

3.6 Materials and Methods

Animals: Female C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, IN) age, 3-4 months (n=45) and 12-13 months (n=45) were housed in groups of 4 per cage on a 12/12-hr reversed light/dark cycle at 21±1°C. Mice had *ad libitum* access to a standard rodent diet (Laboratory Rodent Chow, PMI Feeds, Richmond, IN, USA) and tap water throughout the experiments. All procedures were conducted in accordance with the ethical guidelines and protocols of the Canadian Council on Animal Care and were approved by the University Animal Ethics Committee.

Experimental Procedure: Young and old mice were randomized to one of three exercise conditions: exposure to a single bout of treadmill running with sacrifice 1) immediately (IMM, *n=15*) or 2) two hours (2Hr, *n=15*) after completion of the exhaustive exercise session, or to a 3) a non-exercised sedentary (SED, *n=15*) control that was exposed to treadmill noise and vibrations for 90 min without running. The exercise protocol consisted of a single, 90 min bout of treadmill exercise (10 min warm-up, 30 min at $22 m \cdot min^{-1}$, 30 min at $25 m \cdot min^{-1}$, and 30 min at $28 m \cdot min^{-1}$, 2° slope) (Panlab Mouse Treadmill, Harvard Apparatus Canada) during the dark cycle. Mice were motivated to run by prodding with a nylon test tube brush until the end of the run-to-exhaustion protocol or until reaching volitional fatigue.

Sample Collection

Intestinal lymphocyte (IL) isolation: Intestinal lymphocyte (IL) isolation was performed as described elsewhere (Hoffman-Goetz and Quadrilatero, 2003). Following sacrifice by sodium phenobarbital overdose, cold phosphate buffered saline (PBS) was used to wash the

excised intestinal compartment, Peyer's patches and visible fat was removed and single cell IL suspensions prepared by isolation over a pre-washed nylon wool (0.3g) column. The eluted cells were layered over a Lympholyte-M density gradient medium (Cedarlane Laboratories) and centrifuged to remove debris. The remaining IL pellet (containing iIEL and LP lymphocytes) was suspended in 400µL of PBS. Turk's staining solution (99µL) was used to enumerate intestinal lymphocytes (1µL) by light microscopy.

Determination of Outcome Measures

Western blot analysis of apoptotic proteins: IL were fractionated in lysis buffer on ice for 45 min. Lysates ($1x10^5$) were centrifuged (15 min, 10 000g) and supernatant extracted. A BCA assay was used to determine protein concentration. Protein (40 µg) and selected molecular weight markers (Full Range Rainbow, Amersham Biosciences) were electrophoresed on a 12% SDS-PAGE gel before transfer to PVDF membrane. After electrophoresis, membranes were incubated for 1hr with primary antibody (1:200 in 10% milk-TBST): TNF-α (goat anti-mouse polyclonal IgG), caspase-3 (rabbit anti-human polyclonal IgG), caspase-7 (mouse anti-human monoclonal IgG_1) and Bcl-2 (C-2, mouse anti-human monoclonal IgG_1) (Santa Cruz Biotechnology). Membranes were incubated for 1hr with secondary antibody: horseradish peroxidise-conjugated anti-goat (TNF-α), anti-mouse (caspase-7, Bcl-2) or anti-rabbit (caspase-3) IgG at a concentration of 1:2000 in 10% milk-TBST. ECL Plus detection reagent (Amersham Biosciences) and the ChemiGenius 2 Bio-imaging System were used for protein determination. A biotinylated protein ladder was used to identify the molecular weight of selected proteins (Cell Signalling Technology). Recombinant standards (Cedarlane Laboratories) were run on each gel. Samples from each experimental condition were run on each immunoblot and band densities

were normalised to SED control bands on each immunoblot (units reported as arbitrary densitometric units [A.U.]) for each group.

Flow cytometric analysis: The extent of IL apoptosis was measured using flow cytometry. IL cells (1×10^5 cells) were incubated for 15 min with 2.5µl of Annexin V-FITC (Pharmingen) and 100µl of Annexin binding buffer in the dark at room temperature. Annexin V^+ cells indicate cell membrane expression of the early marker of apoptosis, phosphatidylserine. Following incubation, 400µl of Annexin binding buffer was added and % Annexin V^+ determination was carried out using an Epics XL Flow Cytometer (Beckman Coulter) with an excitation wavelength of 488nm. A detailed description of flow cytometric procedures to characterize IL apoptosis is described elsewhere (Marra et al., 2005).

Plasma Measures of Oxidative Stress

8-iso-$PGF_{2\alpha}$ and Corticosterone: Immediately following sacrifice, a 1-mL syringe containing heparin was used to collect blood via cardiac puncture. Plasma was separated by centrifugation (6 min at 400 rpm) and frozen at -80^0C for determination of 8-iso-$PGF_{2\alpha}$ (8-iso-prostanes) and corticosterone. Plasma 8-iso-$PGF_{2\alpha}$ was assessed by a commercially available kit using direct enzyme immunoassay (EIA) as per the manufacturer's specifications. Samples (100µL sample, 25µL of 10N NaOH) were hydrolyzed for 2 hr at 45°C, neutralized to pH 6-8 with 12N HCl, centrifuged at 14,000 g for 5 min, and incubated with 8-iso-$PGF_{2\alpha}$ antibody for 24 hr at 4°C. Percent absorbance was read at 412 nm at room temperature using a PowerWave 340 microplate spectrophotometer (Biotek Instruments). Plasma corticosterone was measured using a commercially available EIA kit, according to the manufacturer's instructions (Cayman

Chemical). The cold spike protocol was used for the purification of plasma samples and corticosterone concentrations measured using a Power Wave 340 microplate spectrophotometer (Biotek Instruments) at 412nm.

Statistical Analysis: All variables were analyzed using two-way ANOVA with age (two levels: young, old) and exercise challenge (three levels: SED, IMM, 2Hr) as the independent factors, and with cytokine and apoptotic proteins as the dependent factors (SPSS for Windows Version 19; SPSS Inc, Chicago, IL, USA). Tukey's HSD was used as a *post hoc* analysis to identify main effects and interactions between age and acute exercise groups. All results were checked for homogeneity of variance using Levene's test for equality. Differences were accepted as significant from chance alone if $p<0.05$ and all values are expressed as group means ± 1 SEM for respective units.

3.7 Results

Body weight and treadmill running times: Not surprisingly, old mice were significantly heavier than young mice (32.1±3.6g vs. 21.6±1.6g, respectively) ($F_{(1,88)}=325.81$, $p<0.001$) (Table 3.1). Old mice also ran for a shorter time (52.4±3.2 min) than did young mice (90.7±3.2 min) before reaching volitional fatigue $F_{(1,88)}=71.65$, $p<0.001$). No significant interaction between age and exercise condition was observed.

Plasma markers of oxidative stress: Figure 3.2 (Panels A and B) shows the plasma concentrations of 8-iso-$PGF_{2\alpha}$ and corticosterone to acute treadmill exercise in mice. There was a significant effect of exercise ($F_{(1,56)}=10.46$, $p<0.01$) on plasma 8-iso-$PGF_{2\alpha}$ concentration due to higher levels in the IMM (153.6±11.3 pg/ml) compared to SED (102.3±11.1 pg/ml) mice. There

was no difference in plasma 8-iso-$PGF_{2\alpha}$ concentration between young and old mice nor was there an interaction between age and exercise. Since plasma 8-iso-$PGF_{2\alpha}$ is a classic indicator of oxidative stress, it can be inferred that despite the differences in time to volitional fatigue between young and old mice, the exercise bout was similarly stressful for both ages. Exercise was associated with higher plasma corticosterone concentrations ($F_{(1,58)}$=162.70, *p<0.001*) due to elevations in IMM (256.1±9.9 ng/ml) compared to SED concentrations (75.1±10.1 ng/ml). Although corticosterone responses did not differ by age, there was a marginally significant interaction ($F_{(1,58)}$=4.04, *p<0.05*) due to the greater magnitude of post-exercise corticosterone increases in old compared to young mice.

Intestinal lymphocyte counts and apoptosis indices: Old and young mice did not differ significantly ($F_{(1,82)}$= 1.21, *p=0.274*) on IL counts (Old: $3.9 \times 10^7 \pm 1.7 \times 10^6$ cells / 400µl; Young: $4.1 \times 10^7 \pm 1.7 \times 10^6$ cells / 400µl). However, a significant main effect of exercise on IL counts was found ($F_{(2,82)}$=4.96, *p<0.01*) with IL numbers lower in SED ($3.8 \times 10^7 \pm 2.1 \times 10^6$ cells / 400µl) and 2Hr ($3.7 \times 10^7 \pm 2.0 \times 10^6$ cells / 400µl) groups compared to IMM ($4.6 \times 10^7 \pm 2.1 \times 10^6$ cells / 400µl). No significant age x exercise interaction on IL count was found. Exercise did not result in differences in the percentage of Annexin V^+ cells (i.e., apoptotic IL). Old mice had a marginally greater percentage of Annexin V^+ IL cells ($F_{(1,82)}$=4.47, *p=0.038*) compared with young mice (Old: 31.9±1.7 % Annexin V^+; Young: 27.0±1.6 % Annexin V^+). No statistically significant interaction effect between age x exercise was observed for the percentage of Annexin V^+ mouse IL. These results are shown in Table 3.1.

Pro- and Anti-Apoptotic Proteins: The effects of age and exercise on the expression of pro- and anti-apoptotic proteins in mouse IL are shown in Figure 3.3. There was no significant

age effect on the expression of the pro-apoptotic proteins caspase-3 or caspase-7 in mouse IL. However, Bcl-2 expression was significantly lower in the IL obtained from old (1.07±0.08 A.U.) compared to young (1.63±0.09 A.U.) mice ($F_{(1,78)}$=22.68, *p<0.001*). The expression of caspase-3 in mouse IL was significantly affected by exercise ($F_{(2,74)}$=15.18, *p<0.001*) due to higher concentrations at the IMM (1.13±0.08 A.U.) and 2Hr (1.25±0.08 A.U.) post-exercise time points compared to SED (0.67±0.08 A.U.). There was also a non-significant trend for greater caspase-7 expression in mouse IL after exercise ($F_{(2,81)}$=2.80, *p=0.07*). IL Bcl-2 expression was lower after exercise relative to the pre-exercise (SED) condition ($F_{(2,78)}$=7.20, *p<0.001*) (SED: 1.65±0.11 A.U.; IMM: 1.29±0.10 A.U.; 2Hr: 1.11±0.10 A.U.). There were no significant interactions between age and exercise for caspase-3 or caspase-7. There was a small and marginally significant interaction between age and exercise for Bcl-2 expression in mouse IL ($F_{(2,78)}$=3.48, *p<0.05*).

Inflammatory Cytokines: The effect of age and exercise on the expression of the apoptosis-inducing cytokine TNF-α in mouse IL is shown in Figure 3.3. Old and young mice did not differ in the expression of TNF-α in IL. However, IL expression of this cytokine was markedly affected by exercise ($F_{(2,85)}$=22.01, *p<0.001*) with higher levels of TNF-α IMM after exercise (1.20±0.05 A.U.) compared to SED (0.89±0.05 A.U.) and 2Hr (0.79±0.05 A.U.). There were no statistically significant interaction effects between age cohort and exercise for TNF-α expression in mouse IL.

Age Group	Exercise Condition	Body Weight	RTE	% Annexin^{+} IL
Old	SED	31.4 ± 3.3[a]	--	30.4 ± 11.4[a]
	IMM	31.2 ± 3.5[a]	53.6 ± 4.5[a]	32.7 ± 11.3[a]
	2 Hrs	33.7 ± 3.8[a]	51.3 ± 4.5[a]	32.5 ± 9.1[a]
Young	SED	21.8 ± 2.0[b]	--	23.5 ± 8.0[b]
	IMM	21.5 ± 0.9[b]	90.6 ± 4.5[b]	23.8 ± 14.6[b]
	2 Hrs	21.6 ± 1.7[b]	90.7 ± 4.5[b]	24.7 ± 7.6[b]

Table 3.1 Body weight (g), treadmill run-to-exhaustion time (min) and % Annexin V^{+} IL in C57BL/6 mice. Values are means ± one standard error. Superscripts: a vs. b *(p <0.05)*; a vs. a or b vs. b *(p >0.05).* See text for details of statistical analysis.

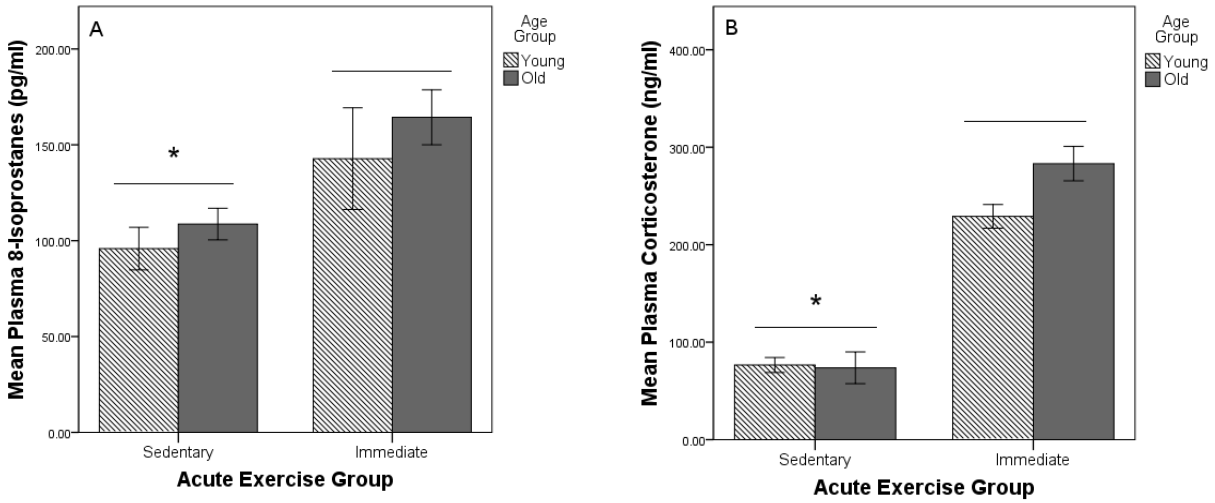
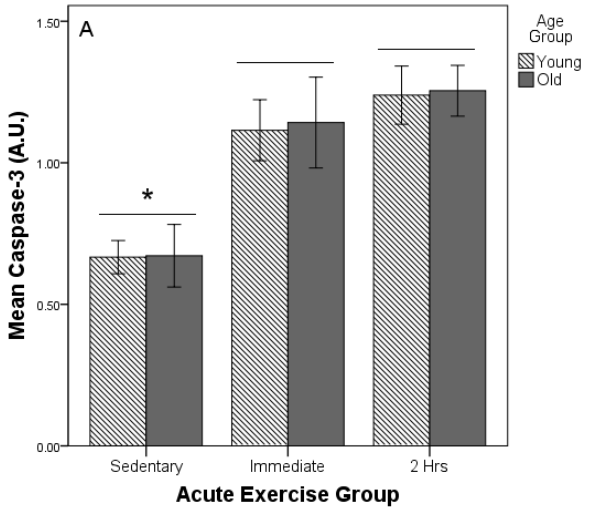
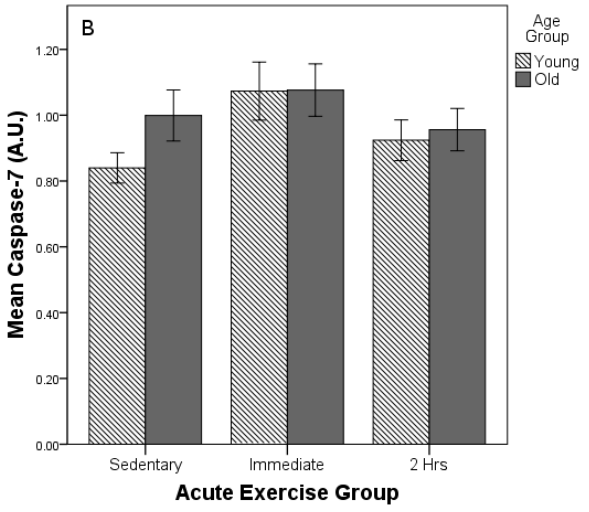


Figure 3.2 Plasma 8-iso-$PGF_{2\alpha}$ (Panel A) and Corticosterone (Panel B) concentrations of mice as a function of age and exercise condition. Values are means ± one standard error. Data were analyzed using 2x2 ANOVA with Tukey's post-hoc. * $p<0.05$. See text for details of statistical results.

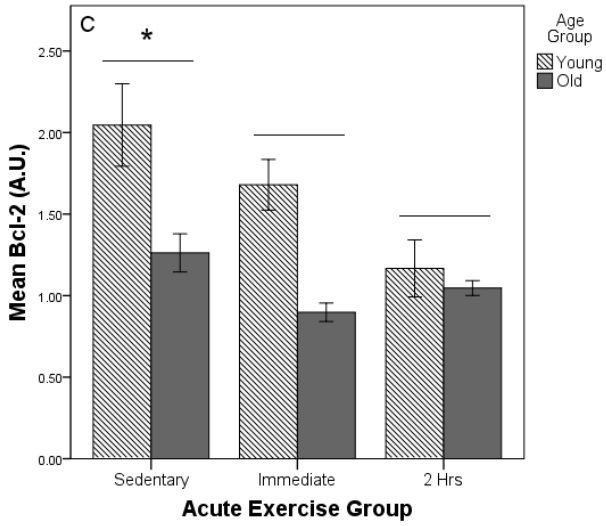
Panel A: Caspase-3



Panel B: Caspase-7



Panel C: Bcl-2



Panel D: TNF-α

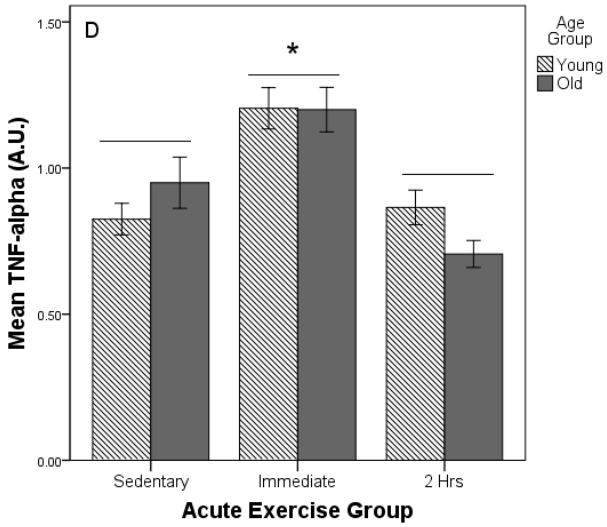


Figure 3.3 Caspase-3 (Panel A), Caspase-7 (Panel B), Bcl-2 (Panel C) and TNF-α (Panel D) expression in intestinal lymphocytes of mice as function of age and exercise condition. Values are means ± one standard error. Data were analyzed using 2x2 ANOVA with Tukey's post-hoc. *$p<0.05$. See text for details of statistical results.

3.8 Discussion

This study evaluated whether old and young C57BL/6 mice differed in their intestinal lymphocyte apoptotic responses to exercise-induced oxidant stress. We hypothesized that older animals would have higher baseline and post-exercise expression of inflammatory and pro-apoptotic indices when compared to young animals. Instead, we found that the expression of the pro-apoptotic proteins, caspase-3 and caspase-7, and the apoptosis-inducing cytokine, TNF-α, in mouse IL did not differ by age in response to exercise challenge. Further, the intrinsic (i.e., caspase-3 and caspase-7 findings) and extrinsic (TNF-α) pathways of apoptosis were both affected by intense exercise in the two age groups.

These findings are novel and indicate that the exercise-induced oxidant stress effect on GI tract lymphocytes does not differ by age. Both age cohorts experienced similar levels of plasma lipid peroxide products (i.e., 8-iso-$PGF_{2\alpha}$). Although oxidant stress has been reported to result in greater apoptosis of splenic lymphocytes from older vs. young individuals (Schindowski et al., 2001), this effect is not uniform across tissues (Navarro et al., 2004). Oxidative stress and its effects also vary by anatomical location in the bowel (Will et al., 2010). Hence, the lack of difference by age in IL apoptotic protein expression after a single bout of intense exercise in mice may reflect differences in the physiologic environment of the bowel compared with blood lymphocytes. For example, flow cytometric analysis of intestinal lymphocytes IL and peripheral blood lymphocytes (PBL) revealed that PBL consist of twice as many $CD4^+$ cells as $CD8^+$ cells, whereas IL (and especially intraepithelial intestinal lymphocytes) display three to five times as many $CD8^+$ cells as $CD4^+$ cells (Waters et al., 1995). Gamberale et al. (2003) 0have shown that exposure of peripheral blood mononuclear cells to fludarabine, a cytotoxic adenosine analogue,

induced a higher level of apoptosis in CD8 T lymphocytes compared to CD4 T cells. Such phenotypic divergences, and corresponding variations in apoptotic susceptibility, may explain different responses to oxidative stress between various tissues.

Ischemia-reperfusion (I//R) injury in the gastrointestinal tract is a problem for the elderly (Shah et al., 1999). I/R injury can arise following intense exercise and is associated with the production of reactive oxygen species, superoxide ions and hydrogen peroxide, products that arise from hypoxanthine production of uric acid (Shah et al., 1999). In healthy tissue, under standard conditions, ROS are scavenged by endogenous antioxidant enzymes (i.e. superoxide dismutase, catalase and glutathione peroxidise) (Shah et al., 1999; Schindowski et al., 2001). However, ROS accumulation is observed in immunosenscence and may be due to age-related reductions in catalase (Shah et al., 1999). However, Shah and colleagues (1999) showed that there were no significant differences in I/R injury in the bowel of aged and young mice as measured by malondialdehyde (an indicator of lipid peroxidation) and by myeloperoxidase activity (as a measure of neutrophil accumulation and inflammation). In our study, both age groups had similar levels of plasma 8-iso-$PGF_{2\alpha}$, experienced similar levels of exercise-induced stress, and did not differ in the expression of apoptosis-inducing enzymes and cytokines (caspase 3, caspase 7 and TNF-α) following aerobic challenge. Nevertheless, the intestine of old mice may be more susceptible to exercise-induced bowel injury than young mice: expression of the anti-apoptotic protein, Bcl-2, was lower in IL from aged compared to younger animals. Bcl-2 is a critical factor involved in the regulation of lymphocyte apoptosis (Wyllie, 2010) and inhibits apoptosis by binding to the mitochondrial membrane or to caspase-activating factors or to active caspase complexes (Kuwana and Newmeyer, 2003). Over-expression of Bcl-2 protects cells

against injury (Mitchell et al., 2011). Whether repeated exercise challenge in old mice results in further depletion of Bcl-2, greater susceptibility to IL apoptosis, and higher risk of I/R injury is an area for future investigation. Such hypotheses follow from the observed age-related differences in post-exercise Bcl-2 depletion in IL. A constant linear decrease in Bc-2 concentration was observed across all post-exercise time-points for young mice. However, in old mice, Bcl-2 levels were elevated from IMM to 2Hr after exposure to oxidant stress. These differences in Bcl-2 reaction kinetics may be a result of immunosenescence. Alternatively, this phenomenon may be due to differences in the 'running duration to recovery time' ratio between old and young mice as a result of longer RTE times in young than in old mice.

An important finding of this study was a higher percentage of IL from old mice expressing Annexin V^+, an indicator of early apoptosis. A similar elevation in phosphatidylserine expression on human lymphocytes from elderly men (mean 70 yrs), compared to young men (mean 29 years), has been reported (Noble et al., 1999). Whether the higher percentage of early apoptotic IL in old mice is due to baseline depletion of Bcl-2 cannot be experimentally determined from our study. Given the anti-apoptotic role of Bcl-2 in preventing the cleavage of caspase zymogens into their active form, (Wyllie, 2010; Kuwana and Newmeyer, 2003) it is possible that age-related reductions in Bcl-2 might contribute to elevated phosphatidylserine expression and lymphocytopenia. However, it is also possible that the elevations in baseline phosphatidylserine expression may be due to immunosenescence-associated impairment in Ca^{2+} influx (Ayub and Hallet, 2004) or other cellular effects of aging, including ROS accumulation (Shah et al., 1999).

There are several limitations with this study. First, the old mice were not necessarily senescent. The average lifespan of C57BL/6 mice is 26-28 months [1] (Goodrick, 1975). Thus, the 'Old' mice, which were 12-13 months old at sacrifice, may correspond more closely - in terms of immunologic and physiologic characteristics - to late-middle age or young elderly people. It is possible that older animals would have greater age effects on apoptotic protein and apoptosis-inducing cytokine expression. Second, the severity of the acute exercise bout may 'overwhelm' any potential differences in oxidant-buffering capacity by age; particularly given age-associated increases in susceptibility to oxidative stress (Schindowski et al., 2001). The magnitude and direction of the inflammatory and GI response to exercise challenge is dependent on the type (e.g. running, cycling, swimming) (Goon et al., 2008), duration (e.g. 30 min, 2Hrs, 10 km, 42km) (Sanchez et al., 2006), and relative intensity (e.g. 45% $VO_{2\ Max}$, 85%MHR) of the exercise protocol (Tuan et al., 2008). The current exercise challenge may not be sufficiently sensitive to tease out the effect of age on apoptosis; using an oxidative challenge of lesser duration or intensity may be required to produce an observable post-exercise difference in IL apoptosis or apoptotic protein expression. Third, IL apoptosis may not lead to gastrointestinal inflammation. Though intestinal inflammation and impaired lymphocyte apoptosis have been linked in inflammatory bowel disease models (Ng et al., 2006), a causal relationship has yet to be examined in healthy human tissue. Fourth, although both young and old mice were fed the same standard 'Laboratory Rodent Chow', it is possible that caloric intake varied between the two age cohorts. Given the effect of energy restriction on adaptive immune function in colonic lamina propria, future studies might consider measuring energy intake as a potential confounding

[1] The lifespan of C57BL/6 mice has been reported to range from 20-22 months (Anisimov, 2009) to 26-28 months (Goodrick, 1975) depending on genetic substrain, gender and housing/breeding conditions.

variable (Nayak et al., 2009). Finally, no measures of rectal or gastrointestinal distress (i.e., bleeding, stool frequency etc.) that typically accompany the study of tissue ischemia/reperfusion injury were recorded. The collection of these qualitative measures was not possible given the constraints of an animal model, including the necessity of sacrifice and intestinal lymphocyte lysate collection immediately post-exercise.

3.9 Conclusion

In conclusion, we observed an increased expression of apoptotic proteins and cytokines in intestinal lymphocytes of old and young mice following an acute aerobic exercise challenge. The expression of Bcl-2, an anti-apoptotic protein, was lower in IL from old relative to young mice. The implications of these findings to gastrointestinal ischemia/reperfusion injury among older individuals engaging in heavy exercise is yet undetermined. However, given the public health messages promoting physical activity as people age, it will be important to determine the intensity of exercise that does no gastrointestinal harm.

CHAPTER 4: Exercise training reduces inflammatory mediators in the intestinal tract of healthy older adult mice

4.1 Objective

The objective of this experiment was to describe the effect of four months of voluntary freewheel running on intestinal lymphocyte (IL) expression of inflammatory cytokines and apoptotic proteins in healthy older (15-16 months) female C57BL/6 mice.

4.2 Hypothesis

Long term, voluntary freewheel running will decrease intestinal lymphocyte expression of pro-inflammatory (TNF-α, IL-1β) and pleiotropic (IL-6) cytokines and apoptotic proteins (caspase-3, caspase-7) in trained older C57BL/6 female mice relative to untrained (sedentary) older C57BL/6 controls.

4.3 Experimental Design

This study examined the effects of long-term voluntary exercise on the expression of inflammatory mediators in the intestine of healthy older (15-16 months) C57BL/6 mice. The experimental design is shown in **Figure 4.1**.

One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to analyze cytokine and apoptotic protein expression in IL of 'wheel running' (WR) and 'no wheel running' (NWR) older mice. Levene's test was used to check for homogeneity of variance. The two levels of freewheel exercise were the independent factors, and selected cytokine and apoptotic proteins were the dependent measures (SPSS Version 19; Chicago, IL, USA). The inflammatory markers chosen included the classic pro-inflammatory (TNF-α, IL-1β) and pleiotropic (IL-6) cytokines

implicated in intestinal inflammation and CRC progression. In addition, the IL expression of two apoptotic proteins (caspase-3 and -7) was assessed because they are activated by TNF-α, IL-1β and IL-6 (Wyllie, 2010). Significant differences were accepted if $p < 0.05$.

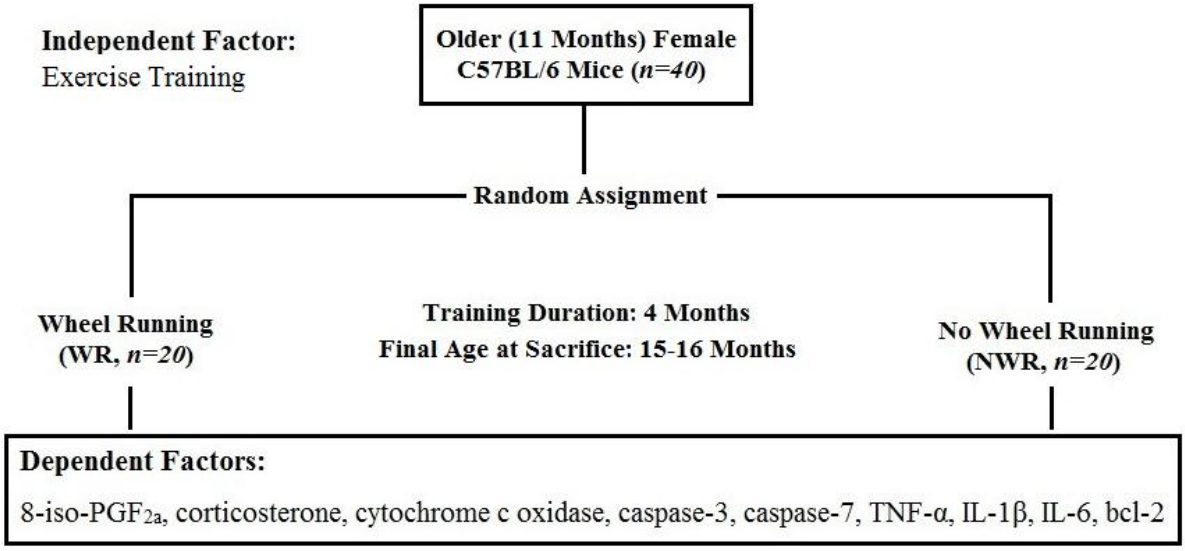


Figure 4.1 Study 2 - Outline of the experimental design.

The work presented in this chapter is under review as:

Packer N, Hoffman-Goetz L. (2011). Exercise training reduces inflammatory mediators in the intestinal tract of healthy older adult mice. *Canadian Journal on Aging*.

4.4 Overview/Summary

Aging is associated with increased intestinal inflammation and elevated risk of chronic diseases including inflammatory bowel diseases and colon cancer; many epidemiologic studies show that regular exercise reduces risk. This study examined the effects of long-term voluntary exercise on the expression of inflammatory mediators in the intestine of older (15-16 months) healthy C57BL/6 mice. Animals were assigned to 4 months of freewheel running (WR; n=20) or to a 'sedentary' (NWR; n=20) control group. Intestinal lymphocytes were analyzed for the expression of (1) pro-inflammatory (TNF-α, IL-1β) and pleiotropic (IL-6) cytokines and (2) pro-(caspase-3/-7) and anti-(Bcl-2) apoptotic proteins. Training was confirmed by skeletal muscle enzyme activity; stress was assessed by plasma 8-iso-$PGF_{2\alpha}$ and corticosterone. Results showed that WR mice had a lower expression of TNF-α, caspase-7, and 8-isoprostanes ($p<0.05$) compared to sedentary controls. This suggests that long-term exercise may 'protect' the bowel from inflammation by reducing inflammatory cytokine and apoptotic protein expression.

4.5 Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality among older Canadians (Canadian Cancer Society, 2011). Despite the complex etiology of CRC, chronic inflammation is a fundamental risk factor (Demarzo et al., 2008). This is evidenced by elevated CRC risk among persons with inflammatory bowel disease (IBD), an increase proportional to the extent and

duration of ulcerative colitis (Kulaylat and Dayton, 2010). Adult Canadians have among the highest IBD incidence rates worldwide (Bernstein et al., 2006). IBD is particularly debilitating among seniors, as 10-15% of incident cases are diagnosed in patients 60 years of age or older (Softley et al., 1988) and the presence of age-related co morbidities may interfere with effective diagnosis and treatment (Swaroop, 2007).

It has been hypothesized that CRC pathogenesis may be influenced by inflammatory immune mechanisms (Terzić et al., 2010). Two inflammatory cytokines, tumour necrosis factor-alpha (TNF-α) (Grimm et al., 2010) and interleukin-1 beta (IL-1β) (Szkaradkiewicz et al., 2010), are elevated in the bowel of CRC patients and elevated TNF-α predicts both the extent of carcinogenesis and likelihood of tumour recurrence (Grimm et al., 2010). Furthermore, immunosenescence (age-related immune changes) may increase cancer risk due to increases in chronic low-grade inflammation (Singh and Newman, 2010). Even healthy elderly individuals demonstrate increased expression of interleukin-6 (IL-6), TNF-α, and IL-1β in peripheral mononuclear cells (Fagiolo et al., 1993) and increased IL-6 and TNF-r1 in plasma (Stowe et al., 2010).

Colorectal cancer has a long asymptomatic period and physical symptoms typically appear only in advanced stages of disease when the prognosis is largely pre-determined and treatment is limited (Gonzalez-Hermoso et al., 2004). Accordingly, screening programs and strategies for risk factor reduction are critical avenues to reduce disease burden in the elderly (Edwards et al., 2010). Since 40-50% of CRC patients die within five years of initial diagnosis (Compton et al., 2000), even a modest delay in disease onset could significantly improve individual and population health outcomes (Luo et al., 2010). Physical activity (PA) is a

promising lifestyle intervention identified to reduce CRC incidence (Colditz et al., 1997) with a relative risk reported of 0.76 (95% CI 0.72 – 0.81) (Wolin et al., 2009) and 0.79 (95% CI 0.72 – 0.87) (Samad et al., 2005) among regular exercisers compared to inactive individuals. The relationship between increased PA and lowered CRC risk follows a strong dose-response curve (Thune and Furberg, 2001) and it is possible that regular exercise may reduce the risk of CRC recurrence (Halle and Schoneberg, 2009).

The mechanisms whereby regular PA protects against inflammatory bowel diseases and CRC in older people are not well known. One possibility is that PA prevents age-related increases in inflammation by altering the intestinal inflammatory cytokine and apoptotic milieu. In young animals, long-term voluntary PA reduces intestinal TNF-α expression (Hoffman-Goetz et al., 2009). This finding has recently been confirmed in elderly humans: long-term (8.6±0.3 months) resistance training reduced plasma TNF-α levels (Córdova et al., 2011). Moreover, Gomez-Merino and colleagues (2007) found that exercise training reduced IL-1β in rat adipose tissue and skeletal muscle (Lira et al., 2009). In response to long term PA, IL-6 increases in the intestinal compartment (Hoffman-Goetz et al., 2010) and in skeletal muscle (Pedersen et al., 2003) of young animals but decreases in older individuals (Nicklas et al., 2008).

The intestinal compartment is populated by a number of immunologically active cells. Lamina propria (LP), intraepithelial (iIEL) and intestinal (IL) lymphocytes play an important role in homeostasis (Lefrancois and Lycke, 2001) by protecting against inflammation (Iliev et al., 2009) and by regulating the secretion of key cytokines (Powrie, 2004).

It is difficult to evaluate the extent of lymphocyte involvement in intestinal homeostasis in healthy adults. Utilizing an animal model circumvents the problem of invasive tissue sampling

from healthy people. Moreover, tissue biopsies are typically limited to individuals with intestinal pathologies and are restricted anatomically to the large intestine (Pironti et al., 2010). Major advantages of using an animal model include: (1) the ability to obtain healthy intestinal tissue for analysis; (2) sufficient tissue collection for assessment of multiple biomarkers and mediators; and (3) detailed physiological measurement and validation of 'training' status. An animal model also allows precise age determination, a key consideration when examining the effects of training in an 'aged' cohort.

The purpose of this study was to describe the effects of long-term voluntary physical activity on the expression of inflammatory cytokines and apoptotic proteins in intestinal lymphocytes of healthy older adult animals. The cytokines chosen were the classic pro-inflammatory (TNF-α, IL-1β) and pleiotropic (IL-6) cytokines implicated in intestinal inflammation, and IBD and CRC progression. In addition, the expression of two apoptotic proteins (caspase-3 and -7) in intestinal lymphocytes was assessed because they are activated by TNF-α, IL-1β and IL-6 (Wyllie, 2010) and induce cell death. We hypothesized that long term, low stress PA in mice would decrease the intestinal expression of pro-inflammatory (TNF-α, IL-1β) and pleiotropic (IL-6) cytokines and apoptotic proteins (caspase-3, caspase-7) relative to untrained (sedentary) age matched controls.

4.6 Materials and Methods

Animals: Female C57BL/6 mice (*n = 40*) (Harlan, Indianapolis, IN, USA), 11-12 months old, were individually housed at 21±1°C on a 12/12-hr reversed light/dark cycle. Mice had *ad libitum* access to standard rodent diet (Lab Rodent Chow, PMI Feeds, IN, USA) and tap water throughout the experiments. All experiments were conducted in accordance with the ethical

guidelines and protocols of the Canadian Council on Animal Care and were approved by the University Animal Ethics Committee.

Exercise-Training Protocol: Mice were matched by weight and randomized to an exercise-training condition: access to in-cage running wheels (WR, *n = 20*) or to a no running wheel cage (NWR, *n = 20*) for 4 months. A magnetic switch attached to each wheel (23 cm in diameter) and an automated computer monitoring system (Vital View Application software, Mini-Mitter, Sunriver, OR) captured the number of completed revolutions. Activity during the dark cycle was recorded as the number of revolutions completed per 15-min interval, converted to distance run (km), and summed by day, week, and month. Total running volume was monitored as an indicator of training status, in combination with skeletal muscle cytochrome *c* oxidase activity and body weight.

Skeletal muscle cytochrome *c* oxidase (CO) activity: Cytochrome *c* oxidase (CO) plays an important role in determining the mitochondrial respiratory capacity of skeletal muscle as it constitutes the last step of ATP generation. Following sacrifice by sodium pentobarbital (0.6–0.8 cc per mouse, i.p.) overdose, soleus (SOL) and plantaris (PLANT) muscle samples were isolated from WR and NWR mice, frozen in liquid nitrogen, and stored at -80°C until assayed. Muscles were cut into 5–10mg segments, mashed and homogenized in buffer [glycerol (50%), sodium phosphate buffer (20mM), 2-mercaptoethanol (5mM), ethylenediaminetetraacetic acid (EDTA, 0.5mM), BSA (10%)] to yield a 50:1 dilution, and sonicated (using a 3 m tip, 2 sec on, 5 sec off for a total of 20 sec at 60 Hz; Vibra Cell, Sonics and Materials, Danbury, CT, USA). Protein content was determined by Lowry assay (Lowry et al., 1951). Muscle homogenates were diluted to 1:500 dilutions in 10mM potassium phosphate buffer. 20µL of reduced cytochrome *c* and

10µL of diluted homogenate were combined with 970µL of warmed (37°C) phosphate buffer. Cytochrome *c* absorbance was determined spectrophotometrically at 550 nm.

Plasma Measures of Stress (8-iso-$PGF_{2\alpha}$ and Corticosterone): Plasma 8-iso-$PGF_{2\alpha}$ and corticosterone were measured to assess stress (oxidative for the former and psychological for the latter). This procedure and rationale have been described extensively elsewhere (Hoffman-Goetz et al., 2010). In brief, immediately following sacrifice, a 1-mL syringe containing heparin was used to collect blood via cardiac puncture. Plasma was separated by centrifugation (6 min at 400 rpm) and frozen at -80°C. Plasma 8-iso-$PGF_{2\alpha}$ was assessed by a commercially available kit using direct enzyme immunoassay (EIA) as per the manufacturer's specifications (Cayman Chemical). Samples (100µL sample, 25µL of 10N NaOH) were hydrolyzed for 2 hr at 45°C, neutralized to pH 6-8 with 12N HCl, centrifuged at 14,000 g for 5 min, and incubated with 8-iso-$PGF_{2\alpha}$ antibody for 24 hr at 4°C. Percent absorbance was read at 412 nm at room temperature using a PowerWave 340 microplate spectrophotometer (Biotek Instruments). The intra-assay coefficient of variation (%CV) was 11.7%. Corticosterone was measured using a commercially available EIA kit, according to the manufacturer's instructions (Cayman Chemical). The cold spike protocol was used for the purification of plasma samples and corticosterone concentrations measured using a Power Wave 340 microplate spectrophotometer (Biotek Instruments) at 412nm. The intra-assay coefficient of variation (%CV) was 12.5.

Determination of Cytokines and Apoptotic Protein Expression in Intestinal Lymphocytes

Intestinal lymphocyte (IL) Isolation: Intestinal lymphocyte (IL) isolation was performed as described (Hoffman-Goetz and Quadrilatero, 2003). Following sacrifice by sodium

phenobarbital overdose, cold phosphate buffered saline (PBS) was used to wash the excised intestinal compartment, Peyer's patches and visible fat was removed and single cell IL suspensions prepared by isolation over a pre-washed nylon wool (0.3g) column. The eluted cells were layered over a Lympholyte-M density gradient medium (Cedarlane Laboratories) and centrifuged to remove debris. The remaining IL pellet (containing iIEL and LP lymphocytes) was suspended in 400μL of PBS. Turk's staining solution (99μL) was used to enumerate intestinal lymphocytes (1μL) by light microscopy. This technique yields high lymphocyte recovery (i.e., 90.9±0.5% $CD45^+$ by flow cytometry analysis).

Western blot analysis of cytokines and apoptotic proteins: Intestinal lymphocytes (IL) were fractionated in lysis buffer on ice for 45 min. Lysates ($1x10^5$) were centrifuged (15 min, 10,000g) and supernatant extracted. A BCA assay was used to determine protein concentration (Lowry et al., 1951). Protein (40μg) and selected molecular weight markers (Full Range Rainbow, Amersham Biosciences) were electrophoresed on a 12% SDS-PAGE gel before transfer to PVDF membrane. Membranes were stained with Ponceau S to confirm quality of transfer and equal loading. After electrophoresis, membranes were incubated for 1 hr with primary antibody (1:200 in 10% milk-TBST): TNF-α (clone: N-19; goat anti-human polyclonal IgG), IL-1-β (clone: Fx02l; mouse anti-rat monoclonal), IL-6 (clone: M-19; goat anti-rat polyclonal IgG), caspase-3 (clone: H-277; rabbit anti-human polyclonal IgG), caspase-7 (clone: 10-1-62; mouse anti-human monoclonal IgG_1) and Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG_1) (Santa Cruz Biotechnology). Membranes were incubated for 1 hr with secondary antibody: biotin-conjugated rabbit anti-goat IgG-B (TNF-α; IL-6), and horseradish peroxidise-conjugated goat anti-mouse IgG-HRP (IL-1β, caspase-7, Bcl-2), or goat anti-rabbit

IgG-HRP (caspase-3) IgG at a concentration of 1:2000 in 10% milk-TBST. ECL Plus detection reagent (Amersham Biosciences) and the ChemiGenius 2 Bio-imaging System were used for protein determination. A biotinylated protein ladder was used to identify the molecular weight of selected proteins (Cell Signalling Technology). Recombinant standards (Cedarlane Laboratories) were run on each gel. Samples from the two exercise conditions were run on each immunoblot and band densities were normalised to control bands on each immunoblot (units reported as arbitrary densitometric units [A.U.]) for each group.

Experimental Design and Statistical Analysis: All variables were analyzed by one-way ANOVA with exercise training (i.e., WR, NWR) as the independent factor, and cytokine and apoptotic proteins as the dependent factors (SPSS for Windows Version 19; SPSS Inc, Chicago, IL, USA). Running volumes (distances) by month were analyzed by repeated measures ANOVA. Levene's test was used to check for homogeneity of variance. Significant differences were accepted if $p < 0.05$. Values are group means ± 1 SEM for respective units (e.g., μmol/min/g; arbitrary densitometric [A.U.] units; g).

4.7 Results

Physiological Indicators of Training and Exercise: Table 4.1 shows the physiologic characteristics [distance run (km), body weight (g), cytochrome c oxidase (μMol/min/g protein) activity in soleus and plantaris muscles] of healthy older C57BL/6 female mice after 4 months of low intensity exercise training. WR and NWR mice did not differ in initial body weights ($p>0.05$). However, after 4 months of exercise training, WR mice were significantly lighter than NWR mice ($p<0.05$). Over the 4 months of wheel running activity the mean distance run per animal was 578.1 km. The average monthly distance run per mouse remained steady for the first

two months and thereafter declined by 20-30 km per month. WR mice also had significantly higher cytochrome *c* oxidase activity in SOL and PLANT compared to NWR mice ($p<0.05$).

Measures of Stress

Plasma 8-iso-$PGF_{2\alpha}$ and Corticosterone: Table 4.1 also shows the plasma concentration of 8-iso-$PGF_{2\alpha}$ and corticosterone in trained and untrained older mice. WR mice had significantly lower plasma 8-iso-$PGF_{2\alpha}$ levels compared with NWR mice [F (1, 29) = 9.36, $p<0.05$]. WR mice did not differ from NWR controls in their plasma corticosterone levels [F (1, 31) = 0.423, $p>0.05$]. Given that plasma 8-iso-$PGF_{2\alpha}$ is an indicator of oxidative stress, it can be inferred that exercise training was associated with lower resting levels of cellular oxidant stress. The lack of a difference in corticosterone levels between the two groups suggests that voluntary low intensity wheel running did not significantly influence psychological or psychosocial stress in the animals.

Western Blot Analysis of Cytokines and Apoptotic Proteins

Pro-Inflammatory and Pleiotropic Cytokines: Figure 4.2 (Panels A-C) shows the effect of long-term (4 months) voluntary wheel-running (i.e., exercise training) on mouse intestinal lymphocyte expression of two pro-inflammatory cytokines, TNF-α and IL-1β, and the pleiotropic cytokine IL-6 (units expressed in arbitrary units [A.U.]). Long term PA was associated with significantly lower expression of TNF-α [F (1, 29) = 9.51, $p<0.05$] in WR compared to NWR mice. Training was also associated with non-significant decreases in IL-1β [F (1, 34) = 2.63, p=0.12] and IL-6 [F (1, 37) = 2.21, p=0.15] expression in the intestinal lymphocytes of older mice.

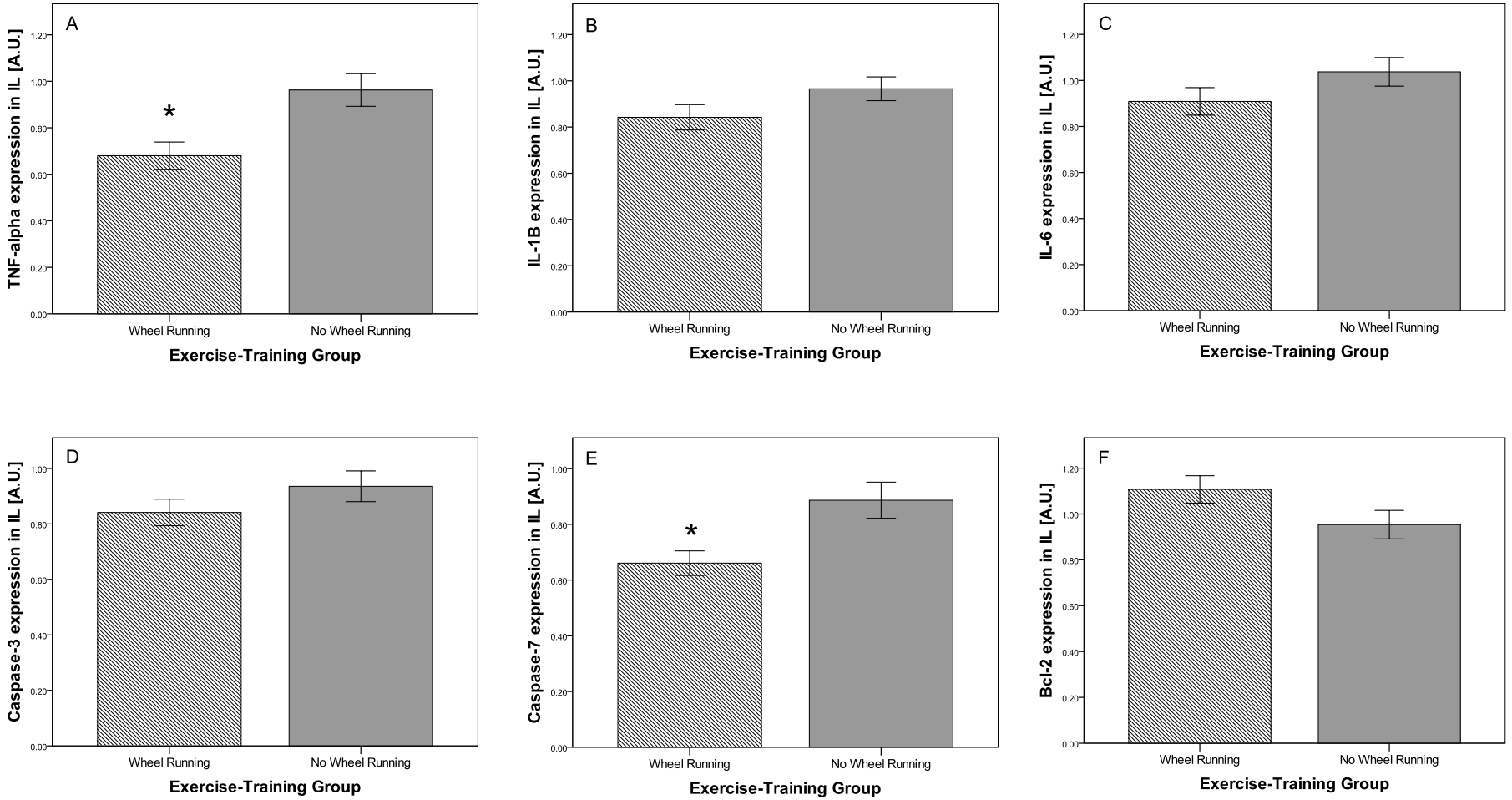
Figure 4.2 (Panels D-F) also shows the effect of long-term (4 months) voluntary wheel-running (i.e. exercise training) on intestinal lymphocyte expression of the pro-apoptotic proteins caspase-3 and caspase-7, and the anti-apoptotic protein Bcl-2. Long term PA was associated with a significant reduction in caspase-7 [F (1, 34) = 7.19, $p<0.05$] and a non-significant decrease in caspase-3 [F (1, 33) = 1.62, p=0.21] expression in intestinal lymphocytes of healthy older mice. Voluntary wheel running for 4 months also led to a non significant increase the expression in intestinal lymphocytes of the anti-apoptotic protein, Bcl-2, [F (1, 36) = 3.17, p=0.08] in healthy older animals.

Group	Running distance (km)			
	Month 1	**Month 2**	**Month 3**	**Month 4**
WR	151.4±10.8	166.3±9.6	133.6±7.0	126.8±11.6
NWR	-	-	-	-

Group	8-iso-$PGF_{2\alpha}$	Corticosterone	Cytochrome C Oxidase		Body Weight	
			Soleus	Plantaris	Initial	Final
WR	167.6±9.2*	53.1±9.2	13.5±0.8*	12.3±.5*	30.4±0.5	31.1±0.6*
NWR	209.4±10.1	45.1±7.8	9.5±0.5	8.0±0.3	30.7±0.7	34.0±0.8

Table 4.1 Body weight (g), running distance (km), 8-iso-$PGF_{2\alpha}$ (pg/ml), corticosterone (ng/ml) and cytochrome *c* oxidase (CO) activity (µMol/min/g protein) in C57BL/6 after 4 months of exercise-training. Values reported as group means ± one standard error; [*] denotes significant ($p<0.05$) group effects.

Figure 4.2 Pro-(TNF-α, IL-1β) inflammatory and pleiotropic (IL-6) cytokine and pro-(caspase-3, -7) apoptotic and anti-(Bcl-2) apoptotic protein expression in IL of aged mice given 4 months of freewheel training vs. untrained controls. *Panel A*: TNF-α; *Panel B*: IL-1β; *Panel C*: IL-6; *Panel D*: Caspase-3; *Panel E*: Caspase-7; *Panel F*: Bcl-2. Values reported as group means ± 1 SEM; [*] denotes ($p<0.05$) group effects.



4.8 Discussion

The purpose of this study was to describe the effects of long-term, voluntary aerobic exercise training on inflammatory cytokine and apoptotic protein expression in the intestinal tract of healthy older adult mice. We hypothesized that intestinal lymphocytes of trained mice would display lower levels of apoptotic proteins (caspase-3, caspase-7) and pro-inflammatory (TNF-α, IL-1β) and pleiotropic (IL-6) cytokines, and higher levels of anti-apoptotic proteins (Bcl-2) compared to untrained (sedentary) age-matched controls. We found that 4 months of wheel running decreased intestinal lymphocyte expression of the pro-inflammatory cytokine TNF-α and the apoptotic protein caspase-7 in this aged cohort. To our knowledge, no other studies have examined the effect of voluntary long term physical activity on the intestinal lymphocyte expression of classical pro-inflammatory and pleiotropic cytokines and apoptotic proteins in healthy aged individuals (either animals or people). Collectively, the results suggest that regular PA may protect against senescent increases in intestinal inflammation (and potentially colorectal cancer risk) though a mechanism of decreased expression of TNF-α and caspase-7 in lymphocytes resident in the bowel.

The cytokine TNF-α, a potent mediator of inflammation and carcinogenesis, induces the inflammatory effects of IL-6 (and increases production of IL-1β) by up-regulating the transcription factor $NF_k\beta$ (Wilson, 2008). Elevated TNF-α is found in the bowel of CRC patients (Grimm et al., 2010) and therapeutic application of anti-TNF-α antibodies prevents CRC development in animal models of colitis (Popivanova et al., 2008). IL-1β is a pro-inflammatory cytokine that promotes tumour formation by inducing cellular proliferation (Jung et al., 2003), apoptosis (Wang et al., 2009), and vasculogenesis (Jung et al., 2003). IL-6 has been shown to prevent apoptosis in myeloid LP cells (Grivennikov et al., 2009) and to promote tumour

progression in animal models of colitis (Strassman et al., 1993). Though IL-6 demonstrates both pro- and anti-inflammatory functions (Pedersen et al., 2003; Pedersen and Fischer, 2007) its effects are primarily pro-inflammatory with increasing age (Dobbs et al., 1999) or in the presence of disease pathology (Ridker et al., 2000). Thus, decreased intestinal lymphocyte expression of these cytokines (TNF-α, IL-1β, IL-6) in response to long-term freewheel training contributes to our understanding of how regular physical activity in older people may decrease the risk of inflammatory diseases of the bowel and colorectal cancer.

Intestinal lymphocyte TNF-α expression was markedly lower in the trained, older animals. Regular physical activity may also decrease intestinal inflammation by reductions in adipose tissue, a major tissue source of non-immune cell derived endogenous TNF-α (Coppack, 2001), or via suppression of TNF-α transcription or translation. Intestinal TNF-α levels are thought to be predictive of risk for later CRC development (Popivanova et al., 2008). Thus, lower TNF-α may reduce the risks for the development of intestinal pathology. This training-induced reduction in TNF-α is remarkable given the reported consistent elevated expression of TNF-α and TNF-r1 in peripheral mononuclear cells (Fagiolo et al., 1993) and plasma (Stowe et al., 2010) of healthy elderly individuals. Despite the older age of our animals, the results mirror those of previous studies which demonstrated that training decreases baseline IL expression of TNF-α (Hoffman-Goetz et al., 2009) in young animals. This was observed despite the fact that young and old mice also differ markedly in their physiologic response to training. In response to 4.5 weeks of resistance exercise (i.e. 14 sessions of electrically evoked training), young (3 months) rats showed a 15.6% increase in wet muscle (*tibialis anterior*) weight, while old (30 months) rats showed no increase (Murlasits et al., 2006). Though both young and old rats showed increased (+968.8% and +409.1%, respectively) HSP72 expression in *tibialis* muscle in

response to prolonged resistance exercise, old rats demonstrated an augmented training response in spite of equal resistance training conditions. Moreover, since TNF-α has been shown to promote carcinogenesis (Grimm et al., 2010) our novel finding may explain, in part, the nature of the protective effect of regular exercise, even at advanced ages. Specifically, 'training' may act to reduce or buffer against intestinal inflammation in aged individuals despite age-related increases in inflammation and TNF-α expression (Fagiolo et al., 1993).

No differences in the intestinal lymphocyte expression of IL-1β or IL-6 were observed although there were non-significant decreases among trained older mice. This is an important observation given the lower TNF-α expression in response to training and the role of TNF-α in inducing IL-1β and IL-6 production (Wilson, 2008). The direction of the trends for both IL-1β and IL-6 support the possibility that TNF-α may be functioning in this manner. Further, the direction of these 'training' effects, albeit not significant, must be considered in light of the observation that healthy elderly adults have elevated expression of IL-6 and IL-1β in immunologically active blood cells (Fagiolo et al., 1993) and higher concentration of IL-6 in plasma (Stowe et al., 2010). Previous studies show that training decreases baseline expression of IL-1β (Gomez-Merino et al., 2007) in white adipose tissue of healthy young mice, while long-term resistance training reduces circulating IL-6 in healthy elderly people (Córdova et al., 2011).

Inflammation induces both the intrinsic and extrinsic pathways of apoptosis (cell death). The former (intrinsic) is mediated by mitochondrial activation of caspases and cytoplasmic translocation, subsequently leading to cell lysis (Wyllie, 2010). These harmful effects are antagonized by cellular anti-apoptotic proteins, such as Bcl-2, which prevent the release, activation, and translocation of mitochondrial caspases (Kuwana and Newmeyer, 2003). Alternatively, the extrinsic pathway proceeds via cytokine (i.e., TNF-α, IL-1β) induced

activation of membrane death receptors (i.e., TNF-R1 and CD95) (Mignini et al., 2008). Collectively, these biological processes explain how cytokine and apoptotic dysregulation can contribute to gastrointestinal pathology and uncontrolled inflammation.

Our study shows that there was a significant reduction in the expression of the pro-apoptotic protein, caspase-7, in the intestinal lymphocytes of trained older mice. This training-induced reduction in the apoptotic protein may be protective in buffering aging-associated increases in the expression of this apoptotic mediator (Zhang et al., 2002). Furthermore, lower caspase-7 expression may be protective, as elevated caspase-7 predicts a higher likelihood of oral squamous cell carcinoma recurrence (Coutinho-Camillo et al., 2010). We did not find a significant reduction in the expression of caspase-3 in intestinal lymphocytes of older healthy animals; however, the direction of the difference between trained and untrained mice was supportive of the finding in young mice given long term training (Hoffman-Goetz and Spagnuolo, 2007). The reason for this lack of significant effect in caspase-3 expression is not clear from our data; age-dependent kinetics of the caspase cascade may be involved (Jiang et al., 2009).

Similar to observations in young animals (Davidson et al., 2006), we confirmed that 4 months of low intensity, voluntary wheel running was sufficient to induce physical, physiological and biochemical changes indicative of 'training' in aged C57BL/6 female mice. The skeletal muscles of 'trained' mice showed increased cytochrome *c* oxidase enzyme activity. Cytochrome *c* oxidase plays a crucial role in cellular energy regulation and directly influences mitochondrial ATP production (Fontanesi et al., 2006). The enzymatic activity of cytochrome *c* oxidase is reduced with increasing age (Bagh et al., 2011) but, in young animals, has been shown repeatedly to increase in response to long-term training (Hoffman-Goetz et al., 2010*)*. Training-

induced increases in skeletal muscle cytochrome *c* oxidase can also provide an antioxidant function 'buffering' against the damaging effects of aging (De Lisio et al., 2011). During the first two months of training, the total running distances of aged mice increased before falling sharply during the third and fourth months. In young mice, there is a sequential increase in running distances across the entire training duration (Hoffman-Goetz et al., 2010). This temporal decrease in wheel running volume among the aged cohort confirms the findings of Turner and colleagues (2005) and may reflect an age-related loss of aerobic capacity (Waters et al., 2008) or an impaired endocrine response to environmental, psychological or physiological stressors (Waters et al., 2010).

Plasma 8-iso-$PGF_{2\alpha}$ is derived from membrane-derived arachidonic acid, a reaction catalyzed by oxidative (e.g., Reactive Oxygen Species) intermediates (Roberts and Morrow, 2000). Corticosterone is a steroid hormone produced by the adrenal gland in response to acute physiological or psychological stress. It has a crucial role in the 'fight or flight' response by stimulating gluconeogenesis and, thereby, accessing energy reserves (Girard and Garland, 2002). Both plasma 8-iso-$PGF_{2\alpha}$ and corticosterone increase throughout the lifespan (Garrido et al., 2010; Montine et al., 2011). We observed that WR and NWR mice did not differ in baseline stress as measured by plasma corticosterone. Moreover, since corticosterone did not differ between WR and NWR, the observed lower levels of plasma 8-iso-$PGF_{2\alpha}$ in WR mice suggests that regular exercise lowers baseline oxidative or cellular stress in older individuals. Given that 8-iso-$PGF_{2\alpha}$ is a marker of inflammation, it possible that this phenomenon might be one mechanism whereby the beneficial effects of exercise are realized, particularly if elevated 8-iso-$PGF_{2\alpha}$ predicts chronic disease progression – including some cancers (Dai and Zhu, 2009).

Collectively, our study findings of decreased TNF-α, caspase-7, and 8-iso-PGF2α in response to long-term wheel running activity support the hypothesis that PA protects against inflammation, even in aged individuals. Moreover, given that TNF-α is a cytokine that 'regulates' the function of other cytokines and markers of apoptosis, training may confer protection against disease initiation and progression by disrupting the harmful activity of this key cytokine. The effects of exercise on TNF-α appear to influence the direction of the caspase-7 and 8-iso-$PGF_{2\alpha}$ effects, particularly since (1) TNF-α binding to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) increases pro-caspase-7 cleavage and apoptosis (Zauli et al., 2003), and (2) reductions in TNF-α reduce baseline oxidative stress, which is reflected in lower levels of 8-iso-PGF2α in plasma (Rizzo et al., 2008).

There are limitations of this study which are important to note. First, we are limited by the extent to which these results can be generalized to a human population. However, in the study of biological aging, animal models allows for research to identify basic 'biomarkers of aging' and improve scientific understanding of disease mechanisms (Huber and Sierra, 2009). As such, animal research is an important step in establishing scientific evidence to guide clinical research and future practice recommendations. Second, 15-16 month old C57BL/6 mice were not extremely aged; however, given a life-expectancy of ~23 months for this strain (Anisimov, 2009) and an average age of 63 years for initial diagnosis of sporadic CRC (Lynch, 1999), this age cohort should provide a useful basis for comparison. Third, as we did not measure intestinal lymphocyte mRNA for the cytokines and apoptotic proteins selected for analysis, only their protein expression. It is possible that some of these proteins may have originated, not from lymphocytes but from other cells in the intestinal tract (such as epithelial cells). Fourth, this study was done in healthy animals in order to investigate possible mechanisms contributing to

the effects of 'training' in a healthy population. Future research will be needed to determine if this protection holds in animal models of human 'disease' or in a clinical population. Finally, our study population was comprised of only female mice. Female C57BL/6 have been shown to be better runners than males (Turner et al., 2005); future research in a male population is necessary to ensure that gender does not confound the protective effect of training.

4.9 Conclusion

In summary, freewheel running for 4 months in healthy aged female C57BL/6 mice was associated with decreased expression in intestinal lymphocytes of the pro-inflammatory cytokine TNF-α and the apoptotic protein caspase-7, and lower plasma levels of the marker of oxidative stress 8-iso-$PGF_{2\alpha}$. These training-induced changes suggest a complex mechanism whereby decreased TNF-α may reduce intestinal inflammation, apoptosis, and oxidative stress. Furthermore, these 'anti-inflammatory' effects of regular exercise raise the possibility that physical activity is protective against the development of intestinal pathology and colorectal cancer, even at later ages. This study used an animal model to explore potential biological mechanisms between PA and inflammatory mediators known to be involved in the etiology of chronic intestinal diseases. This animal research should be considered as hypothesis-generating and future research, focused on biomarkers of aging and IBD, will be needed to determine if PA reduces inflammatory cytokines in the bowel of older, healthy Canadians.

CHAPTER 5: Training preserves the intestinal cytokine response to acute exercise in older mice

5.1 Objective

Evaluate the effect of four months of voluntary freewheel training on intestinal lymphocyte (IL) expression of cytokines and apoptotic proteins immediately after exposure to a single bout of exhaustive treadmill exercise in healthy older (15-16 months) female C57BL/6 mice.

5.2 Hypotheses

In response to acute treadmill challenge, older mice exposed to long-term freewheel running will display a similar intestinal cytokine and apoptotic response as young mice. **Hypothesis 1:** wheel running mice will display elevated IL expression of pro-(TNF-α, IL-1β) and anti-(IL-10) inflammatory cytokines immediately after exercise challenge when compared to older sedentary (no wheel running) controls. **Hypothesis 2:** wheel running older C57BL/6 mice will display elevated IL expression of pro-(caspase-3/-7) and decreased expression of anti-(Bcl-2) apoptotic proteins immediately after exercise challenge, when compared to older C57BL/6 sedentary (no wheel running) controls.

5.3 Experimental Design

This study evaluated the effect of freewheel training on IL expression of pro- and anti-inflammatory cytokines and pro- and anti-apoptotic proteins in response to a single bout of exhaustive exercise in older mice. Previous research has shown that young mice have increased intestinal lymphocyte expression of pro-inflammatory and pro-apoptotic proteins after acute exercise (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). Alternatively, long-term freewheel training decreases IL expression of inflammatory and apoptotic proteins (Gomez-

Merino et al., 2007). Little is known about these responses in older animals. The experimental design is shown in **Figure 5.1**.

Two-way analysis of variance (2x2 ANOVA) was used to analyze the cytokine and apoptotic protein responses of trained and untrained older mice to an acute exercise challenge. Homogeneity of variance was confirmed by Levene's test. The independent factors included freewheel training (two levels: wheel running and no wheel running) and acute treadmill exercise condition (two levels: SED and IMM). Dependent factors included the IL cytokines and apoptotic proteins selected as outcome measures (SPSS Version 19; Chicago, IL, USA). Significant differences were accepted if $p<0.05$.

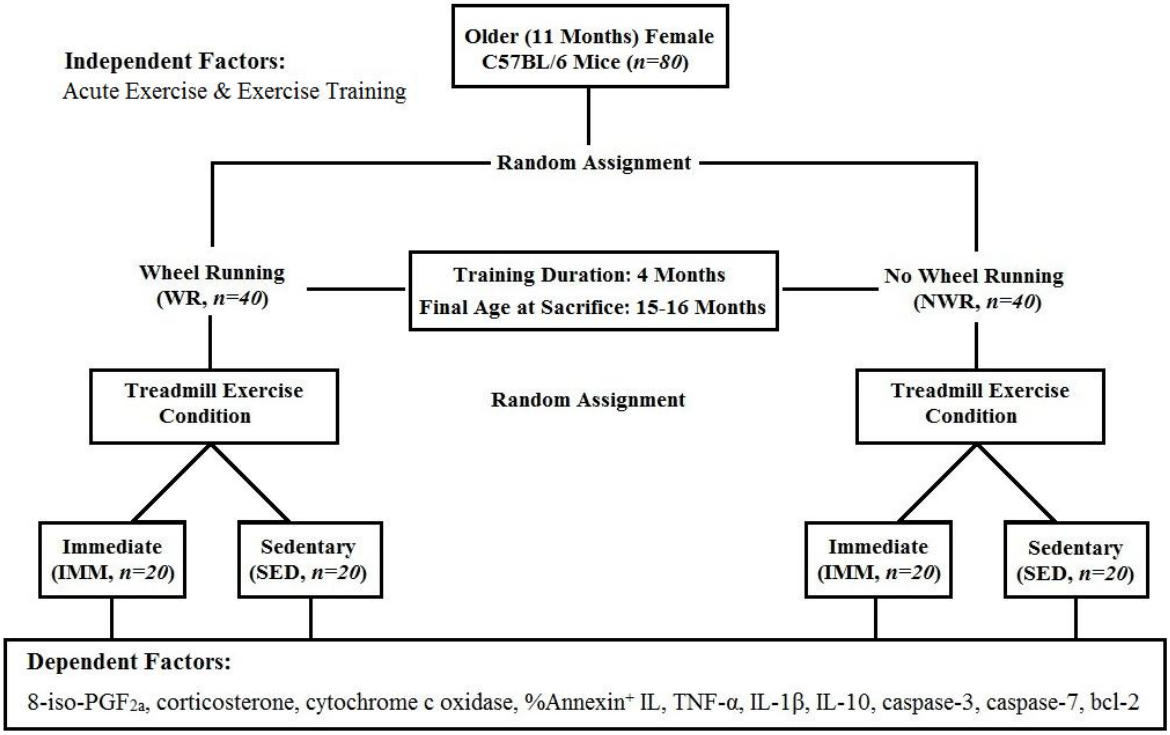


Figure 5.1 Study 3 - Outline of the experimental design.

The work presented in the remainder of this chapter is under review (revision) as:

Packer N, Hoffman-Goetz L. (2011). Training preserves the intestinal cytokine response to acute exercise in older mice. *Medicine & Science in Sports & Exercise*.

5.4 Overview/Summary

Background: Long-term freewheel training in young animals decreases intestinal lymphocyte (IL) expression of inflammatory cytokines and apoptotic proteins. Little is known about these responses in older animals. **Purpose:** To evaluate the effect of training on IL expression of pro- and anti-inflammatory cytokines and pro- and anti-apoptotic proteins after an acute exercise challenge in older mice. **Methods:** Eighty female C57BL/6 mice, aged 11 months at the start of the study, were randomized to two exercise training conditions for 4 months: access to in-cage running wheels (WR, *n = 40*) or to a no running wheel condition (NWR, *n = 40*). After four months of training, WR and NWR mice underwent a single bout of treadmill exercise with immediate sacrifice (WR IMM, *n=20*; NWR IMM, *n=20*) or were sedentary with exposure only to treadmill noise and vibrations without actual running (WR SED, *n=20*; NWR SED, *n=20*). Plasma 8-iso-$PGF_{2\alpha}$ and corticosterone levels and IL cytokine (TNF-α, IL-1β, IL-10) and apoptotic protein (caspase-3, caspase-7, Bcl-2) expression were assessed by Enzyme Immunoassay and by Western Blotting. **Results**: Older (15-16 months) WR mice had greater IL expression of pro-(TNF-α, IL-1β) and anti-(IL-10) inflammatory cytokines and pro-apoptotic (caspase-3, caspase-7) proteins, and decreased expression of anti-apoptotic (Bcl-2) proteins after acute treadmill challenge relative to older WR sedentary mice. Older NWR mice had few changes in IL cytokine or apoptotic protein expression following acute treadmill exercise relative to NWR sedentary mice. **Conclusions**: Long-term freewheel training preserves the intestinal

lymphocyte cytokine and apoptotic protein responses in older mice similar to that previously reported for young animals. Older NWR (untrained) mice have blunted responsiveness (cytokines and apoptotic protein expression) after acute exercise suggestive of immunosenescence.

5.5 Introduction

An active lifestyle is an important predictor of functional capacity, quality of life, positive health outcomes and enhanced immunity (Chodzko-Zajko et al., 2009). The enormous benefits of regular physical activity are recognized across the lifespan, including for the elderly. While regular physical activity conveys broad immune benefits, intense aerobic exercise induces oxidant stress, promotes downstream inflammation, and compromises immunity (Quadrilatero and Hoffman-Goetz., 2005). With an aging population worldwide and a growing segment of older adults engaging in both regular physical activity and competitive high intensity events (e.g., 'Old Timers' hockey, seniors' 10K races), there is a need to characterize inflammatory responses of older, physically active individuals to acute exercise induced oxidative stress (Petersen et al., 2010).

Aging is accompanied by varied functional declines which further complicate the exercise response (Chodzko-Zajko et al., 2009). With advancing age, there are heightened (Hatzinger et al., 2011) as well as blunted (van Eekelen et al., 1992) physiologic and immunologic responses to stress. For example, old (21-24 months) Fisher 344/N rats had a blunted adrenocorticotropic hormone (ACTH) response to hemorrhagic stress that was less than 50% of that demonstrated in young (3-4 months) animals (Hauger et al., 1994). This finding has been explained as a result of age-related decreases in pituitary responsiveness to glucocorticoids (Rohleder et al., 2002) or corticotropin-releasing hormone (Hauger et al., 1994). In contrast, in

response to ACTH stimulation, elderly men ($\bar{X} = 68$ years) and women ($\bar{X} = 63$ years) had elevated post-challenge increases in plasma cortisol compared to young men ($\bar{X} = 33$ years) and women ($\bar{X} = 28$ years) (Vermeulen et al., 1982).

Physical activity itself is a stressful event which activates the hypothalamic-pituitary-adrenal (HPA) axis (Mastorakos and Pavlatou, 2005). Elevated plasma stress hormones directly contribute to inflammation and immune activation. For example, catecholaminergic (β-adrenergic) receptors on lymphocytes induce production of the pleiotropic cytokine, IL-6, and norepinephrine promotes downstream TNF-α (a pro-inflammatory cytokine) production by activating $NF_k\beta$ (Mastorakos and Pavlatou, 2005). Huang et al. (1997) demonstrated experimentally that norepinephrine exposure (dose range: 10^{-6} to 10^{-4} M) induced IL-6 production from rat spleen lymphocyte cultures.

It has also been shown that in response to heavy exercise, elderly individuals have reduced protein synthesis compared to younger persons (Sheffield-Moore et al., 2005). Using real-time PCR, Dennis and colleagues (2008) found significant increases in TIMP1 and α-cardiac actin (ACTC1) mRNA in *vastus lateralis* muscle after resistance exercise in young individuals (32±7 years); in contrast, no significant post-exercise changes in gene expression of either TIMP1 or ACTC1 were reported for elderly individuals (72±5 years). Healthy seniors (62-72 years) had no changes in skeletal muscle IL-1β gene expression after acute exercise whereas a 3.5-fold increase in IL-1β gene expression was observed in younger subjects (20-34 years) (Jozsi et al., 2000). Taken together, these findings suggest that older individuals may have a blunted protein synthesis response to acute exercise stress.

Those who regularly pursue vigorous physical activity face a unique set of challenges. Acute exercise is a strong inducer of the stress response and a single bout of exhaustive exercise generates reactive oxygen (ROS) species (Quadrilatero and Hoffman-Goetz., 2005). This can lead to suppression of immune function and induction of inflammation. These potentially detrimental effects are clearly seen in the gastrointestinal compartment which is densely populated with immunologically active cells (i.e., lamina propria (LP), intraepithelial (iIEL), and intestinal (IL) lymphocytes) involved in cytokine secretion and homeostatic regulation (Lefrançois and Lycke, 2001). Consequent to oxidant stress, high-intensity exercise elicits an inflammatory response indicated by elevated plasma concentrations of the pro-inflammatory cytokines TNF-α and IL-1β (Ostrowski et al., 1999). In contrast, voluntary exercise training confers a number of positive health effects, many of which are independent of age. These include restoration of muscle mass and/or function (Chodzko-Zajko et al., 2009), improved cellular respiratory capacity (Chodzko-Zajko et al., 2009) and greater endogenous antioxidant concentration (Roshan et al., 2011). Regular physical activity may alleviate oxidant stress by increasing anti-inflammatory and decreasing pro-inflammatory cytokine production in lymphocytes. For example, in young C57BL/6 mice, baseline intestinal lymphocyte (IL) expression of pro-inflammatory TNF-α decreases (Hoffman-Goetz et al., 2009) and concentration of anti-inflammatory IL-10 increases (Hoffman-Goetz et al., 2010) with long-term voluntary freewheel running. This anti-inflammatory effect of training occurs in non-lymphoid tissues as well. Gomez-Merino and colleagues (2007) found training-induced decreases in IL-1β in adipose tissue of young male Wistar rats. Indeed, regular physical activity has been consistently shown to improve the immune-cytokine profile across all ages (Chodzko-Zajko et al., 2009) which may abrogate (at least in part) immunoscenescent changes. Healthy sedentary

males (45-64 years) had a significant reduction in serum IL-6 in response to 24-weeks of exercise training (Thompson et al., 2010). Moderate exercise training decreased serum concentration of TNF-α in older males with chronic heart failure (Smart et al., 2011).

The effect of long-term voluntary exercise training on intestinal lymphocyte cytokine and apoptotic protein responses to oxidant stress has not been studied in older animals. Understanding the effects of exercise associated cytokine and apoptotic protein production in older individuals is important for several reasons. First, there is a growing segment of highly active older adults who are increasingly involved in moderate-to-high intensity (aerobic and resistance) exercise (Petersen et al., 2010). Second, with advancing age there is an increase in basal inflammation (Chodzko-Zajko et al., 2009), apoptosis (Zhang et al., 2002) and an impaired stress response (Hatzinger et al., 2011). Third, exercise training is associated with many positive health effects, and may slow the rate of immunosenescence by restoring muscle mass, improving cellular respiratory capacity (Chodzko-Zajko et al., 2009), increasing antioxidant concentration (Roshan et al., 2011*)* and by decreasing and increasing pro-and anti-inflammatory cytokine expression in the gastrointestinal tract (Hoffman-Goetz et al., 2009).

Evidence suggests that some of the age-related declines in cellular function begin with small changes occurring in early to late middle-age (Szweda et al., 2003). Age-related loss of muscle mass and decreased muscle proliferation occur in people as young as 25 years old (Lexell, 1995). These changes accumulate across the lifespan becoming physically observable in late middle-age or old-age as the physiologic 'threshold' for cellular damage is exceeded. In the elderly, the culmination of these changes is thought to be expressed as broad functional declines. Lu et al. (2004) show that increased susceptibility to catastrophic DNA damage occurs between ages 45 and 70. Middle adulthood may thus be the 'ideal' age for preventative health or lifestyle

interventions, including regular exercise. King et al. (2007) demonstrate that the protective effect of regular exercise on functional capacity in late life is promptly recognized if physical activity is begun in middle adulthood.

The purpose of this study was to describe the effects of long-term exercise training on intestinal lymphocyte expression of 1) pro- and anti-inflammatory cytokines and 2) pro- and anti-apoptotic proteins in response to an acute exercise challenge. We hypothesized that older (15-16 months) mice exposed to long term freewheel running would have an increased expression of pro-inflammatory (TNF-α, IL-1β) cytokines and pro-apoptotic (caspase-3, caspase-7) proteins, and a decreased expression of anti-inflammatory (IL-10) cytokines and anti-apoptotic (Bcl-2) proteins in intestinal lymphocyte following acute treadmill exercise. In other words, trained older mice would parallel the intestinal lymphocyte cytokine and apoptotic protein expression (reported elsewhere) observed in young mice after acute treadmill exercise (Hoffman-Goetz et al., 2009; Hoffman-Goetz et al., 2010). We also hypothesized that older untrained mice would be less responsive in their cytokine and apoptotic protein response to acute exercise stress suggestive of immunosenescence (i.e., a blunted response).

We specifically selected 15-16 month old C57BL/6 mice for this study because of the potential for preventative exercise intervention at 'middle-age'. We recognize that this age is not reflective of truly old animals. However, the life expectancy for C57BL/6 mice is quite variable with reported 20-22 (Anisimov, 2009) and 24 (Selman et al., 2006) months under standard housing and breeding conditions. Moreover, 15-16 month old C57BL/6 mice already have a number of senescent changes including thymic involution (Li et al., 2003), elevated ROS in visceral adipose tissue (Zhang et al., 2011) and inflammatory phenotypes in the kidney (Hernández-Corbacho et al., 2011). From an exercise perspective, inclusion of very old animals

presents significant methodological and interpretation challenges. For example, Sinatra et al. (2003) demonstrate in C57BL/6 mice reduced novelty-exploration at 22 months; sharp declines in locomotor behaviour in this strain at 18 months have been reported (Bordner et al., 2011).

5.6 Materials and Methods

Animals. Female C57BL/6 mice (*n = 80*) (Harlan, Indianapolis, IN, USA), 11 months old at the start of the study, were individually housed at 21±1°C on a 12/12-hr reversed light/dark cycle for 4 months until study termination (i.e., at 15-16 months of age). At 16 months female C57BL/6 mice are not capable of maintaining viable pregnancies (Parkening et al., 1980). Mice had *ad libitum* access to standard rodent diet (Lab Rodent Chow, PMI Feeds, IN, USA) and tap water throughout the experiments. All animals were housed and cared for in conformance with the policy of the American College of Sports Medicine on research with experimental animals, the Canadian Council on Animal Care, and the University animal research ethics committee.

Freewheel training. Mice were matched by weight and randomized to exercise training conditions: access to in-cage running wheels (WR or 'trained', *n = 40*) or to a no running wheel (NWR or 'untrained', *n = 40*) condition for four months. An automated computer monitoring system (Vital View Application software, Mini-Mitter, Sunriver, OR) captured the number of completed revolutions via a magnetic switch attached to each wheel (23 cm in diameter). Activity during the dark cycle was recorded as the number of revolutions completed per 15-min interval, converted to distance run (km), and summed by day, week and month.

Acute treadmill exercise. WR and NWR mice were randomly assigned to an acute exercise condition: (1) a single bout of treadmill running with sacrifice immediately (i.e., WR IMM, *n=20*; NWR IMM, *n=20*) after completion of exercise or (2) a no treadmill sedentary

group (i.e., WR SED, *n=20*; NWR SED, *n=20*) exposed only to treadmill noise and vibrations for 90 min before sacrifice. At sacrifice mice were screened for the presence of gastrointestinal tumours and pathologies (i.e., bleeding) and animals with intestinal tumours were excluded. The exercise protocol consisted of a single, 90 min bout of intense treadmill exercise (10 min warm-up, 30 min at 22m·min^{-1}, 30 min at 25m·min^{-1}, and 30 min at 28m·min^{-1}, 2° slope) (Panlab Mouse Treadmill, Harvard Apparatus Canada) during the dark cycle. To avoid carry-over effects of freewheel running, in-cage running wheels were locked 24 hrs prior to the treadmill exercise bout. Mice were motivated to run by gentle prodding with a nylon test tube brush until the end of the run-to-exhaustion protocol or until reaching volitional fatigue.

Skeletal muscle enzyme activity. Cytochrome *c* oxidase (CO) activity was measured in sedentary (no treadmill challenge) mice (WR and NWR) as an indicator of training status; after 10 weeks of exercise training, CO has been shown to be increased by 38% in skeletal muscle (quadriceps) of young mice (De Lisio et al., 2011). Following sacrifice by sodium pentobarbital (0.6–0.8 cc per mouse, i.p.) overdose, *soleus* and *plantaris* muscles were isolated from all non treadmill running (i.e. WR SED; NWR SED) mice. Samples were frozen in liquid nitrogen and stored at -80°C until assayed. Muscles were cut into 5–10mg segments, mashed and homogenized in buffer [glycerol (50%), sodium phosphate buffer (20mM), 2-mercaptoethanol (5mM), EDTA (0.5mM), BSA (10%)] to yield a 50:1 dilution, and sonicated (using a 3 m tip, 2 sec on, 5 sec off for a total of 20 sec at 60 Hz; Vibra Cell, Sonics and Materials, Danbury, CT, USA). Protein content was determined by Lowry assay. Muscle homogenates were diluted to 1:500 dilutions in 10mM potassium phosphate buffer. Reduced cytochrome *c* (20µL) and diluted homogenate (10µL) were combined with warmed (37°C) phosphate buffer (970µL). The decrease in cytochrome *c* absorbance was determined spectrophotometrically at 550 nm.

Stress measures. The stress response of older mice to acute treadmill exercise was measured by plasma 8-iso-$PGF_{2\alpha}$ and corticosterone. In brief, a 1-mL syringe containing heparin was used to collect blood via cardiac puncture immediately following sacrifice. Plasma was separated by centrifugation (6 min at 400 rpm) and frozen at -80°C before it was assessed by direct enzyme immunoassay (EIA) using a commercially available kit as per the manufacturer's specifications (Cayman Chemical). Samples (100µL sample, 25µL of 10N NaOH) were hydrolyzed for 2 hr at 45°C, neutralized to pH 6-8 with 12N HCl, centrifuged at 14,000 g for 5 min, and incubated with 8-iso-$PGF_{2\alpha}$ antibody for 24 hr at 4°C. Percent absorbance was read at 412 nm at room temperature using a PowerWave 340 microplate spectrophotometer (Biotek Instruments). The intra-assay coefficient of variation (%CV) was 11.7%. Corticosterone was measured using a commercially available EIA kit, according to the manufacturer's instructions (Cayman Chemical). The cold spike protocol was used for the purification of plasma samples and corticosterone concentrations measured using a Power Wave 340 microplate spectrophotometer (Biotek Instruments) at 412nm. The intra-assay coefficient of variation (%CV) was 12.5%.

Intestinal lymphocyte isolation. Isolation of intestinal lymphocytes (IL) was performed as described elsewhere (Hoffman-Goetz et al., 2009). Cold phosphate buffered saline (PBS) was used to wash the excised intestinal compartment; Peyer's patches and visible fat were removed and a single cell suspensions prepared by isolation over a pre-washed nylon wool (0.3g) column. The eluted lysate was layered over a Lympholyte-M density gradient medium (Cedarlane Laboratories, Mississauga, Ontario) and centrifuged to remove debris. The remaining pellet (containing iIEL and LP lymphocytes) was suspended in 400µL of PBS. Turk's staining solution

(99µL) was used to enumerate IL (1µL) by light microscopy. This procedure yields a high lymphocyte recovery (i.e., 90.9±0.5% CD45+ IL by flow analysis).

Western Blotting. IL fractionation in lysis buffer (on ice; 45 min) was followed by centrifugation (15 min, 10,000g) of lysates ($1x10^5$ cells). Supernatant was extracted and protein concentration was determined by BCA assay. Protein (40µg) and selected molecular weight markers (Full Range Rainbow, Amersham Biosciences, Piscataway, New Jersey) were electrophoresed on a 12% SDS-PAGE gel before transfer to PVDF membrane. Ponceau S was used to stain membranes, to confirm quality of transfer and equal loading. After electrophoresis, membranes were incubated for 1 hr with primary antibody (1:200 in 10% milk-TBST): TNF-α (clone: N-19; goat anti-human polyclonal IgG), IL-1-β (clone: Fx02l; mouse anti-rat monoclonal), IL-10 (clone: JES5-2A5; rat anti-mouse monoclonal IgG_1), caspase-3 (clone: H-277; rabbit anti-human polyclonal IgG), caspase-7 (clone: 10-1-62; mouse anti-human monoclonal IgG_1) and Bcl-2 (clone: C-2; mouse anti-human monoclonal IgG_1) (Santa Cruz Biotechnology). Membranes were incubated for 1 hr with secondary antibody: biotin-conjugated rabbit anti-goat IgG-B (TNF-α), and horseradish peroxidise-conjugated goat anti-mouse IgG-HRP (IL-1β, IL-10, caspase-7, Bcl-2), or goat anti-rabbit IgG-HRP (caspase-3) IgG at a concentration of 1:2000 in 10% milk-TBST. ECL Plus detection reagent (Amersham Biosciences) and the ChemiGenius 2 Bio-imaging System were used for protein determination. A biotinylated protein ladder was used to identify the molecular weight of selected proteins (Cell Signalling Technology, Pickering, Ontario). Recombinant standards (Cedarlane Laboratories) were run on each gel. Samples from each experimental condition were run on each immunoblot and band densities were normalised to control bands on each immunoblot (units reported as arbitrary densitometric units [A.U.]) for each group.

Analysis. Cytokine and apoptotic protein expression of WR and NWR mice were analyzed by two-way ANOVA with freewheel training (two levels: Wheel Running, No Wheel Running) and acute treadmill exercise (two levels: Sedentary, Immediate) conditions as the independent factors. Intestinal lymphocyte cytokine and apoptotic protein expression and plasma concentration of corticosterone and 8-iso-$PGF_{2\alpha}$ were the dependent factors (SPSS for Windows Version 19; SPSS Inc, Chicago, IL, USA). One-way ANOVA was used to analyze the main effect of training (two levels of independent factor: Wheel Running, No Wheel Running) on cytochrome c oxidase activity in skeletal muscles. Homogeneity of variance was confirmed by Levene's test. Significant differences were accepted if $p<0.05$; values are group means ± 1 SEM for respective units (e.g., μmol/min/g; arbitrary [densitometric] units; g).

5.7 Results

Physiological indicators of training. The training characteristics of older C57BL/6 female mice are shown in **Table 1**. WR and NWR mice did not differ in initial body weights. Not unexpectedly, after four months of freewheel activity, WR mice were significantly lighter than NWR mice ($F_{(1,84)}=15.902$, $p<0.001$). WR mice accumulated an average of 573.91 km over 4 months; the pattern of running volume was steady for the first two months before decreasing by 20-30 km per month thereafter. Skeletal muscle cytochrome *c* oxidase activity was significantly higher in the *soleus* ($F_{(1,37)}=19.443$, $p<0.001$) and *plantaris* ($F_{(1,37)}=50.559$, $p<0.001$) muscles of (trained) WR compared to (untrained) NWR mice.

Stress measures. Plasma concentrations of 8-iso-$PGF_{2\alpha}$ and corticosterone for older WR and NWR mice in response to acute treadmill exercise are shown in **Table 2**. Acute treadmill exercise was associated with significantly increased plasma 8-iso-$PGF_{2\alpha}$ concentration in both WR ($F_{(1,28)}=38.732$, $p<0.001$) and NWR ($F_{(1,30)}=16.865$, $p<0.001$) mice relative to their

respective sedentary (no treadmill) controls. Acute treadmill exercise led to significant elevations in plasma corticosterone in trained ($F_{(1,30)}$=46.091, p<0.001) and untrained ($F_{(1,31)}$=67.751, p<0.001) mice. Given the role of plasma corticosterone and 8-iso-$PGF_{2\alpha}$ as indicators of physiological and oxidative stress, it can be inferred that the acute exercise bout was similarly stressful for both trained and untrained older mice.

Table 5.1 Physiological measures of training status in older (15-16 month) C57BL/6 mice

		Wheel Running (WR)		No Wheel Running (NWR)	
Running Distance (km)	Month 1	151.4±10.8	(n=40)	-	
	Month 2	166.3±9.6	(n=40)	-	
	Month 3	133.6±7.0	(n=40)	-	
	Month 4	126.8±11.6	(n=40)	-	
Body Weight (g)	Initial	30.4±0.5	(n=40)	30.7±0.7	(n=40)
	Final	31.1±0.6*	(n=40)	34.0±0.8	(n=40)
Cytochrome C Oxidase	Soleus	13.5±0.8*	(n=19)	9.5±0.5	(n=20)
	Plantaris	12.3±0.5*	(n=19)	8.0±0.3	(n=20)

Body weight (g), running distance (km), and cytochrome *c* oxidase activity (µMol/min/g protein) in older C57BL/6 after 4 months of exercise-training. Values reported as group means ± one standard error; [*] denotes significant (p<0.05) differences between WR and NWR groups.

Table 5.2 Plasma measures of oxidative stress in response to treadmill exercise in older (15-16 month) C57BL/6 mice

Oxidative Stress Measures		Treadmill Exercise Condition	
		Sedentary (SED)	Immediate (IMM)
8-iso-$PGF_{2\alpha}$	WR (n=30)	167.55±9.23	255.86±10.78*
	NWR (n=32)	209.42±10.12	297.71±18.09*
Corticosterone	WR (n=32)	53.07±9.15	306.54±38.45*
	NWR (n=32)	45.13±10.11	284.40±26.37*

8-iso-$PGF_{2\alpha}$ (pg/ml) and corticosterone (ng/ml) in C57BL/6 after 4 months of exercise-training. Values reported as group means ± 1 SEM; [*] denotes significant ($p<0.05$) difference between SED and IMM groups.

Pro- and anti-inflammatory cytokines. The effects of freewheel training and acute treadmill exercise on the expression of pro- and anti-inflammatory cytokines in mouse intestinal lymphocytes are shown in **Figure 1 (Panels A-C)**. There was no significant main effect of long-term training on the expression of the pro-inflammatory cytokines TNF-α ($F_{(1,69)}=0.01$, $p=0.30$) and IL-1β ($F_{(1,70)}=1.43$, $p=0.24$) or the anti-inflammatory cytokine IL-10 ($F_{(1,73)}=0.05$, $p=0.83$). There was a significant main effect of acute treadmill exercise on intestinal lymphocyte expression of TNF-α ($F_{(1,69)}=29.60$, $p<0.001$) and IL-1β ($F_{(1,70)}=9.02$, $p<0.01$): the expression of these inflammatory cytokines was higher immediately (TNF-α: 1.24±0.05 [A.U.]; IL-1β: 1.09±0.05 [A.U.]) after treadmill challenge compared to the sedentary (TNF-α: 0.82±0.05; IL-1β: 0.90±0.05 [A.U.]) condition. There was also a non-significant trend towards greater IL-10 expression immediately after acute exercise ($F_{(1,73)}=3.52$, $p=0.07$).

There was a significant interaction between freewheel training and acute treadmill exercise for the expression of TNF-α in intestinal lymphocytes of older mice ($F_{(1,69)}$=7.01, p<0.05). This was due to a greater increase in pro-inflammatory cytokine expression in WR mice (SED: 0.68±0.08 [A.U.] vs. IMM: 1.30±0.08 [A.U.]; 91% increase) compared to NWR mice (SED: 0.96±0.08 [A.U.] vs. IMM: 1.18±0.07 [A.U.]; 23% increase). There was no statistically significant interaction between freewheel training and acute exercise for IL-1β expression in intestinal lymphocytes from older mice ($F_{(1,70)}$=0.60, p=0.44). In contrast, a significant interaction effect was seen for intestinal lymphocyte expression of IL-10 ($F_{(1,73)}$=4.13, p<0.05). WR (SED: 0.84±0.07 [A.U.] vs. IMM: 1.09±0.06 [A.U.]; 30% increase) but not NWR (SED: 0.98±0.06 [A.U.] vs. IMM: 0.97±0.06 [A.U.]; 1% decrease) mice had higher IL-10 expression immediately after acute treadmill exercise compared with the sedentary condition.

Pro- and anti-apoptotic proteins. The effects of freewheel training and acute treadmill exercise on the expression of pro- and anti-apoptotic proteins in intestinal lymphocytes from older C57BL/6 mice are shown in **Figure 2 (Panels A-C)**. There was no significant main effect of long-term exercise training on the expression of the pro-apoptotic proteins caspase-3 ($F_{(1,70)}$=0.85, p=0.36) and caspase-7 ($F_{(1,70)}$=1.29, p=0.26) or the anti-apoptotic protein Bcl-2 ($F_{(1,75)}$=0.02, p=0.90). There was a significant main effect of acute treadmill exercise on mouse intestinal lymphocyte expression of caspase-3 ($F_{(1,70)}$=4.66, p<0.05) and caspase-7 ($F_{(1,70)}$=23.79, p<0.001): the expression of these pro-apoptotic proteins was higher immediately (caspase-3: 1.08±0.06 [A.U.]; caspase-7: 1.13±0.05 [A.U.]) after treadmill challenge compared to the sedentary (no treadmill) condition (caspase-3: 0.89±0.06; caspase-7: 0.77±0.05 [A.U.]). Acute treadmill exercise did not alter intestinal lymphocyte expression of Bcl-2 ($F_{(1,75)}$=1.91, p=0.17) across the training conditions.

However, there was a significant interaction between freewheel training and acute treadmill exercise for caspase-3 ($F_{(1,70)}$=4.06, $p<0.05$) due to the greater increase in pro-apoptotic protein expression in intestinal lymphocytes of WR mice in response to acute exercise (SED: 0.84±0.09 [A.U.] vs. IMM: 1.20±0.09 [A.U.]; 43% increase) compared to NWR mice (SED: 0.94±0.09 [A.U.] vs. IMM: 0.95±0.08 [A.U.]; 1% increase). There was a borderline significant interaction between freewheel training and acute exercise for caspase-7 expression in mouse IL ($F_{(1,70)}$=3.85, $p=0.054$); this was due to a post acute exercise increase in the expression of caspase-7 in WR mice (SED: 0.66±0.08 [A.U.] vs. IMM: 1.16±0.08 [A.U.]; 76% increase) compared to little change in NWR mice (SED: 0.89±0.07 [A.U.] vs. IMM: 1.10±0.07 [A.U.]; 24% increase). There was also a significant interaction effect ($F_{(1,75)}$=8.37, $p<0.01$) for intestinal lymphocyte expression of Bcl-2. WR mice (SED: 1.11±0.06 [A.U.] vs. IMM: 0.87±0.06 [A.U.]; 22% decrease) but not NWR mice (SED: 0.95±0.06 [A.U.] vs. IMM: 1.04±0.05 [A.U.]; 9% increase) had a lower expression of anti-apoptotic Bcl-2 immediately following acute treadmill exercise.

Immunoblots. Representative immunoblots for intestinal lymphocyte expression of TNF-α, IL-1β and IL-10 (**Figure 1**) and for caspase-3, caspase-7 and Bcl-2 (**Figure 2**) for each freewheel training and acute treadmill exercise condition (WR SED, WR IMM, NWR SED, NWR IMM) are shown in **Panel D** of **Figure 1** and **Figure 2**, respectively.

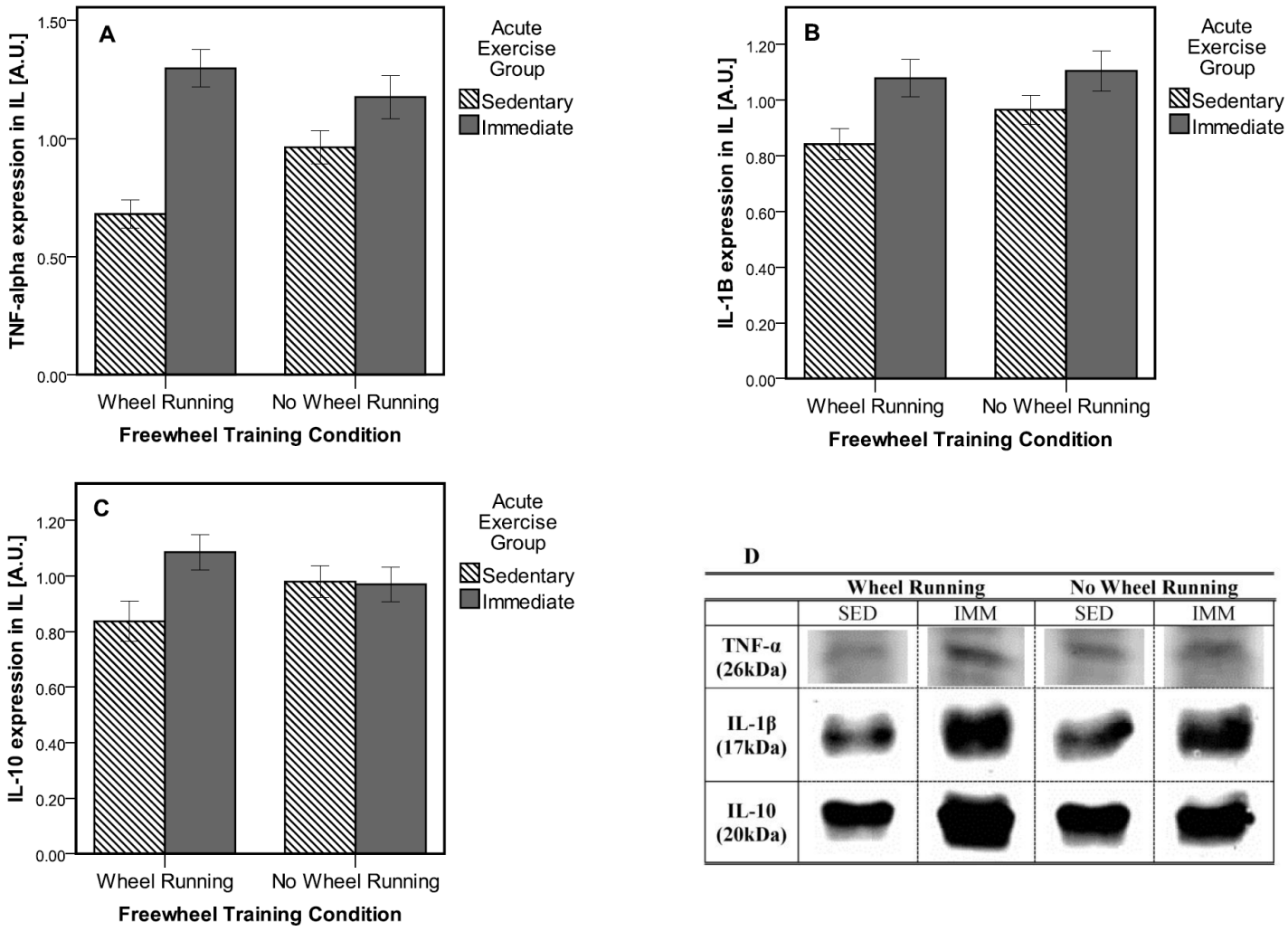


Figure 5.1 Intestinal lymphocyte cytokine expression after acute treadmill exercise in older (15-16 month) WR and NWR mice. *Panel A*: TNF-α; *Panel B*: IL-1β; *Panel C*: IL-10. Values reported as arbitrary units [AU] and group means ± one SEM; See text for details of analysis. *Panel D:* representative immunoblots for TNF-α, IL-1β and IL-10. Cytokines were selected for analysis based on documented biological activity.

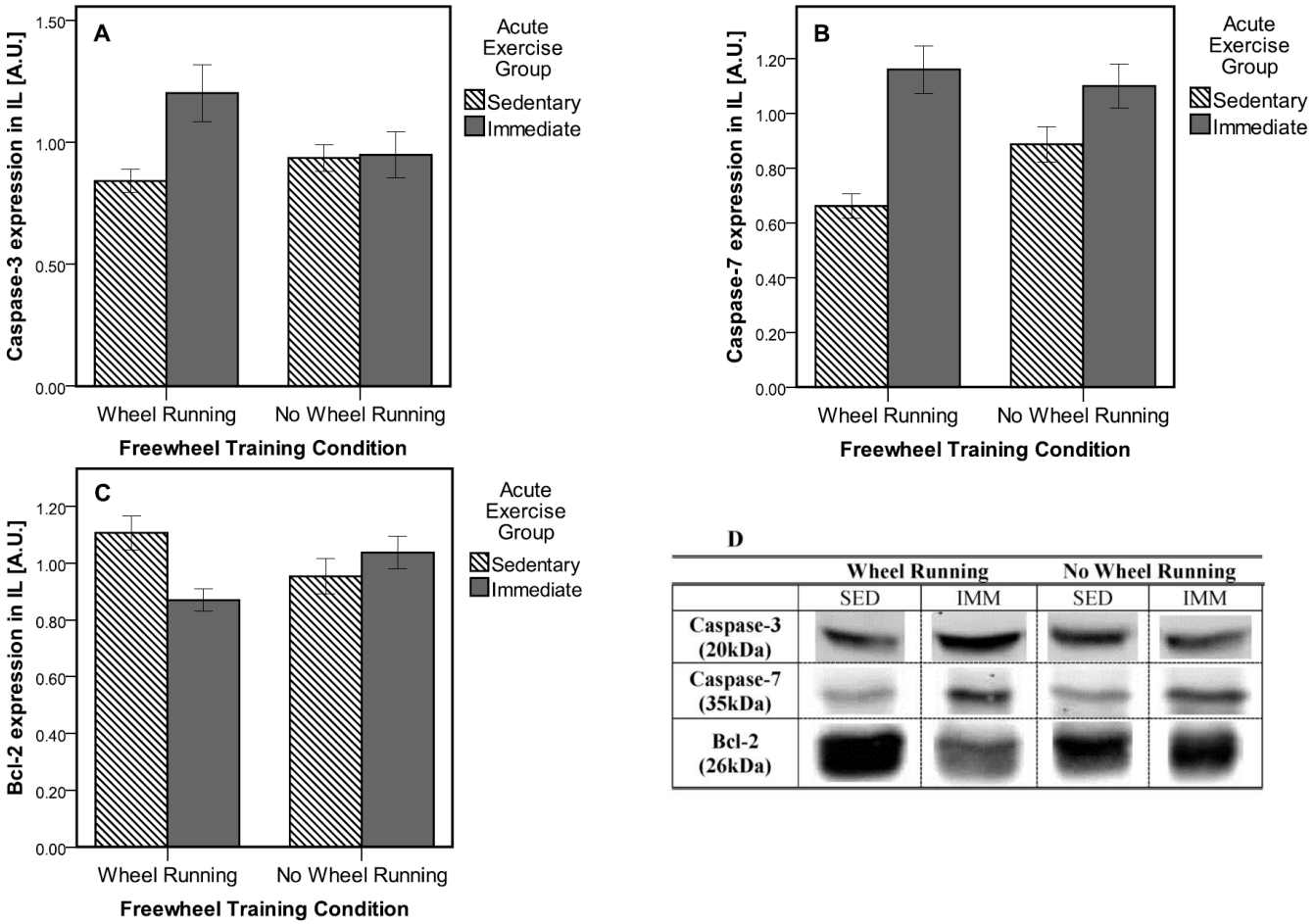


Figure 5.2 Intestinal lymphocyte apoptotic protein expression after acute treadmill exercise in older (15-16 month) WR and NWR mice. *Panel A*: Caspase-3; *Panel B*: Caspase-7; *Panel C*: Bcl-2. Values reported as arbitrary units [AU] and group means ± one SEM; See text for details of analysis. *Panel D:* shows representative immunoblots for Caspase-3, Caspase-7 and Bcl-2. Apoptotic proteins were selected for analysis based on documented biological activity.

5.8 Discussion

The effect of exercise training in older (15-16 month) mice on intestinal lymphocyte cytokine and apoptotic protein expression in response to acute exercise challenge was investigated. No cumulative effect of freewheel training alone on intestinal lymphocyte protein expression in the intestinal lymphocytes of these older animals was found. Acute exercise was associated with higher expression of TNF-α, IL-1β, caspase-3 and caspase-7 in mouse IL. There were interesting significant interactions between freewheel training and acute treadmill exercise for TNF-α, IL-10, caspase-3 and Bcl-2. This was demonstrated by changes in intestinal lymphocyte expression of pro-(TNF-α) and anti-(IL-10) inflammatory cytokines and pro-(caspase-3) and anti-(Bcl-2) apoptotic proteins in response to acute exercise in WR compared to NWR mice. These findings support the hypothesis that older mice undergoing voluntary exercise training have an IL cytokine and apoptotic protein response similar to that reported in the literature for young animals. Older untrained mice showed few changes in the expression of these cytokines and apoptotic proteins after and acute exercise challenge, suggestive of either less responsiveness or a blunted response in line with immunosenescence.

Pro- and anti-inflammatory cytokines. Older WR mice had higher TNF-α, IL-1β and IL-10 expression in intestinal lymphocytes immediately post-exercise. This mirrors findings describing the effect of oxidant stress on lymphocyte cytokine expression in young animals reported previously. In response to acute exercise challenge, Rosa Neto et al. (2009) found increased TNF-α expression in rat adipose tissue and Hoffman-Goetz et al. (2008) showed a 48% increase in mouse intestinal IL-10. Although inflammation is harmful to intestinal tissue, there may be potential physiologic ‘benefits’ to the pro- and anti-inflammatory cytokine responses to oxidant stress. Suzuki et al. (2003) reported that acute exercise induces an immediate increase in

pro-inflammatory cytokines (IL-6, IL-1β); this is followed by an anti-inflammatory response (increased IL-10, IL-1ra and IL-4). This secondary, anti-inflammatory cytokine response was suggested to counter intestinal inflammation and prevent traumatic tissue damage. Petersen and Pedersen (2005) proposed that acute exercise induces an anti-inflammatory environment in three possible ways. First, in response to acute exercise there are elevations in muscle-derived IL-6 which stimulates the production of the anti-inflammatory cytokines, IL-1ra and IL-10. Second, IL-6 inhibits the production of the pro-inflammatory cytokine, TNF-α. Finally, acute exercise increases circulating epinephrine which *in vivo* prevents TNF-α production (Petersen and Pedersen, 2005). Some of the long-term benefits of regular exercise may thus arise from these secondary anti-inflammatory cytokine responses to acute exercise (Mathur and Pedersen, 2008). This compensatory cytokine response may shift lymphocytes towards an anti-inflammatory milieu and protect against chronic inflammation, a key chronic disease risk factor (Mathur and Pedersen, 2008; Petersen and Pedersen, 2005).

Interleukin-10 inhibits the synthesis of pro-inflammatory cytokines TNF-α and IL-1β by regulating the transcription factor $NF_k\beta$ (Haddad and Fahlman, 2002). WR mice had a marked increase in IL-10 expression in response to oxidant stress associated with acute exercise while NWR mice showed little change in IL-10 expression after acute exercise. This finding is novel. Interleukin-10 plays an important antioxidant role by inhibiting the oxidative respiratory burst and preventing ROS-mediated tissue damage (Haddad and Fahlman, 2002). Our results suggest that training not only preserves the cytokine response to oxidant stress in older animals, but may bolster anti-inflammatory reserve (Roshan et al., 2011) or accelerate post-exercise recovery (Pearson et al., 1989) as seen at younger ages.

Regular training of moderate intensity decreases the prevalence and severity of gastrointestinal distress after acute exercise in young athletes (Brouns and Beckers, 1993). It is unknown if this protective effect of training on gastrointestinal symptoms occurs in middle-aged adults. However, a recent prospective cohort study (n=47,228; 18 years follow-up) of male health professionals indicated that highly active individuals (≥57.4 MET hours/week) had decreased risk of developing diverticulitis (RR = 0.75; 95% CI: 0.58-0.95) or diverticular bleeding (RR = 0.54; 95% CI: 0.38-0.77) compared to sedentary (≤8.2 MET hours/week) males (Strate et al., 2009). Another prospective cohort study (n=8,205; 3 years follow-up) in elderly subjects (≥68 years) showed that regular walking (3x/week) decreased the relative risk of gastrointestinal bleeding (RR = 0.60; 95% CI: 0.4 to 0.8) compared to elderly sedentary participants (Pahor et al., 1994). The IL-10 results reported here may have implications for older recreational athletes as the incidence of inflammatory intestinal disorders and acute gastrointestinal distress increases with age (Pahor et al., 1994).

Pro- and anti-apoptotic proteins. Elevated inflammatory cytokine concentration triggers lymphocyte apoptosis as mitochondrial pro-caspases (i.e., caspase-3 and -7) undergo cleavage in response to oxidant stress (Kuwana and Newmeyer, 2003). Active caspases move into the cytoplasm, degrade the plasma membrane and induce lysis (Kuwana and Newmeyer, 2003). Anti-apoptotic proteins, such as Bcl-2, antagonize these harmful effects and prevent the release and activation of mitochondrial caspases. Our results suggest that older WR mice appear to have a similar apoptotic response to oxidant stress as reported elsewhere for young mice (Hoffman-Goetz et al, 2010). Casual consideration of this apoptotic response to acute exercise might be interpreted as maladaptive or harmful. However, apoptosis is an evolutionarily conserved means of host defence and the capacity to induce apoptosis in response to stress

suggests preserved immune function (Kuwana and Newmeyer, 2003). In young mice exhaustive treadmill exercise increases caspase-3 and caspase-7, and decreases Bcl-2 expression in intestinal lymphocytes (Hoffman-Goetz et al, 2010). In healthy young people, caspase activation in cells with high levels of DNA damage decreases cancer risk (Ochs and Kaina, 2000). Thus, the apoptotic responsiveness of WR mice to acute exercise challenge might indicate better clearance potential of damaged lymphocytes; in contrast older NWR mice had increased expression only of caspase-7 without any changes in caspase-3 or Bcl-2 expression in intestinal lymphocytes.

Physiological indicators of training and oxidative stress. Four months of freewheel running induced physiological changes indicative of 'training' in older C57BL/6 mice. WR mice had increased cytochrome *c* oxidase enzyme activity in soleus and plantaris muscles. Long-term aerobic training has been shown to increase cytochrome *c* oxidase activity in young mice (Hoffman-Goetz et al., 2010; De Lisio et al., 2011) but this has not been documented for older mice. However, resistance training increased CO levels in older adults (Parise et al., 2005). The finding is especially noteworthy because cytochrome *c* oxidase activity in skeletal muscle decreases with age and may be indicative of senescent changes in oxidative buffering capacity (Bagh et al., 2011).

Intense treadmill exercise induced oxidative stress in WR and NWR mice demonstrated by elevated plasma corticosterone and 8-iso-$PGF_{2\alpha}$ immediately after the acute exercise bout. Plasma 8-iso-$PGF_{2\alpha}$ is a bio-marker of oxidative stress and is derived from arachidonic acid through a reaction catalyzed by oxidative intermediates (Roberts and Morrow, 2000). Acute exercise induces tissue hypoxia and inhibits SOD2 activity. This leads to increased 8-iso-$PGF_{2\alpha}$ production from multiple cellular origins due to ROS generation. In response to hypoxic (10% O_2) tissue stress, porcine pulmonary artery myocytes generate mitochondrial derived ROS (Gong

et al., 2009). In addition, Schneider et al. (2005) show that cellular exposure to SRM 1648 (an inducer of oxidant stress) increased glutathione and 8-iso-$PGF_{2\alpha}$ in alveolar macrophages. Given ischemia reperfusion in the intestinal-splanchnic interface in response to acute exercise, lymphocyte derived ROS may contribute to the elevated plasma 8-iso-$PGF_{2\alpha}$ observed in this study. Corticosterone is produced by the adrenal gland in response to physiological or psychological stress. This hormone plays a crucial role in the sympathetic response by stimulating gluconeogenesis and accessing energy reserves (Girard and Garland, 2002). Similar elevations in plasma 8-iso-$PGF_{2\alpha}$ and corticosterone in trained (WR) and untrained (NWR) older C57BL/6 mice indicates that animals responded to the acute-exercise challenge with equivalent oxidant stress responses.

This study is not without limitations. First, we measured intestinal lymphocyte protein for the cytokines and apoptotic outcomes of interest and not mRNA expression. We did not measure cytokine IL transcription in response to acute exercise, but only whole protein in the sample. Second, though we have previously shown high leukocyte concentration in intestinal lymphocyte lysates, some proteins may not have originated from lymphocytes but from other intestinal cells. This means that not all of the measured proteins definitively originated from intestinal lymphocytes. Third, our study only examined the effect of one bout of acute treadmill exercise on the intestinal lymphocyte cytokine and apoptotic protein response. Future research might utilize repeated exercise bouts to determine if the oxidant stress response differs between training groups. It is possible that exercise training might protect against repeated acute exercise induced inflammation or alternatively that WR and NWR mice do not differ in their IL response when faced with multiple exercise exposures. Fourth, we did not include young mice for direct comparisons with older animals. Thus, we cannot draw inferences about an “aging” effect as

opposed to an effect observed in older animals. Fifth, animals were 15-16 months at time of sacrifice. As noted previously, this is not extremely old as C57Bl/6 mice have a life-expectancy ranging from 19 to 24 months. Nevertheless, this age cohort provides a useful basis for comparison with a healthy late-middle age or young-elderly adults, when exercise interventions may be most beneficial to slow immunosenescence. We also studied only female mice and cannot generalize to male C57BL/6 mice. The rationale for using female mice was based on published reports on cytokine and apoptosis responses in young female animals and because females of this strain are better runners than males (De Bono et al., 2006). Finally, we only included one post-exercise time point for analysis, additional time points would be necessary to determine the kinetics of the intestinal lymphocyte cytokine and apoptotic protein responses to acute exercise.

5.9 Conclusion

In summary, four months of freewheel running in healthy older (15-16 months) female C57BL/6 mice was associated with a conserved intestinal lymphocyte response to exercise oxidative stress as demonstrated by higher expression of the pro-inflammatory cytokines TNF-α and IL-1β, the anti-inflammatory cytokine IL-10, and the apoptotic proteins caspase-3 and caspase-7, and lower expression of the anti-apoptotic protein Bcl-2. In contrast, untrained mice showed little change in intestinal lymphocyte expression of cytokine or apoptotic proteins to an acute exercise stimulus. This was despite the fact that the acute exercise was associated with increased plasma corticosterone and 8-iso-$PGF_{2\alpha}$. This study used an animal model to explore differences in the acute exercise induced oxidant stress response between trained and untrained older mice. We cautiously propose that voluntary exercise training preserves cytokine and apoptotic responses in

the intestinal tract of older animals and may help to counter blunted or senescent changes in the immune system.

CHAPTER 6: Discussion

6.1 Key Findings

The purpose of these thesis experiments was to describe the effects of *Age* (young vs. old) and *Freewheel Training* (trained vs. untrained) on the expression of: (1) pro- and anti-inflammatory cytokines; (2) pro- and anti-apoptotic proteins; and (3) surface expression of apoptotic markers in mouse intestinal lymphocytes, both at rest (baseline) and in response to acute exercise. **Figure 6.1** provides a summary of key findings across the three studies. Throughout the experiments, age at time of sacrifice, freewheel training status and acute exercise condition were important determinants of cytokine and apoptotic expression in intestinal lymphocytes of C57BL/6 mice.

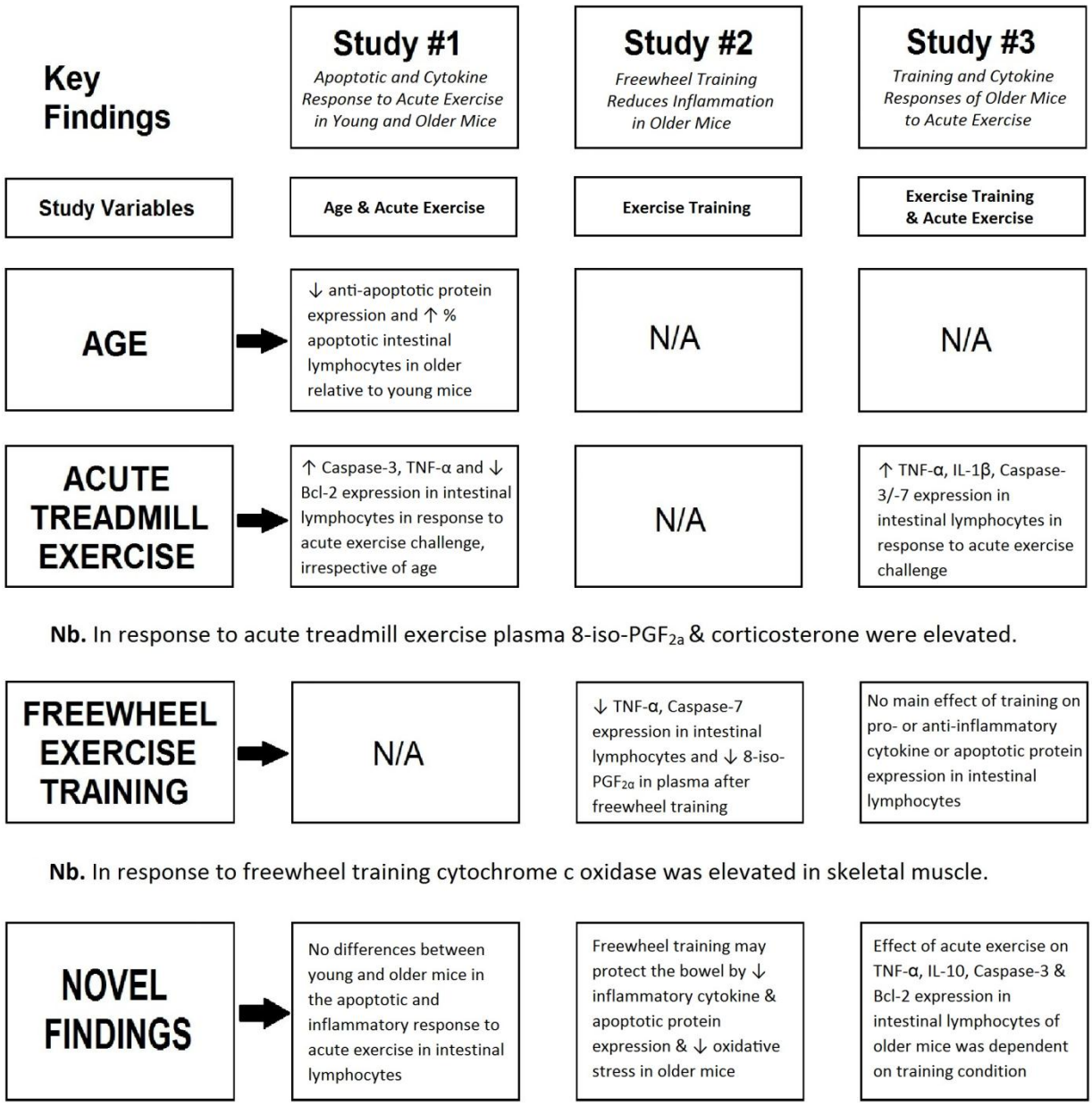
Study #1 demonstrated that young (3-4 months) and middle-aged (12-13 months) mice do not differ in their inflammatory and apoptotic intestinal lymphocyte responses. Oxidant stress exposure was associated with elevated intestinal lymphocyte (IL) expression of apoptosis-inducing cytokines (TNF-α) and pro-apoptotic proteins (caspase-3) and decreased IL expression of anti-apoptotic proteins (Bcl-2) in both age groups. In addition, older mice displayed lower IL expression of anti-apoptotic proteins (Bcl-2) and anti-inflammatory cytokines (IL-4) (Appendix III). Older mice also had higher surface expression of phosphatidylserine (indicative of early stages of cell membrane dysregulation and apoptosis) in IL across all acute-exercise time points. Finally, there was a significant interaction between age and acute exercise for Bcl-2, whereby the 'compensatory' anti-apoptotic response to acute exercise was reduced in older mice.

Study #2 showed that four months of voluntary freewheel training decreases intestinal inflammation, apoptosis and oxidant stress. Older WR (15-16 months) mice displayed lower pro-inflammatory cytokine (TNF-α) and pro-apoptotic protein (caspase-7) expression in intestinal

lymphocytes, and lower expression of stress hormones (8-iso-$PGF_{2\alpha}$) in plasma compared to older NWR (15-16 months) C57BL/6 mice.

Study #3 demonstrated that freewheel running preserved the 'normal' pattern of inflammatory cytokine and apoptotic protein expression in IL of older mice (15-16 months) after an acute exercise challenge. Immediately after acute treadmill exercise, older WR mice displayed significant elevations in: (1) pro-(TNF-α, IL-1β) and (2) anti-inflammatory (IL-10) cytokine and (3) pro-apoptotic (caspase-3, caspase-7) protein expression and (4) decreased anti-apoptotic (Bcl-2) protein expression in IL compared to sedentary (treadmill) controls. In contrast, older NWR mice had little intestinal lymphocyte 'responsiveness' to acute exercise in terms of cytokine and apoptotic protein expression. Only caspase-7 was elevated after acute treadmill exposure. In untrained older mice, this lack of response suggests immunosenescence whereas the findings from trained older mice suggest that training preserves 'normal' immune function.

Figure 6.1 A summary of key findings.



6.1.1 Aging and Immunosenescence

Direct comparison of differences in cytokine and apoptotic protein expression by age was conducted only in *Study #1*. Nevertheless, age at time of sacrifice was an important consideration in the interpretation of results across all three experiments, as different age groups (Young: 3-4 months; Middle-Aged: 12-13 months; Older: 15-16 months) were examined in each study. Despite the absence of a separate 'young' control group for *Study #2* and *Study #3*, the intestinal cytokine and apoptosis results were considered relative to previously published findings which utilized 'young' animal models.

6.1.1.1 Pro-Inflammatory Cytokines and Pro-Apoptotic Proteins

The findings from the first study suggest that the pro-inflammatory cytokine and pro-apoptotic protein response to exercise-induced oxidant stress does not differ by age. This is surprising given research showing that elderly individuals display reduced protein synthesis in the liver (Sheffield-Moore et al., 2005) and in muscle (Jozsi et al., 2000) after heavy resistance exercise compared to young individuals. This age-related reduced responsiveness to physiologic challenge may indicate cellular senescence. *Study #3* showed that untrained older mice demonstrate reduced responsiveness in several inflammatory and apoptotic measures after acute exercise. Other studies suggest that the elderly may have 'blunted' protein synthesis in response to acute exercise stress (Arranz et al., 2010a; Arranz et al., 2010b).

This conflicting result (*Study #1* vs. *Study #*3) may be due to the age of the mice at sacrifice. *Study #1* utilized 12-13 month old C57BL/6 mice. This age group does not reflect older animals as the average life expectancy of the C57BL/6 strain is approximately 20-22 months (Anisimov, 2009). However, there are reports of average life expectancy that range from 19

months (Pelton and Williams, 1958) to 23 months (Storer, 1966; Selman et al., 2006) under standard housing and breeding conditions.

The age of mice in *Study #1* would be more comparable to 'middle-aged' humans with respect to exercise capacity and immune function. In humans, definitions of 'middle-age' vary but typically refer to 45-59 years of age (Frankel et al., 1996). Alternatively, 60 years of age has been used with some consistency to refer to the beginning of old age (Roebuck, 1979). Making rough assumptions of equivalence between the aging processes and lifespan of female humans (84.1 years) (CIA, 2011) and female C57BL/6 mice (20-22 months) (Anisimov, 2009), 13-14 month old mice (*Study #1*) would be similar to 50 year old, 'middle-aged' humans. In contrast, 15-16 month old mice (*Study #3*) would be more similar to 60 year old 'young-old' humans. Thus, it is possible that the mice in *Study #1* were not sufficiently old to display the inflammatory and apoptotic changes or the blunted immune responsiveness seen in the older, immunoscenescent mice from *Study #3*.

The age at which senescent changes begin to be observed cellularly varies greatly between individuals. Research suggests that age-related declines in global function begin with small cellular changes that begin in early to late middle age (Beckman and Ames, 1998; Szweda et al., 2003). For example, age-related loss of muscle mass and decreased muscle proliferation has been reported as early as 25 years of age (Lexell, 1995). Moreover, the rapid increase in osteoarthritis incidence after 40 years of age (Praemer et al., 1999) in predicted by earlier variation in TGF-β signalling (van der Kraan et al., 2011). Studies utilizing gene sequencing and microarray (Erraji-Benchekroun et al., 2005) report that most indicators of senescent cellular vulnerability to DNA damage appear between age 45 and 70 (Lu et al., 2004). Most studies that show impaired protein or cytokine responses to acute exercise have included populations at the

end of this age range (i.e., closer to 70 years). For example, McLachlan and colleagues report reduced anti-tumour (1995a) and cytotoxicity (1995b) functions in monocytes isolated from aged (≥65 years) humans relative to younger ($\bar{X} = 25$ years) individuals.

The fact that 'middle-aged' mice retain the ability to mount an inflammatory and apoptotic response to oxidant stress (*Study #1*) underscores the importance of these physiological responses. Inflammation, as part of the innate immune response, is critical for the control of bacterial pathogens and for the maintenance of mucosal immunity (Strober et al., 2002**)**. This basic immunologic competence decreases with age. For example, the rate of autophagosome formation and lysosome function is dramatically reduced in hepatocytes of old (20-21) compared to young (5-6 months) CBA mice (Terman, 1995). This is likely due to down regulation of the lysosomal glycoprotein receptor 96 (Cuervo and Dice, 2000) suggestive of senescent impairments in cell mediated immune function. In addition, the intensity of the inflammatory response to *Schistosama mansoni* infection in humans follows a progressive decline that begins at 30-40 years of age (Burke et al., 2009) and continues with increasing age (≥60 years) (Comin et al., 2008). This decrease in the protective effects of the innate (inflammatory) response has been hypothesized to arise as a result of aging. Therefore, the decreased 'responsiveness' of untrained older mice (*Study #3*) to acute exercise challenge may be a key indicator of immunosenescence. Speziali et al. (2010) compared the response of young (2 months) and older (18 months) C57BL/6 mice to *Schistosoma mansoni* infection in the liver. In response to infection, young mice displayed an acute phase reaction characterized by cellular inflammation and the formation of a protective fibrotic granuloma; older mice showed none of these changes. In addition, young C57BL/6 mice had massive recruitment and activation of CD4+ T cells in the

liver; older mice had elevated baseline frequencies of $CD4^+$ T cells but no compensatory changes in response to infection

6.1.1.2 Anti-Inflammatory Cytokines and Anti-Apoptotic Proteins

Aging is also associated with dysregulation in anti-inflammatory (Elrefaei et al., 2002) and anti-apoptotic protein expression (Zhang et al., 2003). Arranz et al. (2010b) show *ex vivo* that there is decreased IL-10 expression in peritoneal leukocytes from old (69±4 weeks) and very old (92±4 weeks) extremely long-lived ICR (CD1) female mice compared to middle-aged (44±4 weeks) controls. In addition, in response to ConA stimulation, leukocytes from old and very old animals had impaired T_H1 and T_H2 cytokine responses, including reduced secretion of IL-4. In response to LPS stimulation, dendritic cells isolated from a group of healthy elderly people (*n=10;* range: 60-81 years; gender: 6/4, M/F) displayed marked reductions in IL-10 expression compared to younger persons (*n=10;* range: 21-40 years; gender: 7/3, M/F) (Ciaremella et al., 2011). Moreover, the expression of the anti-apoptotic protein Bcl-2 was markedly lower (-60%, $p<0.05$) in endothelial cells obtained from healthy older (61 ± 1 years) compared with young (age 25±1 years) men (Kushner et al., 2011). Aggarwal and Gupta (1998) report decreased expression of Bcl-2 in CD4+ and CD8+ T cells isolated from aged (65–95 yr; n = 15) compared to young (20–29 yr; n = 15) men.

Due to these senescent changes, older mice may be more susceptible to tissue injury in response to oxidative stress challenge. Findings from *Study #1* were of baseline reductions in intestinal lymphocyte expression of Bcl-2 and IL-4 in aged C57BL/6 mice. Moreover, older mice demonstrated an impaired anti-apoptotic (Bcl-2: *Study #1 & Study #3*) and anti-inflammatory (IL-10: *Study #3*) response to oxidant stress. Considerable Bcl-2 depletion (Kuwana and Newmeyer, 2003) and elevated IL-10 (Haddad and Fahlman, 2002) expression normally

accompanies oxidative stress exposure. These regulatory proteins play a key homeostatic role in buffering the damaging effects of oxidative challenge; the protective effects were not observed in sedentary older (*Study #1*) or untrained older (*Study #3*) mice. Similar age-related impairments in the anti-inflammatory immune response (to infection) have been reported by others (Arranz et al., 2010b). Speziali and colleagues (2010) compared the immune responses of young (2 months) and old (18 months) C57BL/6 mice to *Schistosoma mansoni* infection. Young mice showed elevated liver concentration of the regulatory cytokines IL-4 and IL-10; this was not observed in old mice (Speziali et al., 2010). Elrefaei et al. (2002) report reduced production of IL-10 in spleen cells isolated from old (22 months) C57BL/6 mice in response to *in vitro* infection with E55+ murine leukemia retrovirus compared to young (6 month) animals. Collectively, these data support the hypothesis that there is an age-related decrease in anti-inflammatory cytokine and anti-apoptotic protein expression in response to cellular exposure to various stressful stimuli (Elrefaei et al., 2002).

These senescent impairments may also partly explain the higher percentage of apoptotic IL observed at (1) baseline (*Study #1*) in sedentary older mice and (2) after acute treadmill exercise (*Study #3*) in untrained (NWR) older mice. This result is not surprising given prior research showing oxidant stress results in greater splenic lymphocyte apoptosis in older vs. young individuals (Schindowski et al., 2001). A similar increase in phosphatidylserine expression (i.e., Annexin^{+} or apoptosis) on human lymphocytes from elderly men ($\overline{X}$ = 70 yrs) compared to young men ($\overline{X}$ = 29 years) has been reported elsewhere (Noble et al., 1999). Finally, in response to staurosporine stimulation, endothelial progenitor cells isolated from healthy older (61 ± 1 years) men display significantly higher intracellular concentration of active caspase-3

(+35%, $p<0.05$) when compared to young (age 25 ± 1 years) participants (3.15±0.29 pg/ml vs. 2.33±0.24 pg/ml, respectively) (Kuskner et al., 2011).

6.1.1.3 Summary

When the effects of acute exercise are compiled across each of the three age groups, Young (3-4 months), Middle Aged (12-13 months) and Older (15-16 months), there is a marked reduction in inflammatory cytokine and apoptotic protein expression in intestinal lymphocytes. This is exhibited as a senescent loss of immune 'responsiveness' and is illustrated in **Table 6.1**.

Table 6.1 Decreased 'responsiveness' to acute exercise with increasing age

TNF-α [A.U.]	**SED**	**IMM**	**ΔAE (IMM-SED)**	**ΔAE%**
Young (3-4 mo)	0.83	1.20	+ 0.37 [A.U.]	+44.6%
Middle-Age (12-13 mo)	0.95	1.20	+0.25 [A.U.]	+26.3%
Young-Old (15-16 mo)	0.96	1.17	+0.21 [A.U.]	+21.8%

6.1.2 Potential Biological Mechanisms

6.1.2.1 Baseline Changes

The 'free radical theory of aging' states that the cellular effect of senescence is due to lifetime accumulation of ROS intermediates (Harman, 1956). Many age-related immune changes may be attributed to elevated 'basal' cellular stress that has accumulated over a lifetime. It has been argued that senescent increases in endogenous ROS, in combination with decreases in anti-oxidative defenses, promote increased cellular damage in the elderly (Sasaki et al., 2010). Aged (≥18 months) C57BL/6 mice display increased ROS production as measured by elevated intensity of chemiluminescent signaling in brain slices *ex vivo* (Sasaki et al., 2010). This can

subsequently increase T cell mediated inflammation and apoptosis in the absence of a specific oxidant challenge or discernable disease pathology. Moreover, plasma concentration of 8-iso-$PGF_{2\alpha}$ and corticosterone, biomarkers of oxidative stress, has been shown to increase throughout the lifespan (Garrido et al., 2010; Montine et al., 2011). Chronically elevated 8-iso-$PGF_{2\alpha}$ predicts the progression of age-related diseases, including cancer (Dai and Zhu, 2009). Accordingly, slowing absolute or relative increases in ROS generation is a strategy for preventing senescent declines.

In *Study #1* Bcl-2 expression was decreased in older mice. Hildeman et al. (2003) report an inverse correlation between the levels of Bcl-2 and ROS within T cells. Observation of T cells isolated from C57BL/6 mice indicates that ROS production occurs prior to Bcl-2 down-regulation in response to i.v. injection of *Staphyloccal enterotoxin B* (Hildeman et al., 2003). In this manner, endogenous ROS indirectly sensitize cells towards apoptosis by decreasing T cell concentration of anti-apoptotic proteins such as Bcl-2. Furthermore, IL-4 expression is reduced in old mice, a decrease which may arise from chronic oxidant stress. In healthy individuals, TCR-triggered ROS generation induces IL-4 expression in active T cells. Specifically, oxidative signalling (H_2O_2) triggers influx of Ca^{2+} into T cells which induces IL-4 expression via the transcription factor NF_KB (Kaninski et al., 2010). Given senescent impairments in NF_kB function (Gupta et al., 2005) following chronic NF_kB activation (Arranz et al., 2010a), age-related increases in basal ROS may deplete TCR functionality and T cell capacity for IL-4 production.

Accordingly, ROS increases in senescent cells and tissues would decrease cellular reserve by depleting endogenous stores of anti-apoptotic, anti-inflammatory and anti-oxidant proteins (Bagh et al., 2011). Cellular reserve refers to the maximum level of stress that can be effectively buffered through intracellular biochemical pathways (e.g., citrate synthase, glutathione, GpX)

without the induction of cell death via apoptotic membrane dysregulation. Taken together, the findings of this thesis research provide evidence of decreased homeostatic buffering capacity and decreased cellular reserve with advancing age in C57BL/6 mice. The fact that older mice had higher IL apoptosis at baseline (*Study #1*) and in response to acute exercise (*Study #1* & *Study #3*) may be a reflection of these immunoscenescent changes and may explain the increase in cellular fragility with aging. It is also possible that this fragility may be due to (1) an age-related deficiency in TNF-α induced $NF_K\beta$ dependent anti-apoptotic signalling (Gupta et al., 2005) or (2) elevated Fas ligand (CD95) expression (Potestio et al., 1999).

Age-related decreases in baseline anti-apoptotic (Bcl-2) and anti-inflammatory (IL-4) proteins may increase cell susceptibility to apoptosis. In addition, the age-related inability to mount a compensatory anti-inflammatory (IL-10) and anti-apoptotic (Bcl-2) response to oxidative stress may present an 'open window' for dysregulated apoptosis. Despite the reduced (*Study #3*) or equivalent (*Study #1*) inflammatory and apoptotic response to acute exercise in older animals, the cellular effects of these responses are far more damaging in the elderly. *Study #1* indicates elevated % Annexin V^+ IL in older mice, despite comparable or equivalent inflammatory cytokine and apoptotic expression between young and older animals. It would appear that much of this age-related increase in cellular fragility reflects depleted cellular reserve as a result of baseline decreases in Bcl-2 and IL-4 and impaired Bcl-2 and IL-10 responses to exercise-induced oxidant stress. This can result in greater cellular susceptibility to oxidant stress and may contribute to elevated apoptotic cell death, a physical manifestation of an impaired regulatory mechanism.

6.1.2.2 Reduced Immune Responsiveness

The specific mechanisms for the apparent senescent (blunted) immune responsiveness are unknown. Four potential mechanisms are discussed including: (1) elevated 'baseline' inflammation, (2) impaired response to novel stressors, (3) decreased intracellular transcription and translation, and (4) altered transcription factor activity.

First, aging is characterized by increased inflammatory protein markers reminiscent of an acute phase reaction. Healthy persons (23-87 years of age) display elevated plasma concentration of IL-6 and fibrinogen (an acute phase protein) with increasing age; plasma IL-6 increases by 0.016 pg ml^{-1} per year, from 0.34±0.39 pg ml^{-1} in young (31±5 years) to 1.05±0.77 pg ml^{-1} in old (75±5 years) persons (Hager et al., 1994). This senescent inflammation (Fagiolo et al., 1993) is accompanied by apoptosis in human leukocytes (Noble et al., 1999). Liver, lung and spleen tissue from old (23-27 months) Fisher 344 rats showed elevated pro-apoptotic executioner caspase activity (caspase-3, caspase-6 and caspase-7); greater expression of caspase-2 and caspase-9 was also noted (Zhang et al., 2002).

Second, a senescent increase in baseline inflammation and apoptosis may interfere with responses to novel oxidant stressors (i.e., lack of responsiveness to additional challenge). One possible explanation for this lack of immune responsiveness involves cytochrome *c*, a mitochondrial apoptotic signaling messenger. In elderly Fisher 344 rats, liver concentration of cytochrome *c* is decreased due to continuous cytosolic degradation (Zhang et al., 2002). Cytochrome *c* is needed to induce the intrinsic apoptotic pathway (Jiang et al., 2004); therefore, continuous production and cytosolic release of cytochrome *c* may contribute to senescent increases in baseline apoptosis. The resulting lack of residual stores of

intramembrane/intracellular cytochrome *c* may also account for the inability to mount an apoptotic response to transient increases in oxidant stress.

A third potential mechanism underlying the decreased responsiveness to exercise challenge is that post-exercise increases in transcription and translation are not detectable in (untrained) sedentary older mice. This could reflect constitutively high production of inflammatory proteins under resting conditions, as chronic and senescent elevations in IL-1β or TNF-α inhibit mRNA transcription of apoptotic caspases or inflammatory cytokines (Fagiolo et al., 1993). Although not studied in immune tissue or the gastrointestinal tract, it has been shown that hippocampal injection of IL-1β reduces transcription of mRNA for brain-derived neurotrophic factor (Barrientos et al., 2004).

Finally, changes in expression of the transcription factor $NF_k\beta$ (a known promoter of inflammation and source of tissue-damaging metabolites) occur with age (Lin et al., 2010). Immunoscenescent changes lead to chronic inflammation and apoptosis; this may 'exhaust' the ability of the $NF_k\beta$ pathway to respond to acute exercise, particularly given the observation that senescent increases in ROS can elevate basal $NF_k\beta$ expression (Gloire et al., 2006). In support of this potential mechanism, glomerular cells from aged (24 months) Fisher 344 rats (Wiggins et al., 2010) and peritoneal leukocytes from very old (92±4 weeks) cell cultures obtained from CD1 mice (Arranz et al., 2010a) show constitutive expression of pro-inflammatory phenotypes due to chronic up-regulation of $NF_k\beta$. In addition, human lymphocytes from older individuals display decreased phosphorylation of I_kB_α, and decreased expression of IKK_β, indicative of senescent inefficiency in $NF_k\beta$ signalling (Gupta et al., 2005). Chronic $NF_k\beta$ activation in the elderly may explain the lack of immune 'responsiveness' to cellular challenge (Arranz et al., 2010b). Collectively these results support the hypothesis that chronic inflammation is responsible (at

least in part) for reduced protein transcription and translation in liver and skeletal muscle of elderly individuals in response to heavy resistance exercise **(**Sheffield-Moore et al., 2005; Jozsi et al., 2000).

6.1.3 Acute Treadmill Exercise

The effect of acute treadmill exercise on intestinal cytokine and apoptotic protein expression in IL of older mice was examined in *Study #1* and *Study #3*. Acute treadmill exercise was used to induce oxidative stress, which was confirmed physiologically by elevated plasma 8-iso-$PGF_{2\alpha}$ and corticosterone.

Acute treadmill exercise is commonly used as a stress challenge to evaluate the immune response. The findings reported in this thesis are consistent with earlier studies showing that acute treadmill exercise induces pro-inflammatory (IL-1β, TNF-α) cytokine and pro-apoptotic (caspase-3, caspase-7) protein expression in intestinal lymphocytes of young (3-4 months) mice (Hoffman-Goetz et al., 2010). In addition, elevated phosphatidylserine expression in intestinal (Hoffman-Goetz and Quadrilatero, 2003) and in splenic (Hoffman-Goetz and Spagnuolo, 2007) lymphocytes, and increased expression of anti-(IL-10) inflammatory and decreased expression of anti-(Bcl-2) apoptotic proteins in response to acute exercise have been documented in young mice (Hoffman-Goetz et al., 2010). The findings from *Study #1* and *Study #3* provide support that acute exercise is pro-inflammatory and pro-apoptotic in the intestine of older mice as well. Although intense exercise can induce inflammatory pathologies in the gastrointestinal tract, it can also result in beneficial effects due to secondary anti-inflammatory cytokine responses or leukocytosis of immunologically inactive T cells.

These may be particularly important considerations for immunoscenescent individuals; the number and/or percentage of terminally differentiated (senescent) lymphocytes are elevated in elderly persons. Specifically, peripheral blood T cells from healthy elderly individuals (>65 years) display elevated $CD45RO^{+}CD45Rb^{-}$ phenotypes (indicative of telomeric shortening and terminal T cell differentiation) compared to younger (18-25 years) persons (Ivory et al., 2004). Simpson et al. (2011) propose that lymphocytopenia may selectively target senescent and immunologically unimportant T cells. Acute exercise-induced lymphocytopenia mobilizes senescent T cells from the peripheral tissues to the intestine; this is followed by subsequent apoptosis in peripheral tissues during recovery. Senescent T cells typical contribute only marginally to the innate immune response to novel pathogens and age-related changes in their expression of surface membrane receptors (e.g., elevated β_2-adrenergic receptors) may bias them towards apoptosis in response to acute exercise-induced inflammation (Simpson et al., 2011). This apoptotic 'pruning' is thought to mobilize naïve T cells from the gut-associated lymphoid tissues and improve the immune response of the elderly.

6.1.4 Freewheel Exercise Training

The effect of long-term voluntary freewheel running on the expression of cytokines and apoptotic proteins in intestinal lymphocytes from old C57Bl/6 mice was examined in *Study #2* and *Study #3*. In both studies, training altered intestinal lymphocyte expression of cytokines and apoptotic proteins, either at baseline or immediately after acute exercise. Physiologic confirmation of training status was validated by increased cytochrome c oxidase concentration in skeletal muscle. The results from *Study #2*, important from a chronic disease and immunosenescence viewpoint, were not unexpected. A more novel finding was that freewheel

training preserves immune responsiveness to oxidative stress and maintains anti-inflammatory and anti-apoptotic capacity, despite older age.

6.1.4.1Main Effect of Freewheel Training

Findings from *Study #2* are consistent with the literature which shows that freewheel training results in lower expression of the pro-inflammatory cytokine (TNF-α) and pro-apoptotic protein (caspase-7) in IL (Hoffman-Goetz et al., 2010) and lower expression of 8-iso-$PGF_{2\alpha}$ in plasma (Devries et al., 2008). Additionally, training increases the expression of the anti-inflammatory cytokine IL-10 in mouse IL (Hoffman-Goetz et al., 2010) and the anti-apoptotic protein Bcl-2 in muscle from the soleus and left ventricle of male Sprague-Dawley rats (Vainshtein et al., 2011). Plasma concentration of IL-6 was reduced in response to 20 weeks of treadmill exercise training (60 min day^{-1}, 20 m min^{-1}, 5% grade) in C3(1)SV40Tag mammary tumour prone mice (Murphy et al., 2011). In humans, six months of aerobic exercise training (4 times/week, 45-60 min/session) increased plasma IL-10 ($p=0.039$) in overweight individuals with type 2 diabetes (Kadoglou et al., 2007).

The pathways for the anti-inflammatory effects are likely complex. However, one possible sequence is as follows: with training, concentration of the pleiotropic cytokine IL-6 increases (Petersen and Pedersen, 2005) and functions in an anti-inflammatory manner. $NF_k\beta$ is decreased due to IL-6 induction of IL-10 (Steensberg et al., 2003). Using cardiomyocytes isolated from adult Sprague–Dawley rats, Dhingra et al. (2009) demonstrate that IL-10 inhibits TNF-α-induced $NF_k\beta$ (transcription factor) activation by inhibiting IKK phosphorylation. Moreover, decreased $NF_k\beta$ inhibits a major pathway for the production of many 'classical' pro-inflammatory cytokines (Lin et al., 2010) and pro-apoptotic markers (Dhingra et al., 2009). The decrease in basal inflammation as a function of training results in lower intracellular ROS and

enhances endogenous anti-oxidant concentration (Devries et al., 2008). The sum of these changes may slow the aging process (Harman, 1956; Sasaki et al., 2010) and decrease chronic disease risk (Dai and Zhu, 2009).

6.1.4.2 Restored Immune Responsiveness

6.1.4.2.1 Pro-Inflammatory Cytokines and Pro-Apoptotic Proteins

The results from *Study #3* indicate that freewheel training preserves IL inflammatory cytokine and apoptotic protein expression in response to acute exercise in older (15-16 months) C57BL/6 mice. This finding supports the hypothesis that regular physical activity preserves the ability of aged individuals to respond to oxidant stress (Arranz et al., 2010b). Inflammation is an important part of the innate immune response to bacterial and viral pathogens (Li et al., 2007) and apoptosis plays a key role in preventing the perpetuation of mutated cells, thereby slowing the development of dysplasia/carcinoma. Elderly individuals display increased susceptibility to exogenous/endogenous pathogens and increased cancer risk due to immunoscenescent impairments in the innate immune response (Huang et al., 2008), apoptotic dysregulation and impaired anti-tumour immunity (Goon et al., 2008). Presumably, training slows the rate of this decline and establishes an immune profile similar to young animals (Arranz et al., 2010b).

Specific mechanisms underlying this preservation effect are not well understood. It is possible that the 'anti-inflammatory' effect of training counters senescent 'inflame-aging' (Huang et al., 2008) and preserves the capability of immune cells to induce an acute response. By decreasing baseline transcription of inflammatory and apoptotic proteins through NF_kB modulation, transient increases after oxidant stress are again possible (Arranz et al., 2010a). Impaired function of the $NF_k\beta$ transcription pathway is common with advancing age (Gupta et al., 2005). Ivory et al. (2004) report constitutively higher nucleic expression of $NF_k\beta$ in

peripheral blood lymphocytes obtained from healthy elderly (<65 years) compared to younger (18-25 years) people. Furthermore, in response to exogenous stimulation (pro-inflammatory cytokines), nuclear $NF_k\beta$ concentration in peripheral blood lymphocytes from elderly volunteers was unchanged. Since many inflammatory cytokines and apoptotic proteins are transcribed by this pathway, training-induced decreases in basal NF_kB expression may preserve 'normal' function (Arranz et al., 2010b). For example, intra-cisterna injection of anti-inflammatory IL-1Ra has been shown to prevent the inhibition of brain-derived neurotrophic factor production from neurons of aged (24 months) F344xBN rats in response to intraperitoneal injection of live *E. coli* (Cortese et al., 2011). In this manner the 'anti-inflammatory' effects of training may permit greater production of cytokines and apoptotic proteins after oxidant stress challenge.

6.1.4.2.2 Anti-Inflammatory Cytokines and Anti-Apoptotic Proteins

A novel finding from *Study #3* was that training preserved anti-inflammatory cytokine and anti-apoptotic protein expression in older mouse IL following acute exercise. In trained mice, IL-10 and Bcl-2 expression after acute exercise was similar to that observed in young mice. This suggests that freewheel running improves the homeostatic buffering capacity of intestinal lymphocytes from older animals, thereby improving the ability of these animals to cope with acute oxidant stress. Older WR mice did not have post-exercise increases in the number or percentage of apoptotic IL (i.e., % Annexin V^+ IL), despite elevated expression of inflammatory cytokines and apoptotic proteins immediately after acute exercise. In contrast, older NWR (untrained) mice had post-exercise increases in the number and percentage of apoptotic IL, without significant changes in inflammatory cytokine or apoptotic protein expression in IL. These 'anti-inflammatory' and 'anti-apoptotic' effects of training may be due to lower oxidant stress, inflammation and apoptosis under resting conditions. This environment may permit

normal inflammatory and apoptotic responses to acute exercise consequent to abrogation or continuous depletion of anti-inflammatory or anti-apoptotic reserve.

6.2 Integration

Aging is characterized by a blunted innate immune response. Reductions in anti-inflammatory and anti-apoptotic protein expression after acute exercise may be due to higher baseline oxidant stress and inflammation. In this manner, 'inflame-aging' exhausts the homeostatic buffering capacity that protects cells from transient increases in oxidant stress. This theoretically presents an open window of cell vulnerability. Training maintains the typical inflammatory and apoptotic protein response to acute stress that is impaired with increasing age. This may be one mechanism whereby training conserves innate immune function and slows the progression of immunoscenescent declines. Freewheel training decreases basal inflammation and apoptosis in intestinal lymphocytes and decreases plasma concentration of reactive oxygen species. In this manner, training may slow the effects of aging by decreasing inflammation, apoptosis and oxidative stress under resting conditions, and thereby maintain the apoptotic and cytokine response to acute physiologic (oxidant stress) challenge.

6.3 Implications for Public Health

There is a strong body of evidence supporting the effectiveness of regular exercise for improving health, decreasing chronic disease risk and slowing the rate of immunosenescence. Specifically, the results from this thesis research suggest that overall training may decrease inflammation, apoptosis and oxidant stress. In addition, older WR mice display greater inflammatory cytokine and apoptotic protein expression in IL after acute exercise challenge than older NWR (untrained)

controls. Initially, it might be assumed that acute exercise has the potential to be more damaging to the intestine of trained older mice; however, older WR mice also show secondary anti-inflammatory cytokine expression of IL-10 and compensatory Bcl-2 depletion. This finding indicates that training might actually improve immune-surveillance and tumour cell killing; this may be beneficial in dealing with infections and in slowing the progression of carcinogenesis. It also means that training may reduce chronic inflammation in the gastrointestinal tract due to elevated IL-10; this may have relevance for the pathogenesis of colorectal cancer and inflammation-mediated disease.

6.4 Limitations

There are limitations to the research findings presented in this thesis. Many of which have been discussed in detail in Chapters 3, 4 and 5. Several of these limitations will be highlighted and additional caveats will be discussed below.

There are limitations inherent with animal research, principally in generalizability to humans. Nevertheless, animals provide important models for biological research with which to help identify basic 'biomarkers of aging' and improve scientific understanding of chronic disease (Huber and Sierra, 2009). The use of a healthy mouse model was intended to explore possible mechanisms contributing to the positive effects of 'training' in healthy aged humans. Future research will be needed to determine 'training' effects in animal models of human disease (e.g., ulcerative colitis mice) or in a clinical population (e.g., inflammatory bowel disease patients).

Another limitation is that only female mice were included in the experiments. There are known effects of estrogen and luteal hormones on cellular immune function (De Crée, 2011). In addition, there are menstrual 'phase-dependent' effects on T cell responses to sprint exercise due

to catecholaminergic variation (Botacazou et al., 2006). This may explain, in part, gender differences in the rate of immunoscenescent declines and may justify inclusion of males in future acute-exercise experiments. Nevertheless, female C57BL/6 mice are 'better runners' and more likely to be physiologically trained than male mice (Turner et al., 2005). In addition, future research investigating the effects of training and aging might consider the use of a mouse model with a shorter lifespan (e.g., CD1 mice) as a model of 'rapid' aging. This is pertinent given the fact that C57BL/6 mice, while not extremely long-lived, do have above average life expectancy.

As discussed prior, the old mice in these experiments were not necessarily senescent and showed between-study differences in age. The average life expectancy of C57BL/6 mice is 20-22 months (Anisimov, 2009). Thus, the 'old' mice from *Study #1*, which were 12-13 months at sacrifice, may correspond more closely to 'middle-age' while the 15-16 month 'old' cohort from *Study #3* may be more similar to 'young-old' humans. Future research should examine both the effects of freewheel training and acute treadmill exercise on intestinal inflammation and apoptosis in extremely old mice (i.e., 18-20 months) who display cellular immune decrements indicative of immunosenescence. As mentioned prior, this age cohort is not at the extreme end of their lifespan, of which reports vary from 18.6 months (Pelton and Williams, 1958) to 23.4 months (Selman et al., 2006). In addition, a young control condition was not included in each study due to the extensive literature on the effects of exhaustive exercise and exercise training in young mice. Future research might include a young control group to enable statistical comparison between experimental conditions.

Intestinal lymphocyte protein expression, but not mRNA for the cytokines and apoptotic markers of interest, was measured. Although high leukocyte (% $CD45^+$ IL) concentration in intestinal lymphocyte lysates has been shown elsewhere, some proteins may not have originated

from lymphocytes. This means that not all of the measured proteins definitively originated from intestinal lymphocytes. Future research might consider the use of PCR analysis to measure gene transcription in purified lymphocyte samples. This would empirically validate the specific translational effects of treadmill exercise and the cellular source of cytokines and apoptotic proteins.

An additional limitation is that the absolute intensity of acute treadmill exercise differed between 'young' vs. 'older' and 'trained' vs. 'untrained' C57BL/6 mice. Each of these groups differed in their mean run-to-exhaustion times. This may confound some of the effects of age or training on intestinal inflammation and apoptosis. However, the analysis of treadmill exercise-induced increases in plasma corticosterone and 8-iso-PGF_{2a} suggest that the relative intensity of acute challenge was similar for both age and training conditions. Nevertheless, future research might utilize repeat exercise bouts of shorter duration to determine if the oxidant stress response differs between age or training groups as a function of a less intense treadmill challenge (i.e., one equally tolerated by both age and training conditions).

Finally, there were significant differences in body weight at sacrifice between 'young' and 'older' mice and between 'trained' and 'untrained' mice. Given the documented role of adipose tissue in the production of cytokines, most notably IL-6 (Pedersen and Fisher, 2007), it is possible that group differences in weight may have affected intestinal expression of inflammatory and apoptotic variables (Morisset et al., 2008). Therefore, it is possible that differences in the amount of adipose tissue may have contributed to age and training effects. This is a limitation to *in vivo* experimentation and could likely only be eliminated through the use of isolated lymphocytes grown and aged in culture. Future research using cell cultures may be able to 'tease out' this potential confound.

6.5 Concluding Remarks

Colorectal cancer is the second leading cause of Canadian cancer mortality. Moreover, the incidence of chronic health problems and other diseases of aging will continue to increase as the Canadian demographic changes. Persistent inflammation is a fundamental risk factor in the aetiology of sporadic intestinal carcinoma and in chronic disease pathogenesis. Accordingly, reducing inflammation may decrease disease risk. Many population studies demonstrate reduced odds of developing CRC among physically active persons. The results from this research suggest that training may slow or prevent senescent declines by decreasing inflammation, apoptosis and oxidative stress at baseline, thereby preserving the immune response to acute challenge. This research furthers our understanding of how exercise influences the colonic cytokine milieu, even in the presence of immunoscenescent changes.

APPENDICIES

I – Sample Size Calculations

Sample sizes were calculated based on a power of $1-\beta = 80\%$, and a threshold of significance of $\alpha=0.05$ using previously published data for young C57BL/6 mice given long term freewheel running and acute treadmill exercise (Hoffman-Goetz et al. 2009). Standard deviations and predicted treatment effects are shown for two pro-inflammatory cytokines: TNF-α and IL-6. Published data for use of sample size calculations for anti-inflammatory cytokines are limited; the sample size calculation is based on a single cytokine, IL-1ra. The formula for estimation of sample size [$n = 2(z_{1-\alpha} + z_{1-\beta})^2 \sigma^2 / (\mu_1 - \mu_2)^2$] is from Case and Ambrosius (2007). Sample size per group is 15 mice and (conservatively) 20 mice in the 'pilot' and 'main' studies, respectively.

Intestinal Lymphocyte Data			
Outcome Measure	Standard Deviation	Mean difference* WR IMM vs. NWR IMM	Sample Size
TNF-α	0.07995	0.2241 – 0.1335	12.2
IL-6	0.30703	1.0383 – 0.7483	17.6
IL-1ra	0.20715	1.5171 – 1.3000	14.3

* in arbitrary densitometric units

II – Additional Study Outcomes

Study #1: Young vs. Older (w/ Acute Exercise) – 2x3 ANOVA

IL-1β (2x3 ANOVA)				
		Mean	**SD**	**N**
Young	**SED**	.8671	.34737	14
	IMM	.9153	.32656	15
	2Hrs	.8360	.32754	15
	Total	.8730	.32748	44
Older	**SED**	1.0420	.53336	15
	IMM	1.0153	.34351	15
	2Hrs	.9433	.32714	15
	Total	1.0002	.40483	45
Total	**SED**	.9576	.45406	29
	IMM	.9653	.33322	30
	2Hrs	.8897	.32625	30
	Total	.9373	.37208	89
Age:		$F_{(1,88)} = 2.561$, P = 0.113		
Acute-Exercise:		$F_{(2,88)} = 0.356$, P = 0.702		
Interaction:		$F_{(2,88)} = 0.089$, P = 0.915		

$NF_kβ$ (2x3 ANOVA)				
		Mean	**SD**	**N**
Young	**SED**	1.0857	.50836	14
	IMM	.9487	.45212	15
	2Hrs	1.0147	.45952	15
	Total	1.0148	.46540	44
Older	**SED**	.9567	.33133	15
	IMM	1.1593	.36634	15
	2Hrs	1.0073	.35712	15
	Total	1.0411	.35468	45
Total	**SED**	1.0190	.42330	29
	IMM	1.0540	.41827	30
	2Hrs	1.0110	.40438	30
	Total	1.0281	.41099	89
Age:		$F_{(1,88)} = .079$, P = 0.780		
Acute-Exercise:		$F_{(2,88)} = .087$, P = 0.917		
Interaction:		$F_{(2,88)} = 1.265$, P = 0.288		

INTERLUKIN-4 (2x3 ANOVA)				
		Mean	**Stdev**	**N**
Young	**SED**	1.1293	.20439	14
	IMM	1.0780	.24920	15
	2Hrs	.9573	.23337	15
	Total	1.0532	.23640	44
Old	**SED**	.7407	.37621	15
	IMM	.9567	.28218	15
	2Hrs	.6820	.21802	15
	Total	.7931	.31585	45
Total	**SED**	.9283	.35947	29
	IMM	1.0173	.26875	30
	2Hrs	.8197	.26238	30
	Total	.9217	.30706	89
Age:		$F_{(1,88)}$ = 21.300, P = .000		
Acute-Exercise:		$F_{(2,88)}$ =4.134, P = 0.019		
Interaction:		$F_{(2,88)}$ = 1.858, P = 0.162		

INTERLEUKIN-6 (2x3 ANOVA)				
		Mean	**Stdev**	**N**
Young	**SED**	2.3907	.74700	14
	IMM	2.3913	.85513	15
	2Hrs	2.4015	.50821	13
	Total	2.3943	.70869	42
Old	**SED**	1.5447	.59826	15
	IMM	.9920	.36602	15
	2Hrs	1.4207	.36691	15
	Total	1.3191	.50664	45
Total	**SED**	1.9531	.78940	29
	IMM	1.6917	.96131	30
	2Hrs	1.8761	.65784	28
	Total	1.8382	.81411	87
Age:		$F_{(1,86)}$ = 69.187, P = .000		
Acute-Exercise:		$F_{(2,86)}$ =1.732, P = 0.183		
Interaction:		$F_{(2,86)}$ = 1.696, P = 0.190		

Study #2: Wheel Running vs. No Wheel Running (One-way ANOVA)

ONE-Way ANOVA (WR x NWR)		N	Mean	SD	SEM
CD45RA	Wheel Running	19	66.8158	14.28625	3.27749
	No Wheel Running	25	69.3360	12.68325	2.53665
	Total	44	68.2477	13.29720	2.00463
CD45	Wheel Running	19	78.9842	12.44942	2.85609
	No Wheel Running	25	82.0680	8.79340	1.75868
	Total	44	80.7364	10.50827	1.58418
CDgd	Wheel Running	19	4.1937	1.94800	.44690
	No Wheel Running	23	3.9374	1.87374	.39070
	Total	42	4.0533	1.88853	.29141
CD28	Wheel Running	18	16.3611	7.45229	1.75652
	No Wheel Running	24	15.3483	4.10765	.83847
	Total	42	15.7824	5.72275	.88304
%Annexin+ IL	Wheel Running	17	27.0647	10.93663	2.65252
	No Wheel Running	20	25.0700	10.33467	2.31090
	Total	37	25.9865	10.51404	1.72850
IL-4 (A.U.)	Wheel Running	18	.9900	.31260	.07368
	No Wheel Running	20	.8750	.26363	.05895
	Total	38	.9295	.28978	.04701

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CD45ra	Between Groups	68.567	1	68.567	.382	.540
	Within Groups	7534.503	42	179.393		
	Total	7603.070	43			
CD45	Between Groups	102.662	1	102.662	.928	.341
	Within Groups	4645.560	42	110.609		
	Total	4748.222	43			
CDgd	Between Groups	.683	1	.683	.188	.667
	Within Groups	145.544	40	3.639		
	Total	146.228	41			
CD28	Between Groups	10.550	1	10.550	.317	.577
	Within Groups	1332.196	40	33.305		
	Total	1342.747	41			
%Annexin+ IL	Between Groups	36.562	1	36.562	.325	.573
	Within Groups	3943.061	35	112.659		
	Total	3979.623	36			
IL-4 (A.U.)	Between Groups	.125	1	.125	1.513	.227
	Within Groups	2.982	36	.083		
	Total	3.107	37			

Note: CDgd = gamma delta T cells

Study #3: Wheel Running vs. No Wheel Running (w/ Acute Exercise) – 2x3 ANOVA

CD45RA (2x2ANOVA)				
		Mean	**SD**	**N**
WR	**SED**	66.8158	14.28625	19
	IMM	65.4737	11.42224	19
	Total	66.1447	12.77590	38
NWR	**SED**	69.3360	12.68325	25
	IMM	70.4522	13.82432	23
	Total	69.8708	13.11174	48
Total	**SED**	68.2477	13.29720	44
	IMM	68.2000	12.88860	42
	Total	68.2244	13.02210	86
Training:		$F_{(1,85)} = 1.734$, P = 0.192		
Acute-Exercise:		$F_{(1,85)} = 0.002$, P = 0.968		
Interaction:		$F_{(1,85)} = 0.186$, P = 0.667		

IL-4 (2x2 ANOVA)				
		Mean	**SD**	**N**
WR	**SED**	.9900	.31260	18
	IMM	1.1211	.28170	19
	Total	1.0573	.30039	37
NWR	**SED**	.8750	.26363	20
	IMM	1.0185	.27350	20
	Total	.9468	.27492	40
Total	**SED**	.9295	.28978	38
	IMM	1.0685	.27872	39
	Total	.9999	.29089	77
Training:		$F_{(1,76)} = 2.846$, P = 0.096		
Acute-Exercise:		$F_{(2,88)} = 4.533$, P = 0.037		
Interaction:		$F_{(2,88)} = 0.009$, P = 0.923		

III – Representative Statistical Analysis

Chapter 3: Univariate Analysis of Variance (2x3 ANOVA)

Between-Subjects Factors

		Value Label	N
Age_Group	1.00	Young	43
	2.00	Old	39
Acute_Exercise	1.00	Sedentary	25
	2.00	Immediate	29
	3.00	2 Hrs	28

Descriptive Statistics

Dependent Variable:Caspase_7

Age_Group	Acute_Exercise	Mean	Std. Deviation	N
Young	Sedentary	.8400	.16633	13
	Immediate	1.0733	.34182	15
	2 Hrs	.9240	.23946	15
	Total	.9507	.27456	43
Old	Sedentary	.9992	.26916	12
	Immediate	1.0764	.29819	14
	2 Hrs	.9562	.23251	13
	Total	1.0126	.26666	39
Total	Sedentary	.9164	.23157	25
	Immediate	1.0748	.31576	29
	2 Hrs	.9389	.23243	28
	Total	.9801	.27095	82

Levene's Test of Equality of Error Variances[a]

Dependent Variable:Caspase_7

F	df1	df2	Sig.
1.533	5	76	.190

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Age_Group + Acute_Exercise + Age_Group * Acute_Exercise

Tests of Between-Subjects Effects

Dependent Variable:Caspase_7

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.574[a]	5	.115	1.625	.163
Intercept	77.943	1	77.943	1102.687	.000
Age_Group	.086	1	.086	1.210	.275
Acute_Exercise	.396	2	.198	2.802	.067
Age_Group * Acute_Exercise	.090	2	.045	.638	.531
Error	5.372	76	.071		
Total	84.719	82			
Corrected Total	5.946	81			

a. R Squared = .097 (Adjusted R Squared = .037)

Estimated Marginal Means

1. Age_Group - Dependent Variable:Caspase_7

Age_Group	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Young	.946	.041	.865	1.027
Old	1.011	.043	.926	1.096

2. Acute_Exercise - Dependent Variable:Caspase_7

Acute_Exercise	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Sedentary	.920	.053	.814	1.026
Immediate	1.075	.049	.976	1.173
2 Hrs	.940	.050	.840	1.040

3. Age_Group * Acute_Exercise - Dependent Variable:Caspase_7

Age_Group	Acute_Exercise	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Young	Sedentary	.840	.074	.693	.987
	Immediate	1.073	.069	.937	1.210
	2 Hrs	.924	.069	.787	1.061
Old	Sedentary	.999	.077	.846	1.152
	Immediate	1.076	.071	.935	1.218
	2 Hrs	.956	.074	.809	1.103

Chapter 4: One-way ANOVA (Independent Factor: Freewheel Training Condition)

Descriptives

Mean TNF-alpha (A.U.)

	N	Mean	Stdev	SEM	95% CI		Minimum	Maximum
					Lower	Upper		
Wheel Running	15	.6800	.22788	.05884	.5538	.8062	.23	.95
No Wheel Running	15	.9627	.27212	.07026	.8120	1.1134	.50	1.34
Total	30	.8213	.28545	.05212	.7147	.9279	.23	1.34

Test of Homogeneity of Variances

Mean TNF-alpha (A.U.)

Levene Statistic	df1	df2	Sig.
.179	1	28	.676

ANOVA

Mean TNF-alpha (A.U.)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.599	1	.599	9.514	.005
Within Groups	1.764	28	.063		
Total	2.363	29			

Chapter 5: Two-way Analysis of Variance

Between-Subjects Factors

		Value Label	N
Training Group	1.00	Wheel Running	29
	2.00	No Wheel Running	31
Acute Exercise Group	1.00	Sedentary	30
	2.00	Immediate	30

Descriptive Statistics

Dependent Variable: TNF-alpha (A.U.)

Training Group	Acute Exercise Group	Mean	Std. Deviation	N
Wheel Running	Sedentary	.6800	.22788	15
	Immediate	1.2971	.29738	14
	Total	.9779	.40685	29
No Wheel Running	Sedentary	.9627	.27212	15
	Immediate	1.1756	.36249	16
	Total	1.0726	.33460	31
Total	Sedentary	.8213	.28545	30
	Immediate	1.2323	.33378	30
	Total	1.0268	.37116	60

Levene's Test of Equality of Error Variances[a]

Dependent Variable: TNF-alpha (A.U.)

F	df1	df2	Sig.
.972	3	56	.413

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Exercise_Training + Acute_Exercise + Exercise_Training * Acute_Exercise

Tests of Between-Subjects Effects

Dependent Variable: TNF-alpha (A.U.)

Source	Type III SS	Df	Mean Square	F	Sig.
Corrected Model	3.243[a]	3	1.081	12.395	.000
Intercept	63.372	1	63.372	726.563	.000
Exercise_Training	.097	1	.097	1.114	.296
Acute_Exercise	2.578	1	2.578	29.560	.000
Exercise_Training * Acute_Exercise	.611	1	.611	7.008	.011
Error	4.884	56	.087		
Total	71.391	60			
Corrected Total	8.128	59			

a. R Squared = .399 (Adjusted R Squared = .367)

Estimated Marginal Means

1. Training Group

Dependent Variable: TNF-alpha (A.U.)

			95% Confidence Interval	
Training Group	Mean	Std. Error	Lower Bound	Upper Bound
Wheel Running	.989	.055	.879	1.098
No Wheel Running	1.069	.053	.963	1.175

2. Acute Exercise Group

Dependent Variable: TNF-alpha (A.U.)

			95% Confidence Interval	
Acute Exercise Group	Mean	Std. Error	Lower Bound	Upper Bound
Sedentary	.821	.054	.713	.929
Immediate	1.236	.054	1.128	1.345

3. Training Group * Acute Exercise Group

Dependent Variable: TNF-alpha (A.U.)

				95% Confidence Interval	
Training Group\	Acute Exercise Group	Mean	Std. Error	Lower Bound	Upper Bound
Wheel Running	Sedentary	.680	.076	.527	.833
	Immediate	1.297	.079	1.139	1.455
No Wheel Running	Sedentary	.963	.076	.810	1.115
	Immediate	1.176	.074	1.028	1.324

IV – Additional Study Methods

$NF_k\beta$ [2] is a transcription factor responsible for regulation of inflammatory and apoptotic processes. $NF_k\beta$ can be activated by pro-inflammatory cytokines and cellular stress (such as an acute-exercise stress or ROS) and plays a role in the promotion of inflammation-associated carcinogenesis – as such, it is expected that $NF_k\beta$ may become a potential pharmacologic target to prevent intestinal inflammation and CRC (Wang et al., 2010). For the assessment of the transcription factor $NF_k\beta$, nuclear fractions were obtained from intestinal lymphocyte suspensions incubated with lysis buffer for 1 hr. Samples were centrifuged and supernatant recovered as the nuclear fraction before electrophoresis on a 12% SDS-PAGE gel and subsequent transfer to a PVDF membrane. Before the membrane was incubated with primary antibody for $NF_k\beta$ (clone: C-20; rabbit anti-human polyclonal IgG) for 1hr, it was blocked with 10% skim powdered milk and TBS-T. The antibody was then washed with TBS-T, and incubated with secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG in 10% milk-TBS-T) for 1hr and washed again. Visualization of the immunoblots was by Western Blotting Detection Reagents and a ChemiGenius 2 Bio-Imaging System. Protein molecular weight on the immunoblot was evaluated by biotinylated protein ladder and secondary antibody (anti-biotin HRP) comparison. Detection was verified by loading $NF_k\beta$ standards (as a positive control) alongside the protein samples, standard procedure in this lab (Spagunolo and Hoffman-Goetz, 2007; Hoffman-Goetz et al, 2009). Samples from each experimental condition were run on each immunoblot and band densities were normalised to NWR SED control bands on each immunoblot (units were reported as arbitrary densitometric units [a.u.]).

[2] $NF_k\beta$ was not reported in Chapter 3 because of a significant loss of samples due to contamination (i.e., a technical issue). Cost and time considerations prevented us from obtaining additional samples.

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