IMMUNOLOGICAL CHANGES IN RESPONSE TO EXERCISE

IMMUNOLOGICAL CHANGES IN RESPONSE TO ACUTE EXERCISE, CONSIDERING PUBERTY AND SEX

by

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ABSTRACT

This research was conducted to elucidate age-, puberty-, and sex-related effects on immunological changes in response to acute exercise. Compared with young men, 9- and 10-yr-old boys experienced smaller natural killer (NK) cell and interleukin-6 (IL-6) changes and a faster neutrophil and IL-6 recovery in response to exercise (Chapter 3). In these boys, exercise-induced neutrophil and NK cell changes were also more sensitive to carbohydrate (CHO) intake. The possible interaction of puberty and CHO intake on NK cell responses to exercise was then investigated in boys at the same chronological age (Chapter 4). The magnitude of change in NK cells was greatest in boys at advanced stages of puberty, and responses to CHO intake were most sensitive in pre-pubertal boys. To further investigate age-, puberty-, and sex-related differences in leukocyte and cytokine responses to exercise, boys and girls ages 12 and 14 were studied (Chapter 5). Neutrophil, lymphocyte, NK cell, and IL-6 responses were more pronounced in 14-yr-old *versus* 12-yr-old and in mid- *versus* early-pubertal girls. Age- and puberty-related differences in neutrophil recovery were also observed in the boys. Sex differences (girls > boys) in lymphocytes and NK cells were observed among 14-yr-old, but not 12-yr-old and among mid-, but not late-pubertal subjects. Finally, the effects of sex, menstrual phase (MP) and oral

contraceptive (OC) use on leukocyte and IL-6 responses to exercise were studied in adults (Chapter 6). Compared with men, women experienced larger increases in lymphocytes, but MP did not influence immune changes. A novel observation was that OC use increased the magnitude of exercise-induced changes in leukocyte subsets. Collectively, the studies in this thesis represent the first comprehensive examination of age-, puberty-, and sex-related effects on immunological changes in response to exercise and provide the first reports of immune-related responses to CHO intake during exercise in children.

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I would like to thank my family for their continued support and for their interest in what I do. Some day I will explain it all to you!

To my supervisor, Dr. Oded Bar-Or, my sincerest *thank you*. Thank you for taking a chance on an unknown undergraduate student in an unknown area of research. Thank you for your support and understanding when I needed it most. It has been a tremendous honour and privilege learning from you, and no words on a page can fully and accurately describe what you mean to me.

Finally, and ***most importantly***, I thank my wife, Lisa, for everything. You put your dreams on hold so I could achieve mine. This thesis is as much a product of your sacrifice, understanding and love as it is of my time and effort.

DEDICATION

This thesis is dedicated to the memory of my father, Carson Timmons,

who taught me the value of hard work.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CBC	complete blood count
CD	cluster designation
CHO	carbohydrate
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
EPI	epinephrine
FACS	fluorescence activated cell sorter
FOL	follicular phase of menstrual cycle
GH	growth hormone
HIV	human immunodeficiency virus
HR	heart rate
HRT	hormone replacement therapy
Ig	immunoglobulin
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
LUT	luteal phase of menstrual cycle
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC	major histocompatability complex
MP	menstrual phase
NEP	norepinephrine
NK cells	natural killer cells
OC	oral contraceptives
OCT	oral contraceptive therapy
sIgA	salivary immunoglobulin A
T2DM	type 2 diabetes mellitus
TNF-α	tumor necrosis factor-α
$\dot{V}CO_2$	carbon dioxide output
$\dot{V}O_2$	oxygen consumption
$\dot{V}O_{2max}$	maximal oxygen consumption
WanT	Wingate anaerobic test
WBC	white blood cells

FORMAT AND ORGANIZATION OF THIS THESIS

This thesis was prepared in the "sandwich thesis" format as outlined in the McMaster University School of Graduate Studies March 2003 publication, "Guide for the Preparation of Theses", with supplementary requirements as described in "Thesis Preparation Guide", provided by the Department of Kinesiology. This thesis consists of four original research papers that are either published or currently under peer-review. Each of these chapters is formatted according to the requirements of the journal in which it was published or submitted for publication. These chapters are preceded by an introduction (Chapter 1), which sets the context for the complete body of research. In addition, a brief chapter (Chapter 2) has been included to detail specific aspects of study design and methodology. A concluding chapter (Chapter 7) summarizes and discusses the main findings of this thesis and includes future research directions in this area. Finally, Appendices are included to supplement Chapters 2 and 7.

CONTRIBUTIONS TO MULTI-AUTHORED PAPERS

CHAPTER 3

PUBLICATION

Timmons BW, Tarnopolsky MA, Bar-Or O 2004 Immune responses to strenuous exercise and carbohydrate intake in boys and men. *Pediatric Research* 56: 227–234

Contributions:

The experiment was designed by B.W. Timmons, assisted by O. Bar-Or. B.W. Timmons collected the data, assisted by M. Kubacki. The data were analyzed by B.W. Timmons, assisted by M.A. Tarnopolsky. B.W. Timmons wrote the paper with assistance from the co-authors. The primary supervisor for this study was O. Bar-Or.

CHAPTER 4

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The experiment was designed by B.W. Timmons, assisted by the co-authors. B.W. Timmons collected the data, assisted by M DeJonge, M. Kubacki, R. Trott, J-H. Lee, M. Hamadeh, and A. Mark. The data were analyzed by B.W. Timmons. B.W. Timmons wrote the paper with

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CHAPTER 5

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CHAPTER 6

PUBLICATION

Timmons, B.W., Hamadeh, M.J., Devries, M.C., and Tarnopolsky, M.A. Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling: *Journal of Applied Physiology* (In press).

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CHAPTER 1: INTRODUCTION

A detailed characterization and understanding of immunological changes in response to exercise in humans hold several important health implications. In addition to its defining role in protection against pathogens, the immune system is intimately linked to health disorders such as obesity, Type 2 diabetes mellitus (T2DM), and cardiovascular disease. The incidence of these diseases in adults is associated with physical inactivity (Kelley & Goodpaster, 2001; Kohl, 2001; Ross & Janssen, 2001). Likewise, physical inactivity among children and adolescents is a serious concern in today's society and is strongly associated with high rates of obesity (Tremblay & Willms, 2003) and a parallel occurrence of "adult-type" health problems such as T2DM (Pinhas-Hamiel *et al.*, 1996; Sinha *et al.*, 2002) and vascular dysfunction (Reed *et al.*, 2004; Woo *et al.*, 2004). To what extent the early onset of these adult-type disorders during childhood is linked to development of the immune system is unknown. That regular exercise during childhood may influence normal growth and development of the immune system, and thus etiology of disease, is, therefore, of considerable interest. However, compared to the body of literature compiled from adults (Garrey & Bryan, 1935; Keast *et al.*, 1988; Mackinnon, 1999; McCarthy & Dale, 1988; Nash,

1994; Pedersen & Hoffman-Goetz, 2000; Shephard, 1997; Simon, 1984), our understanding of immunological changes in response to acute and chronic exercise in children and adolescents is deficient.

An improved understanding of "normal" immunological responses to acute exercise in healthy children and adolescents is also of clinical significance. What is a safe exercise prescription for children with, or recovering from, an immune-related disease? How do we distinguish normal exercise-induced changes in a child's immune system from abnormal changes? What interventions, nutritional or otherwise, can or should be incorporated into the prescription of exercise to promote immune health in growing children? Answers to these questions are critical to pediatric health professionals. This thesis was an attempt to address some of these important questions and to improve our understanding of immunological responses to acute exercise in children and adolescents.

1.1. Overview of the Immune System and "The Relevant Players"

1.1.1. Overview

The human immune system has evolved to distinguish self from non-self. Comprised of cellular and soluble factors, which act synergistically to extinguish pathogens, the immune system contributes to the body's homeostasis and maintenance of health. Traditional thinking of the immune system separates cellular and soluble components into

specific (i.e., adaptive) and non-specific (i.e., innate) immunity (Male, 2001). Specific immunity is characterized by antigen specificity and "memory", which allows the host to mount a faster and stronger response following repeated exposures to the same antigen (Dutton *et al.*, 1998; Male, 2001). Non-specific immunity provides early detection and clearance of pathogen, thus serving as a first line of defense against infection (Male, 2001). However, it is becoming increasingly evident that considerable "cross-talk" occurs between the innate and adaptive arms of immunity (Castriconi *et al.*, 2004; Eberl *et al.*, 2004; Trinchieri *et al.*, 1993). It is also becoming clear that our immune system plays an integral role in the pathogenesis of health complications such as insulin resistance (Moller, 2000) and atherosclerosis (Hansson *et al.*, 2002), states unrelated to "foreign" micro-organisms *per se*. Although a thorough discussion of the immune system is beyond the scope of this thesis, selected components relevant to the experiments conducted are briefly introduced below[1].

1.1.2. Phagocytes

Phagocytes include **neutrophils** and **monocytes** that can ingest (i.e., phagocytize) microbes and degrade them, thus acting as a first line of defense against infection (i.e., non-specific immunity). Neutrophils

[1]Information in sections 1.1.2. to 1.1.7. is derived from Roitt et al. (Roitt *et al.*, 2001) unless otherwise indicated.

constitute the majority of the total blood leukocyte pool (~60-70% in adults) and migrate into damaged tissue during the early stages of inflammation. The term "monocytes" refers to mononuclear phagocytes that circulate in the blood stream. Macrophages are tissue-specific monocytes (e.g., Kupffer cells in the liver) and are strategically situated to maximize contact with pathogenic micro-organisms allowing for efficient antigen presentation to lymphocytes. Compared to monocytes and macrophages that may survive for months or years, neutrophils are relatively short-lived (2-3 days).

1.1.3. *Lymphocyte subsets*

The two major subsets of lymphocytes are **T cells** and **B cells**, which represent the adaptive arm of the immune system. Direct phenotyping of T and B cells is possible by identification of surface molecules with monoclonal antibodies (mAb). The term "CD" (Cluster Designation) refers to the group or cluster of mAb that recognizes particular surface molecules (e.g., CD3 is found on all T cells and CD19 is a characteristic B cell marker). Thus, expression or lack of expression of surface molecules can provide insight into the lineage, maturation, and activation status of a particular cell. As previously mentioned, one of the main attributes of lymphocytes is memory. Changes in functional memory or activation status of T cells can be evaluated by expression of different molecular weight isoforms of the leukocyte common antigen, CD45. Thus,

memory cells are typically defined as $CD45RO^{+}CD45RO^{-}$ and naïve cells as $CD45RO^{-}CD45RA^{+}$ (Dutton *et al.*, 1998). T lymphocytes may be further distinguished into helper ($CD3^{+}CD4^{+}$) or cytotoxic ($CD3^{+}CD8^{+}$) cells. T helper cells recognize specific antigen in association with major histocompatability complex (MHC) II molecules and are required for the induction of an immune response. In contrast, T cytotoxic cells recognize antigen associated with MHC I molecules and are critical in the elimination of cells infected with virus. The CD4:CD8 ratio provides a clinically useful indication of immunosuppression and risk of infection (Hansbrough *et al.*, 1984).

1.1.4. Natural killer (NK) cells

NK cells are large granular lymphocytes that represent a population of cells with natural cytotoxicity. In other words, NK cells represent one component of innate immunity that can destroy certain virally-infected and tumor cells, without prior sensitization (i.e., non MHC-restricted). The natural cytotoxicity associated with NK cells marks them as key players in anti-cancer defense mechanisms (Brittenden *et al.*, 1996). The widely accepted CD classification of NK cells is the lack of CD3 and co-expression of CD16 and CD56 (i.e., $CD3^{-}CD16^{+}CD56^{+}$). However, within the category of NK cells there are distinct subsets with unique phenotypic and functional properties (Cooper *et al.*, 2001). The emerging heterogeneity in NK cell biology has implicated these cells as

important mediators of the cross-talk between innate and adaptive immunity (Assarsson *et al.*, 2004; Carnaud *et al.*, 1999; Zingoni *et al.*, 2004). Because of their role in communication between the innate and adaptive arms of immunity and because they are the most responsive cell type to the stress of exercise (Pedersen & Hoffman-Goetz, 2000), NK cells will be given considerable attention throughout this thesis.

1.1.5. *Interleukin-6 (IL-6)*

IL-6, like many cytokines, demonstrates functional pleiotropy and redundancy, which is to say that one cytokine can have a variety of biological effects on cells or tissues and that different cytokines can have similar and overlapping effects (Kishimoto *et al.*, 1995). Traditionally, IL-6 has been classified as a pro-inflammatory cytokine, produced and secreted by activated monocytes to induce acute phase protein production in the liver. It was later demonstrated that IL-6 possesses crucial anti-inflammatory properties (Pedersen *et al.*, 2003b; Xing *et al.*, 1998) and is involved in bone metabolism (Kurihara *et al.*, 1990; Manolagas, 1998). Moreover, IL-6 has known metabolic effects during exercise. For example, IL-6 release from contracting skeletal muscle is enhanced when exercise is performed with low muscle glycogen content (Steensberg *et al.*, 2001a), and IL-6 infused during exercise seems to contribute to glucose production and clearance (Febbraio *et al.*, 2004), but not fat

metabolism (Hiscock *et al.*, 2005). Thus, exercise-induced changes in IL-6 are of particular interest throughout this thesis.

1.1.6. Interleukin-8 (IL-8)

IL-8 plays a key role in early inflammatory events as a chemoattractant for neutrophils. Production of IL-8 at the site of inflammation is induced by pro-inflammatory cytokines like IL-1 and tumor necrosis factor-α (TNF-α). Interestingly, IL-8 is constitutively expressed by human myoblasts and can be up-regulated by incubation with TNF-α (De Rossi *et al.*, 2000). Like IL-6, the expression of IL-8 in skeletal muscle is associated with changes in the cell's energy status (Chan *et al.*, 2004). Plasma levels of IL-8 are also positively associated with fat mass in obese humans (Straczkowski *et al.*, 2002), suggesting an important role in the low-grade inflammation associated with obesity (Das, 2001).

1.1.7. Tumor necrosis factor-α (TNF-α)

TNF-α is a prototypical pro-inflammatory cytokine and is important in the initiation of the inflammatory response to infection (Baumann & Gauldie, 1994). TNF-α is another example of an extremely multi-functional cytokine. Muscle protein synthesis is inhibited by TNF-α (Lang *et al.*, 2002), which may contribute to muscle atrophy associated with ageing (Greiwe *et al.*, 2001). TNF-α is produced and secreted by adipocytes (Hotamisligil *et al.*, 1995; Kern *et al.*, 1995) and is associated

with insulin resistance (Moller, 2000). Further, TNF-α can activate cellular pathways that lead to programmed cell death (i.e., apoptosis).

1.2. Growth and Development of the Immune System

1.2.1. Overview

Concentrations and functions of immune components do not remain static across the lifespan. Growth and development of the immune system during childhood have been studied for over 60 years (Kato, 1935). Initially, interest was in simple numeration of leukocyte populations, but as more precise methodologies (e.g., flow cytometry) became available, our understanding of age-related changes in the immune system expanded (Thomas & Linch, 1983; Yanase *et al.*, 1986). Concurrently, a number of studies have reported age- and sex-related differences in functional immune status (e.g., *in vitro* cytokine production) during childhood. Identification of reference values for various leukocyte sub-populations and their function in newborns, infants, children and adolescents is essential to permit longitudinal tracking of immune-related disease progression (e.g., HIV) and medical treatments (e.g., corticosteroids). The following sections address selected aspects of the immune system as they relate to chronological and biological age, sex and menstrual cycle phase.

1.2.2. Changes with chronological age

Much of the work on age-dependent changes in immune parameters has focused on the neonate and infancy period of growth. Indeed, some of the most significant development of the immune system occurs in the early months to years of life (Buck *et al.*, 2002; Calado *et al.*, 1999; de Vries *et al.*, 2000; Denny *et al.*, 1992; Yachie *et al.*, 1989), including NK cell development (Abo *et al.*, 1982; Yabuhara *et al.*, 1990). However, as the focus of this thesis is on older children and adolescents (9- to 14-yr-old), changes during the infancy and early childhood period will be largely ignored to allow a more concentrated discussion of immune development during later childhood. Particular publications have been chosen because they report the spread of individual data over different ages rather than group comparisons, but should not be considered an exhaustive review of the literature.

Throughout childhood, the total white blood cell (WBC) count consistently decreases (Erkeller-Yuksel *et al.*, 1992; Heldrup *et al.*, 1992; Hicks *et al.*, 1983; Hulstaert *et al.*, 1994; Shahabuddin *et al.*, 1998; Shearer *et al.*, 2003; Yanase *et al.*, 1986). Similarly, but to a greater degree, the total peripheral lymphocyte pool decreases during childhood (Erkeller-Yuksel *et al.*, 1992; Hicks *et al.*, 1983; Hulstaert *et al.*, 1994; Shahabuddin *et al.*, 1998; Shearer *et al.*, 2003; Yanase *et al.*, 1986). An interesting phenomenon which emerges during childhood is that while

absolute lymphocyte counts are decreasing, the relative proportions of various lymphocyte subsets may increase or decrease or remain stable. For example, the proportion of $CD4^+$ cells has been reported to either decrease (Heldrup *et al.*, 1992; Osugi *et al.*, 1995; Robinson *et al.*, 1996; Yanase *et al.*, 1986) or remain stable (Erkeller-Yuksel *et al.*, 1992; Hulstaert *et al.*, 1994; Shahabuddin *et al.*, 1998; Shearer *et al.*, 2003), whereas the proportion of $CD8^+$ cells either increases with age (Osugi *et al.*, 1995; Shahabuddin *et al.*, 1998; Shearer *et al.*, 2003; Yanase *et al.*, 1986) or remains stable (Erkeller-Yuksel *et al.*, 1992; Heldrup *et al.*, 1992; Hulstaert *et al.*, 1994; Robinson *et al.*, 1996). In light of the unidirectional change in the total lymphocyte pool, the absolute number of $CD4^+$ cells usually decreases gradually with age, but the number of $CD8^+$ cells may remain constant, thus highlighting the need for cautious interpretation when only relative values of lymphocyte subsets are provided. Consequently, the CD4:CD8 ratio decreases with age in most (Osugi *et al.*, 1995; Robinson *et al.*, 1996; Shahabuddin *et al.*, 1998; Yanase *et al.*, 1986), but not all (Erkeller-Yuksel *et al.*, 1992; Hulstaert *et al.*, 1994) studies. Thus, a child's age is an important consideration when interpreting the clinical utility of this ratio, as previously proposed (Hansbrough *et al.*, 1984). Moreover, while mean values of a given cell population change over time, there can be a simultaneous narrowing in the range of values, suggesting that individual variability in certain cell

populations diminishes, at least into the early years of puberty (Robinson *et al.*, 1996). A more consistent effect of age on immune development is a decrease in both proportion and total number of B cells (Erkeller-Yuksel *et al.*, 1992; Heldrup *et al.*, 1992; Hicks *et al.*, 1983; Hulstaert *et al.*, 1994; Robinson *et al.*, 1996; Shahabuddin *et al.*, 1998; Shearer *et al.*, 2003; Yanase *et al.*, 1986).

In addition to significant age-related changes in cell proportions and numbers, there are also functional changes that occur throughout childhood. Functional development of the immune system is characterized by a gradual decrease in the proportion of $CD45RA^+$ cells and a reciprocal increase in the proportion of $CD45RO^+$ cells (Erkeller-Yuksel *et al.*, 1992; Hulstaert *et al.*, 1994; Osugi *et al.*, 1995; Shearer *et al.*, 2003). This age-related change in memory status results from antigenic experience and is associated with maturation of cytokine production. In general, cytokine production in response to *in vitro* stimulation progressively increases throughout childhood (Elsasser-Beile *et al.*, 1995; Krampera *et al.*, 1999; Smart & Kemp, 2001), but remains lower in children than in adults (Chipeta *et al.*, 1998; Elsasser-Beile *et al.*, 1995; Gasparoni *et al.*, 2003; Krampera *et al.*, 1999; Lilic *et al.*, 1997). Further, the activation threshold for cytokine production may be higher in children *versus* adults (Muller *et al.*, 1996). The apparent inability of young children to produce cytokines in response to stimulation is

associated with a lower proportion of $CD45RO^{+}$ memory lymphocytes (Chipeta *et al.*, 1998; Gasparoni *et al.*, 2003).

As a proportion of the total lymphocyte pool, NK cells are low during infancy and tend to increase with age in some (Abo *et al.*, 1982; Bartlett *et al.*, 1998; Osugi *et al.*, 1995; Yanase *et al.*, 1986), but not all (Erkeller-Yuksel *et al.*, 1992; Heldrup *et al.*, 1992; Hulstaert *et al.*, 1994; Shearer *et al.*, 2003; Yanase *et al.*, 1986) studies. Some of the discrepancy regarding changes in NK cells with age is likely related to choice of surface antigen to represent the NK cell population. Not all studies used the now accepted $CD3^{-}CD16^{+}CD56^{+}$ phenotype, and it is unknown whether cells expressing high or low levels of CD16 or CD56 are affected in a similar fashion by the ageing process. In light of this apparent age-related development of NK cells, it has been argued that the susceptibility to malignancy during early, compared to later, childhood may be related to deficient natural cytotoxicity (Page & Ben Eliyahu, 1999). However, despite a low proportion of NK cells during early life, a higher total lymphocyte count partially offsets any absolute NK cell deficiency. In addition, several NK cell characteristics including cytotoxicity, recycling and target binding achieve adult-like status by the first year of life (Yabuhara *et al.*, 1990). Taken together, these may be mechanisms for the child to help compensate for a low proportion of NK cells.

Like during childhood, the adolescent period (i.e., teenage years) is associated with a decline in total B cell count (Bartlett *et al.*, 1998), which may be a phenomenon attributed to boys and not girls (Rudy *et al.*, 2002). Age-related increases in the number of $CD4^+$ and $CD8^+$ memory cells (Rudy *et al.*, 2002) and in the proportion of NK cells (Bartlett *et al.*, 1998) are also found during adolescence. Changes in lymphocyte subsets during adolescence into an adult-like phenotype may also depend on gender (Tollerud *et al.*, 1990). Unlike the childhood period, however, the total WBC count increased during adolescence from the age of 12 to 18 years, according to one study (Bartlett *et al.*, 1998).

In contrast to the abundance of studies reporting age-related changes in cellular immunity, there has been little effort to describe changes in plasma or serum levels of cytokines as a function of age. One study reported that IL-6 increased and TNF-α decreased from 3 to 17 years of age (Sack *et al.*, 1998), whereas another study did not find an effect of age on IL-6, IL-8 or TNF-α levels from 1 to 18 years of age (Lo *et al.*, 2004). Several studies that measure cytokine levels in children with a particular disease will often report comparable levels from "healthy" control children. Such comparisons provide an alternative means of informally assessing "normal" cytokine values during childhood. Interpretation from multiple studies, however, is problematic because of inter-study differences in blood collection and storage methods, assay procedures,

and technical error. Notwithstanding these limitations, a number of studies from Cooper and colleagues (Nemet *et al.*, 2002; Nemet *et al.*, 2003b; Nemet *et al.*, 2003a; Nemet *et al.*, 2004b; Scheett *et al.*, 1999; Scheett *et al.*, 2002; Tirakitsoontorn *et al.*, 2001) have measured IL-6 and TNF-α in children with the same assay and show no substantial variation in resting concentrations with chronological age. The lack of normative data for cytokines during childhood may be due to a previous shortage of sufficiently sensitive cytokine assays capable of detecting very low quantities of cytokine typically found in humans.

1.2.3. *Changes with biological age*

While the effects of age *per se* on the immune system are fairly well documented, many studies are weakened by the practice of compiling values from a wide range of ages into a single group average. For example, a widely referenced publication on age-related changes in lymphocyte subpopulations (Erkeller-Yuksel *et al.*, 1992) classified one group of children by an age range of 7 to 17 years. This method, although not uncommon, effectively ignores the entire period of puberty and any potential influence of puberty-related events on the outcome measures.

It is somewhat surprising that more attempts have not been made to categorize data according to biological development (e.g., pubertal status) during childhood, considering that *in vitro* studies demonstrate regulation of cellular and soluble components of the immune system by

sex steroids. Exposure to testosterone is associated with inhibition of immunoglobulin (Ig) production by blood mononuclear cells (Kanda *et al.*, 1996) and an increase in the proportion of $CD4^+$ and $CD8^+$ cells, but a decrease in the proportion $CD4^+CD45RA^+$ cells (Athreya *et al.*, 1993). On the other hand, estrogen may enhance NK cell activity (Sorachi *et al.*, 1993) and $CD4^+$ cytokine production (Gilmore *et al.*, 1997), without influencing the absolute number of $CD4^+$ cells (Athreya *et al.*, 1993).

This author is aware of only one publication that related immunological measures to a marker of biological age (Bartlett *et al.*, 2001). In this study of "inner-city" children, Tanner stages (defining characteristic not reported) were positively correlated with absolute levels of $CD19^+$ B cells and the proportion of $CD3^+$ T cells in boys. However, these relationships may have been skewed because very few numbers of subjects were categorized as late or post-pubertal (i.e., Tanner 4 and 5). In contrast, no significant Tanner relationships were reported for girls. Again, very few girls were in advanced stages of puberty and were, therefore, classified as pre- (n=77) or post-menarcheal (n=7) for comparison purposes. Absolute numbers of total WBCs, total lymphocytes, $CD3^+$, $CD3^+CD4^+$, and $CD3^-CD16^+CD56^+$ cells and the proportion of $CD3^-CD16^+CD56^+$ cells were higher in pre- *versus* post-menarcheal girls.

An important consideration in the study of pubertal influences on immune development is the need to separate biological from chronological effects. An ideal experimental paradigm, therefore, would test a group of children at the same chronological age, but who vary in biological development, thus partially controlling for previous antigenic experience. Clearly more work is needed to describe development of the immune system as a function of puberty in boys and girls.

1.2.4. Influence of sex and menstrual phase

Sex-related influences on the immune system during adulthood should be obvious when one considers the sexual dimorphism in the prevalence and presentation of several auto-immune diseases. For example, the prevalence of diseases such as multiple sclerosis, rheumatoid arthritis, myasthenia gravis, scleroderma, systemic lupus erythematosus, and Sjogren's syndrome ranges from 60 to 95% in favor of females (Whitacre, 2001). With few exceptions, there has been little interest in, or evidence for, differences in components of the immune system between boys and girls during childhood. In a large group of 8- to 12-year-old children, Bartlett and colleagues (Bartlett *et al.*, 1998) found that, compared with boys, girls had higher proportions and absolute counts of total T cells and $CD4^+$ cells, but a lower proportion of NK cells. During adolescence, however, it has been reported that girls have higher total $CD4^+$ counts (Bartlett *et al.*, 1998; Rudy *et al.*, 2002) and either

higher (Rudy *et al.*, 2002) or lower (Bartlett *et al.*, 1998) B cell counts. Adolescent girls also appear to have higher proportions of total T lymphocytes and $CD4^+$ cells and a lower proportion of B cells (Bartlett *et al.*, 1998; Tollerud *et al.*, 1990), a lower proportion of NK cells (Bartlett *et al.*, 1998), and a higher NK cell count (Rudy *et al.*, 2002). Interestingly, differential changes in cell populations have been noted between boys and girls during adolescence. For example, the proportion of $CD4^+$ cells was similar among boys and girls ages 12 to 14 years and 15 to 16 years, but higher among girls in a 17 to 19 age group (Tollerud *et al.*, 1990). Conversely, the proportion of $CD8^+$ cells was higher in girls *versus* boys at ages 12 to 14 years, 15 to 16 years, but equal at 17 to 19 years (Tollerud *et al.*, 1990). The changes observed during the adolescent period, which also corresponds with puberty, appear to serve as a transition into adult-like status.

In adults, there is considerable evidence to support sex-related differences in several immune functions. Compared with men, women experience a differential regulation of cytokine responses under stimulated (Giron-Gonzalez *et al.*, 2000; Schwarz *et al.*, 2000) and unstimulated (Cannon *et al.*, 1998; Lynch *et al.*, 1994) conditions. Sex differences in cytokine regulation may depend on the phase of the menstrual cycle in which women are tested (Schwarz *et al.*, 2000). It has also been shown that women display higher levels of some serum Igs, but not others

(Giron-Gonzalez *et al.*, 2000). Resting NK cell cytotoxicity appears similar between men and women (Shakhar *et al.*, 2000), but lower in women when adjusted for the number of NK cells in the assay (Yovel *et al.*, 2001). Further, women experience greater adrenergic-induced suppression of NK cell cytotoxicity, which appears to be more associated with estradiol than progesterone (Shakhar *et al.*, 2000).

The effects of menstrual cycle phases on immune function are also of particular interest as symptoms of some auto-immune diseases change in severity according to phase of the menstrual cycle (Whitacre, 2001). In general, the luteal phase of the menstrual cycle characterized by a high level of progesterone and a moderate level of estrogen is associated with a heightened immune system. In other words, the capacity of immune cells to produce cytokines is greater (Bouman *et al.*, 2001; Konecna *et al.*, 2000), corresponding to a greater number of circulating cells (Bouman *et al.*, 2001; Faas *et al.*, 2000). Further, the concentration of serum IL-6 (Konecna *et al.*, 2000) and plasma IL-1 activity (Lynch *et al.*, 1994) are reported to be greater in the luteal, compared with the follicular, phase of the cycle. In contrast, other studies have found that the follicular phase is associated with greater cytokine production from immune cells (Lynch *et al.*, 1994; Schwarz *et al.*, 2000) and either higher (Angstwurm *et al.*, 1997) or similar (Chiu *et al.*, 2000) serum IL-6 levels, and similar Ig levels (Giron-Gonzalez *et al.*, 2000). Higher levels of IL-6 during the follicular phase

correspond to low progesterone (Angstwurm *et al.*, 1997) and estrogen (Chiu *et al.*, 2000) levels. Further, higher estradiol levels during the luteal phase were positively correlated with secretion of IL-6 and TNF-α from mononuclear cells (Schwarz *et al.*, 2000).

With respect to NK cells, greater stress-induced suppression of NK cell cytotoxicity has been reported during the luteal, compared with the follicular, phase (Shakhar *et al.*, 2000), while no differences between phases were noted for resting, unstimulated NK cytotoxicity (Shakhar *et al.*, 2000). Changes in the stress-induced NK cell cytotoxicity across menstrual phases may be due, in part, to changes in cell number (Yovel *et al.*, 2001).

1.2.5. *Summary*

Advancing age through childhood and adolescence is associated with significant changes in the proportion, number and function of several immune cells, but relatively little is known about cytokine changes. Theoretically, biological development (i.e., puberty) should significantly impact normal immune development; however the available information in this regard is sparse. Sex appears to play an important role in immune development beginning in the adolescent years and into adulthood, likely related to reproductive maturation. Taken together, the above observations of the immune system under resting conditions provide a framework against which to evaluate how the immune system responds to

exercise. In the next section, the general effects of exercise on the immune system in adults are summarized, and possible mechanisms are provided.

1.3. General Effects of Exercise on the Immune System

1.3.1. Overview

Exercise immunology, as a scientific discipline, gained considerable attention in the early 1990s. Figure 1.1 provides a historical perspective of the number of exercise immunology publications since 1976. These frequency data were generated by performing a Pub Med database internet search with the keywords "exercise" and "immune" and limited to human studies. Although this procedure will have severely underestimated the true number of publications, it clearly identifies the pattern of recent interest in this area. The reasons for this upsurge in exercise immunology papers are several-fold. Early investigators were interested in athlete populations and the incidence of infections due to chronic levels of intense physical training (Simon, 1984). Investigations into the potential role of moderate-intensity exercise training to improve immune status in the elderly are also common (Woods *et al.*, 2002), and more recently some researchers (Connolly *et al.*, 2004; Moldoveanu *et al.*, 2000; Ullum *et al.*, 1994) have taken a molecular approach to understand functional responses (e.g., gene expression) at the single cell level. In addition, cytokine responses to exercise have recently received

considerable attention, in particular IL-6 (Pedersen *et al.*, 2001; Pedersen *et al.*, 2003a; Steensberg, 2003). In the following sections, the general effects of acute exercise on the immune system are discussed in terms of exercise duration, exercise intensity, sex and menstrual cycle phase.

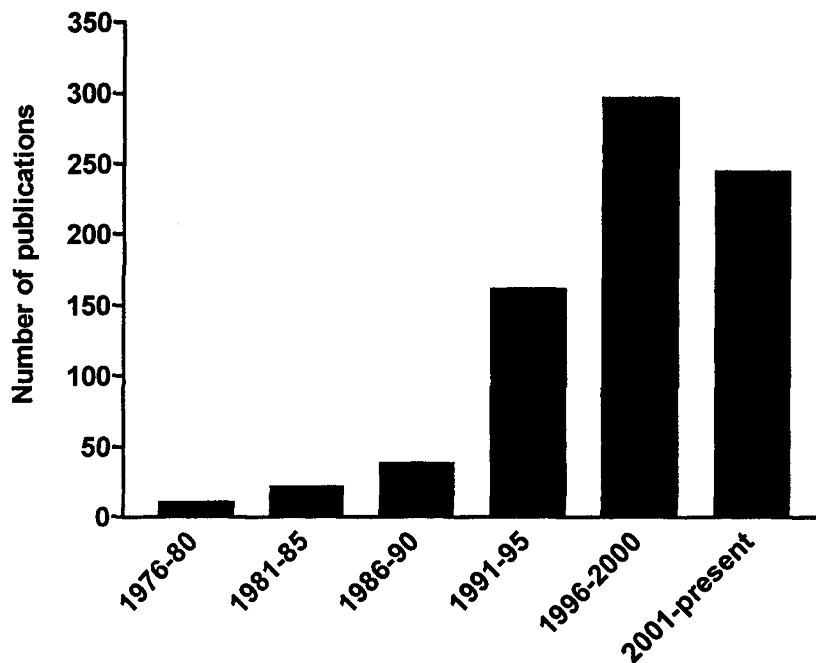

Figure 1.1 *Publication frequency of exercise immunology-related articles since 1976 organized in 5-year periods.*

1.3.2. Influence of exercise duration

There are few formal investigations into the effects of exercise duration on the immune system (Kendall *et al.*, 1990; MacNeil *et al.*, 1991). However, studies with multiple blood sampling times during constant-load exercise allow for a kinetic analysis of duration effects. With the onset of exercise, most leukocytes are rapidly mobilized into the

general circulation. In fact, exercise-induced recruitment of WBCs can be detected within seconds of activity (McCarthy & Dale, 1988), and 30 sec of "all-out" cycling induce changes in most leukocyte populations comparable to those observed following more prolonged, sub-maximal exercise (Nieman *et al.*, 1992). As exercise continues, the concentrations of total WBCs and neutrophils continue to increase such that blood levels at 60 min of exercise are greater than at 30 min (Shek *et al.*, 1995; Shinkai *et al.*, 1992). Like neutrophils, there are early increases in lymphocyte and lymphocyte subsets, including NK cells, in the early minutes of exercise. Unlike neutrophils, lymphocytes do not continue to increase, and if exercise is maintained beyond ~90 min these cells begin to decrease in concentration (Shek *et al.*, 1995). In fact, changes in NK cells measured after 120 min of exercise performed at 65% of maximal oxygen uptake ($\dot{V}O_{2max}$) are identical to those observed after only 30 min of exercise performed at the same intensity (Kendall *et al.*, 1990). During very long duration exercise (e.g., ultra marathon), concentrations of circulating lymphoid cells can revert back close to (Nieman *et al.*, 2003b) or below (Gabriel *et al.*, 1994; Gannon *et al.*, 1997; Nieman *et al.*, 2002) pre-exercise values. This kinetic analysis of the leukocytosis of exercise is important in terms of understanding mechanisms of cell mobilization. The contributing factors to the leukocytosis of exercise are probably multi-factorial, but a model of hormonal regulation of cell mobilization (McCarthy

& Dale, 1988; Pedersen & Hoffman-Goetz, 2000) is accepted by most investigators. In this context, it is thought that exercise-induced changes in catecholamines and cortisol are the two main mechanisms of leukocyte recruitment.

Several lines of evidence support a primary role for epinephrine in the leukocytosis of exercise. First, exogenous administration of epinephrine (Eriksson & Hedfors, 1977; Martin, 1932; Tvede *et al.*, 1994) into humans results in significant increases in neutrophils and lymphocytes. Importantly, these epinephrine-induced alterations in the peripheral blood pool occur within minutes of administration (Eriksson & Hedfors, 1977), consistent with the immediate effects of exercise. Interestingly, the effects of exercise and epinephrine infusion on the relative proportions of lymphocyte subsets in the circulation are identical (Tvede *et al.*, 1994). Second, administration of a chemical blockade (e.g., propranolol) of β-adrenergic receptors significantly attenuates the anticipated exercise-induced increase in neutrophils and lymphocytes (Ahlborg & Ahlborg, 1970; Foster *et al.*, 1986; Murray *et al.*, 1992). Thus, the expression of β_1 and β_2 adrenergic receptors sensitizes immune cells to the increased concentrations of epinephrine during exercise. Therefore, immune cells with a greater surface density of adrenergic receptors, particularly the β_2 type, are most sensitive to changes in epinephrine associated with exercise. NK cells have the highest density

of β-adrenergic receptors on their surface with $CD4^+$ cells having the lowest (Landmann, 1992; Maisel *et al.*, 1990). Consequently, the magnitude of immune cell changes with exercise is greatest for NK cells (Pedersen & Hoffman-Goetz, 2000).

The effects of cortisol on immune changes seem relevant only during prolonged exercise (i.e., > 90 min). A continued increase in neutrophil levels (i.e., neutrophilia) and a decline in lymphocytes (i.e., lymphopenia) have been attributed to an increase in blood cortisol concentration, particularly during the recovery period when catecholamine levels quickly return to resting levels (McCarthy & Dale, 1988). Indeed, glucocorticoid administration promotes a redistribution of T and B lymphocytes into bone marrow and out of the peripheral circulation (Fauci, 1975; Fauci & Dale, 1975), while simultaneously recruiting neutrophils into the circulation from marginated pools and the bone marrow (Nakagawa *et al.*, 1998). That lymphocytes decrease and neutrophils increase during prolonged, but not short-duration, exercise is therefore consistent with the immunological effects of cortisol.

As previously mentioned, there are most likely several other factors that contribute to the leukocytosis of exercise. One of these may be the hemodynamic effects of exercise. A considerable increase in cardiac output at the onset of exercise, resulting in increased peripheral blood flow, may cause a shearing effect and physical detachment of cells from

the endothelium into the peripheral circulation (Foster *et al.*, 1986; McCarthy & Dale, 1988). Other local responses of the cardiovascular system may also help explain exercise-induced increases in immune cells. It is possible that epinephrine exerts effects on blood redistribution from the periphery to specific compartments (e.g., lungs) containing marginated pools of cells and a dilatation of visceral and skeletal muscle blood vessels serving to "flush-out" marginated leukocytes (McCarthy & Dale, 1988).

So far, this section has focused on the effects of exercise duration on cellular components of the immune system. Soluble factors like cytokines also increase in response to exercise, the magnitude of which depends, in part, on exercise duration (Pedersen, 2000). The literature, however, is void of systematic investigations into the impact of exercise duration *per se* on changes in the cytokines IL-6, IL-8 and TNF-α. With strenuous exercise, a cascade of cytokine responses can be mapped, with IL-6 apparently the most responsive (Pedersen & Toft, 2000). Interestingly, epinephrine infusion does not appear to influence circulating IL-6 concentrations in humans (Steensberg *et al.*, 2001b). Although there are contradictory findings with respect to TNF-α (Pedersen & Hoffman-Goetz, 2000), it appears that exercise must be of moderate to high intensity and performed for a prolonged period of time (e.g., a marathon) in order to demonstrate significant changes in plasma levels of TNF-α

(Camus *et al.*, 1997; Ostrowski *et al.*, 1999; Rhind *et al.*, 1995). Relatively few studies have measured IL-8 following exercise, but systemic levels of this cytokine can increase following prolonged endurance exercise (Nieman *et al.*, 2003b; Nieman *et al.*, 2004b; Ostrowski *et al.*, 2001; Suzuki *et al.*, 2000). It has also been concluded that exercise of moderate intensity and duration does not influence IL-8 levels in general, and that any IL-8 production in response to exercise may be a local phenomenon (Moldoveanu *et al.*, 2001). Such a hypothesis is supported by more recent work indicating an increase in IL-8 expression in contracting skeletal muscle (Chan *et al.*, 2004; Nieman *et al.*, 2003a; Nieman *et al.*, 2004a). The mechanism(s) responsible for exercise-induced increases in cytokine levels are less well understood, but may relate to micro-trauma (Pedersen & Hoffman-Goetz, 2000) and energy status (Gleeson, 2000) of the working muscle.

Recovery of the immune system following exercise is proportional to exercise duration and is characterized by a continued increase in neutrophil concentrations with a simultaneous suppression of most lymphocyte subsets below normal resting values (Pedersen *et al.*, 1998). This time of lymphopenia, or more specifically suppression of NK cell cytotoxicity, has been coined the “open window” (Pedersen & Ullum, 1994) and may be a time when the risk of infection is greatest. In general, most components of the immune system return to pre-exercise levels

within 24 hrs after the end of exercise, regardless of exercise duration, but prolonged suppression of NK cells for up to 7 days has been reported (Shek *et al.*, 1995). It is, however, very difficult to completely control extraneous variables and, thus, assess long-term recovery from an acute bout of exercise.

Given its significant effects on the distribution of neutrophils and lymphocytes in the peripheral circulation, cortisol has been implicated in the continued immune perturbations observed during recovery from exercise (McCarthy & Dale, 1988; Pedersen & Hoffman-Goetz, 2000). It is important to note, however, that considerable inter-individual variability can exist in the cortisol response to exercise. For example, a study categorized subjects as "responders" or "non-responders" based on their cortisol changes following an exercise task (Shinkai *et al.*, 1996). Those subjects with the largest exercise-induced increase in cortisol also had the largest decreases in T cell subsets (i.e., $CD4^+$ and $CD8^+$) during the recovery period, but neutrophil profiles were similar among responders and non-responders, thus questioning the role of cortisol on the well-documented post-exercise neutrophilia (Shinkai *et al.*, 1996). It may be, therefore, that the post-exercise neutrophilia is driven by other factors that also change in response to exercise including cytokines, and evidence suggests that IL-6 (Suwa *et al.*, 2000; Yamada *et al.*, 2002) and IL-8 (Opdenakker *et al.*, 1998) contribute to physiological neutrophilia.

1.3.3. Influence of exercise intensity

In general, the more intense the exercise task the greater the resultant immune perturbations. Indeed, a prevailing theory in exercise immunology is that high-intensity exercise is detrimental to the immune system, at least in the short term, but that moderate-intensity exercise imparts significant benefits to immune health (Pedersen & Hoffman-Goetz, 2000). The "J-curve" hypothesis, as originally proposed (Heath *et al.*, 1992), defines the relationship between risk of an upper respiratory infection and intensity of physical training. This relationship may also be applied to the short-term effects of acute exercise on immunological changes as diagrammed in Figure 1.2. In this context, the intensity of exercise determines the magnitude of change in cell concentration during and following exercise.

Figure 1.2 *Proposed relationship between immune competence, in terms of cell number and function during and following acute exercise, and exercise intensity.*

With light exercise of walking-like intensity, only minor (Nieman *et al.*, 1993; Tvede *et al.*, 1993) or no (Strasner *et al.*, 1997) perturbation in NK cells arises. As exercise intensity, expressed as a percentage of $\dot{V}O_{2max}$, increases, there is a corresponding graded effect on the magnitude, and in some cases direction, of immune cell changes. Thus, the exercise-induced increases in NK cells, other lymphocyte subsets, neutrophils and total WBCs are directly proportional to intensity (Gabriel *et al.*, 1992; Nieman *et al.*, 1993; Nieman *et al.*, 1994; Strasner *et al.*, 1997; Tvede *et al.*, 1993). Even relatively small gradients in exercise intensity (e.g., from 60 to 70% $\dot{V}O_{2max}$) can have a significant impact on neutrophils, T cytotoxic cells and NK cells (Gabriel *et al.*, 1992). Interestingly, recovery of NK cell concentrations appears to be independent of exercise intensity (Gabriel *et al.*, 1992; Nieman *et al.*, 1993; Strasner *et al.*, 1997; Tvede *et al.*, 1993), whereas NK cell cytotoxicity may be significantly depressed following high-intensity ($\geq$ 75% $\dot{V}O_{2max}$), but not moderate-intensity ($\leq$ 50% $\dot{V}O_{2max}$) exercise (Nieman *et al.*, 1993; Strasner *et al.*, 1997; Tvede *et al.*, 1993). In contrast, the overall leukocytosis and neutrophilia during the recovery period are greater following high- *versus* moderate-intensity exercise (Gabriel *et al.*, 1992; Nieman *et al.*, 1994; Tvede *et al.*, 1993). From a mechanistic perspective, the factors that contribute to the leukocytosis of exercise of various durations, as discussed above, regulate differential immune effects of exercise intensity. Exercise-

induced cell mobilization is closely related to changes in stress hormone concentrations, in particular epinephrine, and epinephrine levels increase exponentially with intensity of exercise, when expressed as a percentage of one's $\dot{V}O_{2max}$ (Kjaer & Dela, 1996). Moreover, cortisol is also sensitive to the intensity of exercise, provided exercise duration remains constant (Gabriel *et al.*, 1992; Nieman *et al.*, 1994).

1.3.4. *Influence of sex and menstrual phase*

Inclusion of females in exercise immunology studies is becoming more common, but well-controlled comparisons between sexes and between phases of the menstrual cycle are still lacking. Studies that have compared men with women have concluded similar findings: no effect of sex, with few exceptions. In studies by Moyna and colleagues (Moyna *et al.*, 1996b; Moyna *et al.*, 1996a) and by Venkatraman and colleagues (Meksawan *et al.*, 2004; Venkatraman *et al.*, 1997; Venkatraman & Pendergast, 1998) no sex differences were reported in immunological responses to acute exercise in active and sedentary males and females. However, the phase of the menstrual cycle in which testing occurred for the females was not controlled. A recent study (Nieman *et al.*, 2001) was specifically interested in possible sex differences in immune changes following a marathon race and compared 12 women to 86 men. No sex differences were found. However, the average age of the entire group was ~42 years, and no mention was given as to the age of the women.

There was also no indication as to what phase of the menstrual cycle testing was performed or whether the women maintained a regular menstrual cycle.

Regardless of what appears to be convincing evidence that the immune system of men and women responds similarly to the stress of exercise, notwithstanding numerous methodological limitations, there indeed exists sparse examples of sex differences in immunological responses to exercise. In 1960, De Lanne et al. (De Lanne *et al.*, 1960) reported that, following 30 min of cycling exercise at the same relative intensity, increases in WBCs and lymphocytes were greater in six healthy women *versus* six healthy men. Following muscle-damaging eccentric exercise (i.e., muscle lengthens during force production), women experience a distinct pattern of inflammatory cell infiltration into skeletal muscle, compared with men. Early in recovery from this type of exercise, women respond with a greater influx of neutrophils into the active muscle (MacIntyre *et al.*, 2000), whereas by 24 hours the degree of inflammation is considerably less in women, compared with men (Stupka *et al.*, 2000). Therefore, interpretation of sex differences in immunological responses to exercise may depend on the type of muscle contraction in so far as the immune-directed recovery from unaccustomed eccentric exercise appears to be faster in women.

Sex hormones are obvious candidates contributing to any observed differences between men and women. The effects of sex hormone fluctuations within women on immunological responses to exercise, however, are not well understood. In 20 female athletes followed over three consecutive menstrual cycles resting salivary Ig A (sIgA) levels remained stable, although salivary progesterone concentrations increased throughout the menstrual cycle, as expected (Burrows *et al.*, 2002). That training levels did not change over the study period is the likely reason why sIgA levels remained stable. In another study (Gonzalez *et al.*, 1998), the cytokine response to 80 min of treadmill walking at 32% $\dot{V}O_{2max}$ plus cold stress was determined in nine healthy women. No changes in IL-6 or TNF-α were found during exercise or between menstrual phases (early follicular and mid luteal). However, such a low intensity of exercise would not be expected to induce significant changes in cytokine levels (Moldoveanu *et al.*, 2001). Taken together, there is a clear lack of understanding pertaining to the influence of the menstrual cycle on immunological responses to exercise.

1.3.5. Influence of carbohydrate (CHO) intake

That high-intensity, prolonged exercise leads to significant suppression of the number and function of various immune cells during recovery initiated a search for strategies to improve immune status under these conditions. Of an assortment of nutritional countermeasures to

exercise-induced immunosuppression (Nieman & Pedersen, 2000), CHO intake has consistently resulted in significant modification of immune perturbations (Nieman, 2001). Several studies have now shown a variety of effects on both cell number and function during and following acute bouts of exercise. Compared with water consumption, CHO intake during exercise attenuates the rise in neutrophil number (Bishop *et al.*, 2002; Henson *et al.*, 2000; Nagatomi *et al.*, 1994; Nieman *et al.*, 1997a; Nieman *et al.*, 1999; Scharhag *et al.*, 2002). A more variable effect on neutrophil function has been observed with either a maintenance (Bishop *et al.*, 2002; Nagatomi *et al.*, 1994; Scharhag *et al.*, 2002), an attenuation (Henson *et al.*, 2000; Nieman *et al.*, 1998a) or no effect (Nieman *et al.*, 1997a). Part of this variability may be due to what aspect of neutrophil function is tested. CHO intake also attenuates changes in monocyte (Henson *et al.*, 1999; Nieman *et al.*, 1997a) and lymphocyte number (Henson *et al.*, 1999; Nieman *et al.*, 1997a) and in lymphocyte function (Green *et al.*, 2003; Henson *et al.*, 1998). NK cells are also attenuated in number (Henson *et al.*, 1999; Nieman *et al.*, 1997b; Nieman *et al.*, 1999) and in function (Henson *et al.*, 1999), while stimulated NK cell cytotoxicity is maintained (McFarlin *et al.*, 2004). With respect to immune recovery following exercise, CHO intake appears to speed the return of neutrophils (Bishop *et al.*, 2002; Green *et al.*, 2003; Henson *et al.*, 1998; Nieman *et al.*, 1998a; Nieman *et al.*, 1999; Scharhag *et al.*, 2002) and lymphocytes

(Henson *et al.*, 1998; Henson *et al.*, 1999), but not NK cells (Henson *et al.*, 1999; McFarlin *et al.*, 2004; Nieman *et al.*, 1997b) to pre-exercise values.

The increase in plasma levels of several inflammatory-related cytokines is also attenuated with CHO intake (Bishop *et al.*, 2002; Nehlsen-Cannarella *et al.*, 1997; Nieman *et al.*, 1998b; Starkie *et al.*, 2001), as is their expression in skeletal muscle following exercise (Febbraio *et al.*, 2003; Nieman *et al.*, 2003a). However, exercise may have to be of a certain threshold of intensity for CHO intake to demonstrate a significant impact, since CHO intake during less intense exercise tasks is not associated with immune changes (Bishop *et al.*, 1999).

The mechanisms by which exogenous CHO affects circulating immune cells are unclear. As previously discussed, hormonal changes associated with exercise likely explain a large portion of the exercise-induced leukocyte trafficking. One hypothesis proposed that maintenance or increases in blood glucose levels, due to CHO intake, results in a blunted stress hormone (e.g., epinephrine and cortisol) response (Nieman, 1998). As a result of lower epinephrine and cortisol concentrations, there is a reduced effect on the sequestered cell populations, and less mobilization. In most immune studies, CHO intake has not proven to influence catecholamine responses in any significant measure (Henson *et al.*, 1999; Nieman *et al.*, 1997b). However, CHO does appear to lower the

cortisol response (Bacurau *et al.*, 2002; Bishop *et al.*, 2002; Green *et al.*, 2003; Henson *et al.*, 1999; Nieman *et al.*, 1997b).

With respect to CHO-attenuated cytokine responses, evidence is accumulating that cytokine changes during exercise may reflect the metabolic status of the active muscle tissue. In particular, low muscle glycogen content enhances the IL-6 (Bishop *et al.*, 2001; Chan *et al.*, 2004; Steensberg *et al.*, 2001a) and IL-8 (Chan *et al.*, 2004) response to exercise. Thus, the utilization of exogenous CHO as an alternative fuel source during exercise may spare muscle glycogen, thereby reducing upregulation of cytokine expression.

1.3.6. *Summary*

Exercise immunology is an expanding field that has focused on young adults. The immune system responds rapidly to exercise with mobilization of cells from marginated pools. The trafficking of cells is likely due to systemic changes in stress hormones, but may also include local factors. The duration and intensity of exercise are primary determinants of the magnitude and direction of change in immune cells and cytokines. Strong evidence for sex and menstrual phase effects on immunological responses to exercise is lacking. CHO intake during exercise is associated with several effects on the immune system that may, in part, be related to substrate availability. The following section describes the

effects of exercise on the immune system that are specific to children and adolescents.

1.4. Effects of Exercise on the Immune System of Children and Adolescents

1.4.1. Overview

Despite an expanding field of exercise immunology, study participants have been mostly young healthy adult males and investigations devoted to children remain sparse. Several reasons can likely explain this paucity of literature, including the ethical issues related to peripheral blood collection in younger individuals, the cost associated with immunological measurements, and the simple fact that few laboratories in the world are interested in pediatric exercise science. Given the age- and puberty-related changes in the immune system under resting conditions, it would seem prudent to highlight the possible effects of growth on the immunological responses to exercise in children and adolescents. The few available publications in this area have varied considerably in the intensity and duration of exercise employed.

1.4.2. Influence of brief, intense exercise

From a historical perspective, the earliest data reporting a leukocytosis of physical exercise in young children appear to be those of Christensen and Rothstein (Christensen & Rothstein, 1979). In a way, these authors were the first to determine the effect of exercise intensity on

immune changes, by comparing the total leukocytosis and neutrophilia following circumcision, chest physical therapy, and heel puncture in newborn babies. The most robust increases in circulating total WBCs and neutrophils were observed following circumcision (~46% and ~48%, respectively), the "most violent physical activity", with a full recovery of cells to resting levels by 60 min after the procedure (Christensen & Rothstein, 1979).

Other investigators have examined children's responses to high-intensity exercise in a more standardized manner. Two groups (Boas *et al.*, 1996; Wolach *et al.*, 1998b) tested children's responses to the Wingate anaerobic test (WanT). The WanT is a 30-sec "all-out" cycling task, characterized by excellent validity and reproducibility (Bar-Or, 1987). Moreover, the standardized testing procedures facilitate inter-study comparisons. Figure 1.3 is a plot of the exercise-induced change (Δ) in NK cells from rest determined after exercise in the two pediatric studies, with those of one study on young healthy adult males, also using the WanT (Nieman *et al.*, 1992). Based on this figure, the magnitude of change in NK cells increases with advancing age, at least during childhood, with the greatest exercise-induced increases observed in teenagers. The 10-, 14-, and 16-year-old age groups from Boas et al. (Boas *et al.*, 1996) correspond to pre, mid and post-pubertal subjects, respectively. Interestingly, the adult response was comparable to that of

the youngest male subjects, but greater than the girls. This similarity between youngest and oldest males is interesting considering that the average power output over the 30 sec was higher in the adults (~9.2 Watts/kg body weight) than in the children (~6.7 Watts/kg body weight). Another study with teenagers (Christensen & Hill, 1987) investigated the effects of 10 min of all-out stair-running on immune changes and found that total WBCs and lymphocytes increased by 41% and 67%, respectively. A 5-min of stair-climbing task in adults resulted in more than a 100% increase in lymphocytes (Edwards *et al.*, 1984).

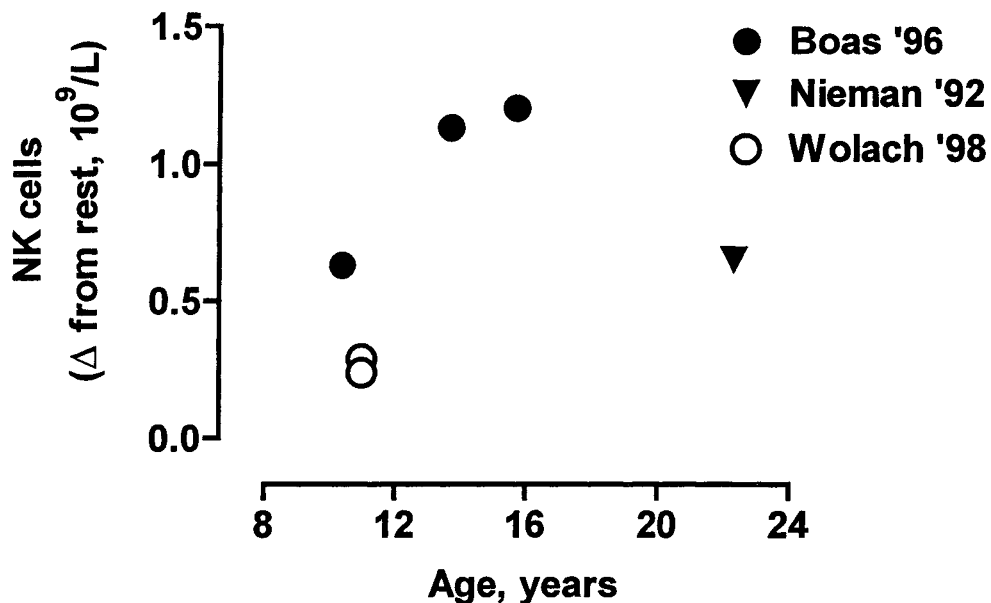

Figure 1.3 *Relationship between magnitude of increase in NK cells from rest determined after a Wingate anaerobic test and chronological age. Data from Boas et al. 1996, Nieman et al. 1992, and Wolach et al. 1998.*

Maximal exercise testing (i.e., $\dot{V}O_{2max}$) provides another example of high-intensity exercise of relatively short duration (~10-12 min). With this approach, Boas and colleagues (Boas *et al.*, 2000) reported very large increases in NK cell counts. Moreover, age accounted for almost 40% of the variance in the NK cell response (Figure 1.4). However, this age effect could have been ascribed to the fact that older kids were stronger and able to exercise longer, thus achieving a higher intensity of exercise and a greater mobilization of cells. Boas and colleagues did not report exercise times.

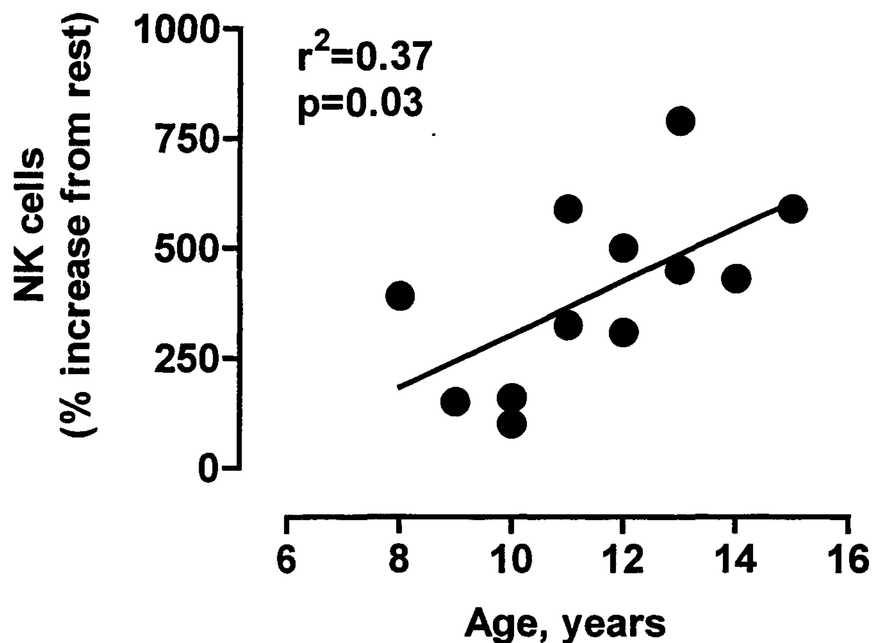

Figure 1.4 *Relationship between percent increase in NK cells from rest determined after a test of maximal aerobic power and chronological age in males. Data is redrawn from Boas et al. 2000.*

Physiological recovery from brief, intense exercise is faster in children than in adults (Hebestreit *et al.*, 1993; Hebestreit *et al.*, 1996). Although direct age comparisons of immune recovery kinetics have not been made, the studies of Boas et al. (Boas *et al.*, 1996) and Nieman et al. (Nieman *et al.*, 1992) provide some insight into this issue. One hour after the end of the WanT, neutrophil counts returned to pre-exercise levels in the adults (Nieman *et al.*, 1992) and in the pre- and post-pubertal children (Boas *et al.*, 1996). Consistent with the known effects of high-intensity aerobic exercise, lymphocyte counts were suppressed at one hour into recovery by ~34% in adults (Nieman *et al.*, 1992), ~24% in post-pubertal boys, and ~11% in the pre-pubertal boys (Boas *et al.*, 1996). Interestingly, NK cells were considerably more suppressed (~54%) in the adults (Nieman *et al.*, 1992), compared with an ~27% decrease in post-pubertal and only an ~14% reduction in the pre-pubertal boys (Boas *et al.*, 1996). That neutrophils returned to pre-exercise levels challenges a possible influence of cortisol on the observed changes in lymphocytes.

1.4.3. *Influence of aerobic-type exercise*

Undoubtedly, the majority of exercise immunology-related studies in the pediatric population have used aerobic tasks. Duration of exercise has varied from 20 to 90 min in both the laboratory and field setting, with a variety of sub-maximal exercise intensities employed (Eliakim *et al.*, 1997; Nemet *et al.*, 2002; Nemet *et al.*, 2003a; Nemet *et al.*, 2004a; Perez *et al.*,

2001; Scheett *et al.*, 1999; Shore & Shephard, 1998; Sun *et al.*, 1990; Tirakitsoontorn *et al.*, 2001; Wolach *et al.*, 1998a). However, no study has directly compared the responses of children to those of adults under identical experimental conditions. Notwithstanding this methodological limitation, some studies (Eliakim *et al.*, 1997; Shore & Shephard, 1998) concluded that the responses of their child subjects were comparable to those previously reported for adults. Figure 1.5 is a plot of the exercise-induced Δ in NK cells from rest determined immediately after exercise reported by a number of pediatric (Eliakim *et al.*, 1997; Nemet *et al.*, 2003a; Nemet *et al.*, 2004a; Perez *et al.*, 2001; Shore & Shephard, 1998) and adult (Brenner *et al.*, 1996; Kendall *et al.*, 1990; Klokker *et al.*, 1995) studies, as a function of chronological age. Adult studies were chosen to match the exercise duration and intensity of the child studies as closely as possible. Despite the limitations with this approach, the responses of the youngest children are generally smaller than those of the teenagers, but also less than many of the adult studies. What, if any, impact puberty may have on this apparent relationship is unclear, and no study has formally addressed the effects of puberty on immune changes in response to prolonged, aerobic exercise in children.

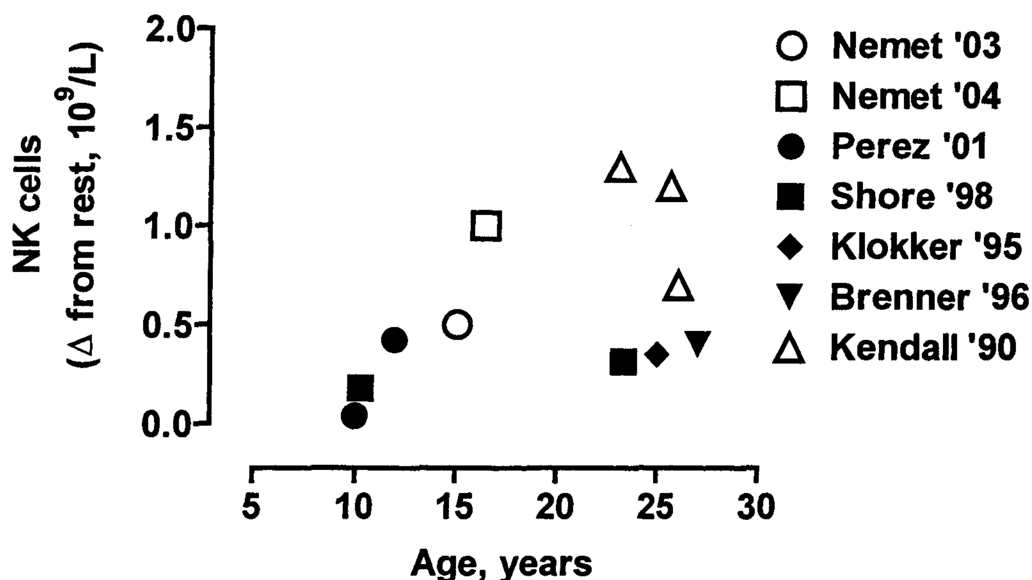

Figure 1.5 *Relationship between magnitude of increase in NK cells from rest determined immediately after aerobic-type exercise and chronological age. Data from Brenner et al., 1996 (30 min cycling @ 50% $\dot{V}O_{2max}$), Kendall et al., 1990 (30 min cycling @ 65% $\dot{V}O_{2max}$), Klokker et al., 1995 (20 min cycling @ 60% $\dot{V}O_{2max}$), Nemet et al., 2003 (90 min water polo), Nemet et al., 2004 (90 min wresting), Perez et al., 2001 (90 min soccer and 20 min cycling @ ~75% $\dot{V}O_{2max}$), and Shore and Shephard, 1998 (30 min cycling @ ~70% $\dot{V}O_{2max}$).*

It is also important to note that many of the pediatric studies included a mix of boys and girls in their subject pool, and to this author's knowledge attempt has been made to consider sex differences in immune changes with exercise among children and adolescents. However, one can cautiously compare the responses of adolescent girls (Nemet *et al.*, 2003a) to adolescent boys (Nemet *et al.*, 2004a) since these studies were conducted by the same laboratory. Although total WBCs increased with exercise to a similar degree in boys (1.9-fold) and girls (1.8-fold), the

change in NK cells was greater in boys (3.4-fold) *versus* girls (2.5-fold). Whether this is a true sex difference in the NK cell response to exercise or a methodological artifact remains to be determined.

As with the literature reporting brief, intense exercise effects, studies using aerobic-type exercise have not adequately followed the post-exercise recovery of immune changes. In one study (Shore & Shephard, 1998), neutrophil counts returned to pre-exercise levels, but lymphocyte subsets including NK cells remained elevated 30 min after a 30-min cycling task. This is in contrast to a maintained elevation in neutrophils and a depression in lymphocyte subsets 30 min following exercise in adults, as reported in the same paper (Shore & Shephard, 1998). However, the adults had cycled for an additional 30 min, thereby making interpretation of the age differences tentative at best.

Reports of cytokine changes in response to exercise in children and adolescents are relatively new, and our understanding of their biological impact is limited. Moreover, there have been no studies specifically designed to compare cytokine responses to exercise between children and adults. Figure 1.6 is a plot of the exercise-induced Δ in IL-6 from rest determined after exercise in a number of pediatric (Nemet *et al.*, 2002; Nemet *et al.*, 2003a; Scheett *et al.*, 1999; Tirakitsoontorn *et al.*, 2001) and adult (Connolly *et al.*, 2004; Starkie *et al.*, 2001) studies . Again, adult studies were chosen to approximate the duration and intensity of exercise

in the child studies. Notwithstanding the unusually high increase in IL-6 from one study (Nemet *et al.*, 2002), the youngest children generally experience a lower IL-6 response to exercise than reported in adults.

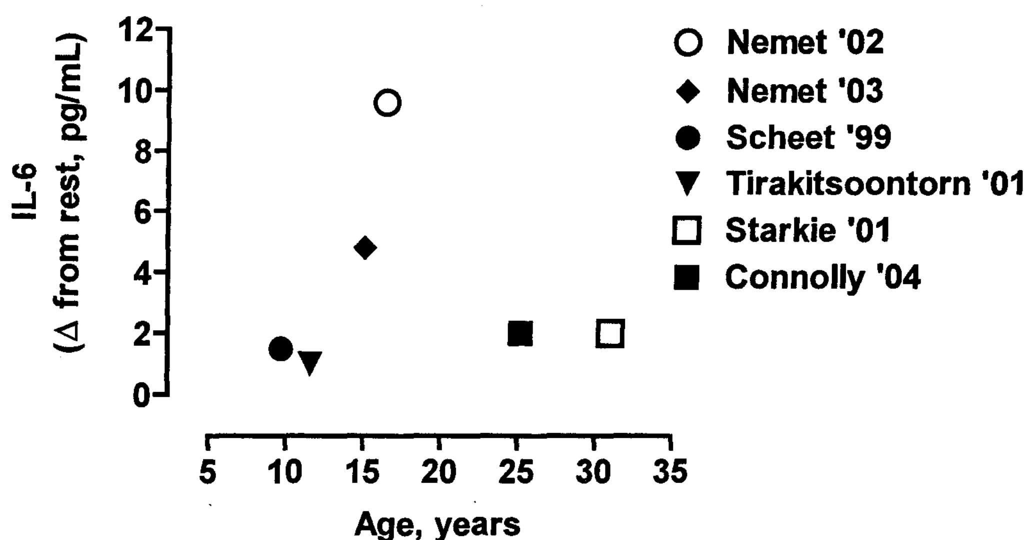

Figure 1.6 *Relationship between magnitude of change in IL-6 from rest determined after aerobic exercise and chronological age. Data from Connolly et al., 2004 (30 min cycling @ 80%* $\dot{V}O_{2max}$*), Nemet et al., 2002 (90 min wrestling), Nemet et al., 2003 (90 min water polo), Scheet et al., 1999 (90 min soccer), Starkie et al., 2001 (30 min cycling @ ~70%* $\dot{V}O_{2max}$*), and Tirakitsoontorn et al. 2001 (20 min cycling @ ~75%* $\dot{V}O_{2max}$*).*

Similar to IL-6, TNF-α levels have been reported to increase following exercise in young children (Scheett *et al.*, 1999; Tirakitsoontorn *et al.*, 2001) and adolescent boys (Nemet *et al.*, 2002), but not adolescent girls (Nemet *et al.*, 2003a). The absolute increase in concentration, however, ranged from only 0.25 to 1.1 pg/mL with the largest increase observed in the oldest subjects (Nemet *et al.*, 2002). It is therefore

unclear what, if any, biological significance these small changes might represent. Like in adults, few pediatric studies have identified the effects of exercise on IL-8. However, systemic IL-8 levels have been reported to increase following an exercise test for bronchial asthma in children (Boznanski & Rudzka, 1998). That exercise events such as a marathon run seem necessary to induce significant changes in IL-8 levels in adults, more prolonged and intense exercise may also prove to be an important determinant of changes in IL-8 in children.

1.4.4. Influence of CHO intake

The influence of CHO intake on immunological changes in response to exercise in children has not been studied under placebo-controlled conditions. One study (Nieman *et al.*, 2000) reported immune changes in elite adolescent tennis players who drank ~9 mL/kg body weight/hr of a 6% commercial sports drink (Gatorade) during practice. Although neutrophil counts increased significantly, the concentration of lymphocytes and NK cells actually decreased with exercise and remained below pre-exercise levels 60 min into recovery (Nieman *et al.*, 2000). However, since there was not a comparable trial with only water consumption, it is impossible to separate CHO and exercise effects in this study. There are no reports of the effects of CHO intake on immunological responses to exercise in children younger than 14-years-old.

1.4.5. Summary

Limited evidence suggests that the degree of exercise-induced perturbation in some cellular and soluble immune components may be less in children than in adults. The effects of puberty and sex on immune-related changes with aerobic exercise are not known. The available data also suggest that the recovery of the immune system following exercise may be faster in children than in older adolescents and adults. There are no controlled studies investigating the effects of CHO intake during exercise on immune changes in children or adolescents. In order to better understand the potential benefits of exercise on immune health in children and adolescents, an improved understanding of age-, puberty-, sex-, and CHO-related effects on immunological changes in response to exercise seem warranted.

1.5. Objectives

1.5.1. General objective

The general objective of this thesis was to advance the state of knowledge regarding immunological changes in response to exercise performed with and without CHO intake during childhood, adolescence and young adulthood.

1.5.2. Specific objectives

The specific objectives of the studies included in this thesis were to:

1) determine age-related differences in acute immune changes to exercise, the recovery of leukocytes and cytokines following exercise, and the effects of CHO intake on immune changes to exercise (Chapter 3);
2) determine the influence of puberty on NK cell responses to exercise and CHO intake (Chapter 4);
3) determine the effects of age, puberty and sex on changes in various cellular and soluble components of the immune system in response to exercise (Chapter 5);
4) determine the influence of sex, menstrual cycle phase, and oral contraceptive (OC) use on immunological changes in response to endurance exercise (Chapter 6).

1.6. Hypotheses

1.6.1. General hypothesis

The general hypothesis of this thesis was that the magnitude and direction of various immunological changes in response to acute exercise would depend on age, puberty and sex, with a further modification by the ingestion of CHO during exercise.

1.6.2. Specific hypotheses

The specific hypotheses of the studies included in this thesis were that:

1) exercise-induced changes in the immune system would be smaller, recovery of immune changes faster, and that CHO intake would have a minimal effect on immune changes in boys *versus* men (Chapter 3);
2) the NK cell response to exercise would be greater with progressing pubertal status and that CHO effects would be more pronounced in pre-pubertal boys (Chapter 4);
3) perturbations to, and recovery of, the immune system would be smaller and faster, respectively, in younger *versus* older children and that immunological changes in response to exercise would vary between boys and girls (Chapter 5);
4) immunological changes in response to exercise would vary between men and women, between phases of the menstrual cycle, and between OC users and non-users (Chapter 6).

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CHAPTER 2: PROJECT DESIGN, STUDY SUBJECTS, AND METHODOLOGICAL CONSIDERATIONS

The study of children and adolescents demands special circumstances and considerations for the researcher that are not encountered in adult research. Besides ethical issues, the pediatric scientist is faced with the challenge of accounting for factors related to growth (e.g., body size) and maturity (e.g., puberty). The study of immunological responses to exercise in children and adolescents is no different. Unlike the tempo and timing issues of normal biological development, however, the immune system also develops in accordance with antigenic experience. Thus, the development of a child's immune system is under both internal (i.e., biological) and external (i.e., environmental) influences. The following sections describe the considerations of, and rationale for, general methodological aspects of this thesis. Details of methodology are further described within each subsequent chapter.

2.1. Project Design

The studies in this thesis were all cross-sectional in nature. As a first step toward understanding age-related differences in immunological changes in response to exercise, 9- and 10-yr-old boys were compared to

university-aged men (Chapter 3). Upon revealing specific differences between boys and men, a larger-scale study was designed to further investigate the effects of age, puberty and sex (Chapters 4 and 5). During the tenure of this thesis, an opportunity developed to evaluate immune-related responses to endurance exercise between men and women, between phases of the menstrual cycle, and between users and non-users of oral contraceptives (Chapter 6). The latter three adult comparisons were, therefore, a logical extension to the pediatric studies.

2.2. Study Subjects

For the most part, subjects participating in these studies were recreationally active, but not trained athletes. However, due to the demanding requirements of this project (e.g., high-intensity exercise, venous blood collection, time scheduling, etc.), it became difficult to secure interested children and their parents. Therefore, local sports teams were approached, and additional subjects (n=9) who trained for competitive sport (triathlons) were recruited. These child athletes were four 12-yr-old girls, four 14-yr-old girls, and one 14-yr-old boy. All adult subjects were recruited from the McMaster University student body, which is the normal practice for most exercise-related studies.

Detailed subject characteristics are provided in each of the subsequent chapters. For Chapters 4 and 5, we deliberately recruited boys and girls at two distinct chronological ages, 12 and 14 years. Table

2.1 outlines the final number of subjects in each age and pubertal group from Chapters 4 and 5. The age of 12 years was chosen to ensure that a wide range of pubertal stages would be represented (e.g., Karpati *et al.*, 2002). More 12-yr-old boys were recruited in order to sub-divide these subjects into separate groups representing various stages of physical maturity. Previous large scale studies of children's responses to exercise have tested only 12-yr-old children to represent the full range of physical maturity (e.g., Armstrong *et al.*, 1999). The age of 14 years was arbitrarily chosen as an adolescent age group that would be likely to represent a range of pubertal development, and in the case of females would be likely to represent sexually mature individuals (i.e., regular menstrual cycles). For obvious technical, time and financial considerations, larger groups of females representing more stages of puberty were not recruited.

Table 2.1 *Final number and pubertal status of 12- and 14-yr-old boys and girls in Chapters 4 and 5.*

Gender	Age	Tanner Stage	Number of Subjects
GIRLS	12	2	4
		3	8
		4	1
		5	1
	14	3	3
		4	7
		5	1
BOYS	12	1	7
		2	7
		3	3
		4	1
		5	2
	14	3	2
		4	3
		5	8
		TOTAL	**58**

From an immunological perspective, the chronological age of 12 years carries important implications. According to Scammon's curves of post-natal growth, the age of 12 years is an approximate time when physical development of the lymphoid system (e.g., lymph gland, thymus gland, tonsils, etc.) reaches its maximum (~200% of adult status), rapidly declining thereafter (Scammon, 1930 as referenced in Malina *et al.*, 2004). The age of 12 years is also closely associated with rapid progression into puberty and attainment of menarche for boys and girls, respectively (Malina *et al.*, 2004). These two observations, therefore, highlight the temporal relationship between increasing concentrations of androgens and estrogens and thymic involution, which has been supported experimentally (e.g., Olsen *et al.*, 1998). Thus, the choice of subjects at the chronological age of 12 and 14 years is likely to represent a range of immune development.

2.3. Flow Cytometry

Data in Chapters 3, 4 and 5 were derived, in part, from direct immunofluorescence analysis of specific lymphocyte subsets. To accomplish this, a whole blood, lyse-wash procedure was employed, which is described in detail in each of the above-mentioned chapters. The analysis of lymphocyte subsets employed a double-platform approach; A FACScan flow cytometer was used to determine the proportion of cells positive (or negative) for a particular antigen and a Coulter counter

determined the absolute number of lymphocytes in the same sample. Together, the absolute number of the particular lymphocyte subset was determined by simple multiplication of subset proportion by lymphocyte count. Appendix A provides an example of the gating procedure for some of the lymphocyte subsets presented in Chapter 5.

2.4. High-sensitivity (HS) ELISA Determination of IL-6

In Chapter 3, an enzyme-linked immunosorbent assay (ELISA) from ENDOGEN (Woburn, MA, U.S.A.) was used to determine IL-6 concentrations. The results of this analysis are described in detail within the METHODS and RESULTS sections of that chapter. This approach, however, caused some concern among other researchers within the field who dismissed the results as "negative" (Cooper *et al.*, 2004). Their concerns were based on the sensitivity of the chosen ELISA kit, which they suggested may not have been sufficient to detect small changes in IL-6 concentrations. They further suggested that a high-sensitivity (HS) ELISA from R&D Systems would be a more appropriate kit to use. Although we believed the findings to be real and consistent with the existing literature, a re-analysis of the same samples was performed with the suggested HS ELISA kits. These samples had been stored at –70°C for three years at the time of re-analysis. Given that CHO intake did not impact the IL-6 response to exercise in either boys or men (Chapter 3), samples from the water trial only were processed. This also allowed for a

comparison with the data in Chapter 5 (see Appendix E). Appendix B provides the results of re-analysis (Figure B.1). Using the HS ELISA kit, an identical pattern to that originally described in Chapter 3 was statistically confirmed, albeit with lower absolute concentrations from the HS ELISA *versus* ENDOGEN kit. More importantly, however, was that the absolute exercise-induced increase in IL-6 (~0.5 pg/mL) remained extremely small in the children, which was quite comparable to the absolute change identified with the ENDOGEN ELISA (~0.6 pg/mL). In addition, these results are not confounded by prolonged storage time, because IL-6 frozen at –20°C is stable for several years (Kenis *et al.*, 2002).

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CHAPTER 3: IMMUNE RESPONSES TO STRENUOUS EXERCISE AND CARBOHYDRATE INTAKE IN BOYS AND MEN

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3.1. Abstract

Few studies describe immune responses to exercise in children, compared to adults, and none have investigated the influence of carbohydrate (CHO) intake. We hypothesized less perturbation and a faster recovery of the immune system with exercise in children, regardless of supplemental energy. Twelve boys (9.8 ± 0.1 yr) and 10 men (22.1 ± 0.5 yr) cycled for 60 min at 70% $\dot{V}O_{2max}$ while drinking 6% CHO (CHO-T) or flavored water (FW-T). Blood samples were collected before (PRE), immediately after (POST) and 60 min after (REC) exercise. Boys, compared to men, had smaller (p<0.05) increases in total leukocytes (28 vs. 38%), natural killer (NK) cells (78 vs. 236%) and NK T cells (42 vs. 128%) at POST, averaged across beverage trials. Exercise did not increase tumor necrosis factor-α (TNF-α), but significantly (p<0.05)

increased interleukin-6 (IL-6) in men (189%), but not in boys (11%). In both trials, lymphocytes and T cells at REC were suppressed ($p<0.05$), relative to PRE, in men (-21%), but not in boys (4%). IL-6 remained elevated ($p<0.001$) in men at REC, with no change from POST in boys. In boys, but not in men, CHO significantly ($p<0.05$) attenuated increases in neutrophil, lymphocyte, and NK cell counts at POST. Neutrophils at REC in CHO-T were lower ($p<0.05$) than in FW-T in men (~25%) and in boys (~17%). CHO had no effect on TNF-α or IL-6 in either group. Our results indicate a distinct pattern of the immune response to exercise and CHO intake in boys, compared to men.

3.2. INTRODUCTION

Strenuous exercise can significantly influence several elements of the human immune system. The magnitude and direction of changes in immune cell counts, for example, are mediated by the intensity of the activity (1,2), the nutritional status of the subject (3,4) and changes in stress hormones, in particular epinephrine during exercise (5) and cortisol following exercise (6). Although most immune cells increase in concentration during exercise, a brief period of immunosuppression, termed the "open window", occurs during recovery from strenuous exercise and may increase susceptibility to infection (7). Another component of the immune system responsive to exercise are cytokines. Cytokines serve in a network to help regulate signaling of innate and

specific immune responses (8). Traditionally, cytokine responses to exercise, in particular interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), have been interpreted in the context of immune-mediated host defense (9). However, exercise-induced increases in plasma levels of IL-6 and TNF-α are not always associated with simultaneous increases in their intracellular production or excretion from immune cells (10-13). Therefore, changes in these low molecular weight proteins with exercise likely serve roles in addition to their control of inflammation.

It is important to note, however, that our current understanding of exercise-induced changes in components of the human immune system is derived primarily from adults, and there is a paucity of data on children. With respect to immune cell counts, a few studies have reported similar responses to aerobic exercise between children and adults (14-16). However, these studies did not make direct comparisons between children and adults under identical, experimental conditions, and closer inspection of the data (14-16) would suggest that the magnitude of change in various immune cells was smaller in younger subjects, compared to older ones. However, convincing proof of age-related differences in the immune response to prolonged exercise is lacking. In addition, we are aware of no study that has directly compared the inflammatory cytokine response to exercise between young children and adults, but limited evidence suggests that muscle damage associated with exercise, which produces

an inflammatory response, may be less in children than in adults (17,18). Moreover, to our knowledge, no study has directly compared the recovery of immune cells or cytokines following exercise in children with that in adults. Considering that children tend to have a faster physiological (e.g., heart rate and ventilation) recovery from exercise than do adults (19), immune cells may also recover more quickly than adults thereby limiting the "open window" period.

Recently, there has been growing interest in nutritional strategies to modify the immune response to acute exercise in adults (reviewed in ref. 20). For example, compared with water, carbohydrate (CHO) ingestion during exercise can attenuate the expected increase in neutrophils (21,22) mononuclear cells (23,24) and several inflammatory cytokines (25-28). Furthermore, the post-exercise recovery of immune cells tends to be faster with the ingestion of CHO, compared with water (21,23,24), which may reduce the "open window" period. CHO-induced attenuation of leukocyte trafficking is likely due to a blunted stress hormone (e.g., epinephrine and cortisol) response mediated by maintained blood glucose concentrations (29). CHO-mediated changes in cytokine levels indicate a reduced inflammatory response. Although debatable, some indirect evidence (30) suggests that the epinephrine response to sustained exercise may be smaller in children than in adults and, as discussed above, children may also have a smaller inflammatory response to a given

amount of exercise. Therefore, the potential benefits of CHO ingestion on immune changes in children may be limited due to an already blunted stress hormone and an inherently smaller inflammatory response. However, no study has investigated the effects of CHO ingestion during exercise on immune changes in young (i.e., pre- and early pubertal) children to address this issue.

A clearer comprehension of the acute effects of exercise on the developing immune system may have important clinical implications for children with, or recovering from, an immune-related disease. Therefore, the objectives of the present study were to determine possible age-associated differences in 1) the acute immune response to exercise, 2) the recovery of leukocytes and cytokines following exercise, and 3) the effects of CHO ingestion on the immune response to exercise. To address our objectives, we used a model of high intensity exercise to produce significant perturbations in commonly reported aspects of the cellular immune system including natural killer (NK) cells, which are the most responsive immune cell to exercise (6) and two commonly reported inflammatory cytokines, IL-6 and TNF-α. We hypothesized that in boys, compared with men, exercise-induced changes in these elements of the immune system would be smaller, recovery of immune changes would be faster, and that CHO ingestion would have a minimal effect on immune changes.

3.3. METHODS

Subjects. Twelve boys and 10 men volunteered for this study approved by the McMaster University Research Ethics Review Board. Table 1 summarizes their physical and fitness characteristics. All subjects were healthy with no history of allergies and were not taking any medication. To determine pubertal status of the boys, a parent and the child assessed pubic hair development according to Tanner (31). Boys were either Tanner stage 1 (n=8) or 2 (n=4). After the purpose, procedures and risks of the study were explained, written informed consent was obtained from the men. The boys agreed verbally to participate and their parent then signed a written informed consent.

Preliminary session. An initial visit was conducted one week prior to the experimental trials in order to measure body height (Harpenden Stadiometer, CMS Weighing Equipment LTD.), body weight (BW; BWB-800, TANITA), and percent body fat (bio-electric impedance-101A, RJL Systems) of each subject. To determine maximal O_2 uptake ($\dot{V}O_{2max}$), a maximal exercise test was conducted, as previously described (19), on a mechanically braked cycle ergometer (Fleisch-Metabo). Measurements of $\dot{V}O_2$ and CO_2 production ($\dot{V}CO_2$) were made continuously using a metabolic cart (Vmax29, SensorMedics). A Polar heart rate (HR) monitor (Vantage XL, Polar Electro) was used to record HR throughout the test.

Experimental sessions. To account for the confounding effects of diet on immune responses to exercise (3,4), subjects were asked to record their nutrient intake the day before their first trial. Physical activity was also recorded during this 24-h period, and strenuous activity was avoided by all subjects. Recorded food intake and activity were then repeated the day prior to their next visit. Two experimental trials were conducted 1-2 weeks apart. Upon arrival to the laboratory (~07:30), subjects consumed a small standardized breakfast (toast and sugar-free jam, ~2.4 kcal/kg; water, ~3.4 mL/kg). Fluid intake for the session was calculated from a naked body weight taken after subjects finished their breakfast and emptied their bladder. Subjects then rested (~20 min) before a pre-exercise blood sample was drawn from an arm or hand vein by venipuncture. Thirty minutes before the start of exercise, they were given their first drink (4 mL/kg). The same volume was consumed every 15 min until the end of exercise and every 20 min during the first 60 min of recovery. We chose this drinking schedule as it has been used in previous adult studies investigating the effects of CHO ingestion on immune responses (22). Subjects cycled at a power output equivalent to 70% of their predetermined $\dot{V}O_{2max}$ for two 30-min bouts separated by a 5-min rest period. Exercise began 30 min after the first blood sample and the target intensity was achieved within the first 5 min by analysis of expired gas. Before, during and after exercise, each subject consumed either a 6%

CHO-electrolyte solution (4% sucrose, 2% glucose, ~18 mmol/L Na^+, ~3 mmol/L K^+) or water (identical in flavor, sweetness and electrolyte concentration, but without CHO) for a total of 40 mL/kg BW. Additional, expired gas samples were collected at steady state from minutes 12-15 and 27-30 of each exercise bout. Immediately after exercise, a second blood sample was drawn within 30 sec while subjects remained seated on the cycle ergometer. HR was monitored throughout the session. Subjects remained seated in the laboratory for 60 min after exercise, but were allowed to empty their bladder if necessary. At the end of the 60-min recovery period, a final blood sample was drawn. Except for CHO intake before, during and after exercise, the CHO (CHO-T) and flavored water (FW-T) trials were identical and performed in a counterbalanced order, with subjects blinded to the contents of their drink.

Glucose analysis. Whole blood (2 mL) treated with EDTA was centrifuged at 2000 g for 10 min and the plasma was stored at –70°C until analyzed. Plasma glucose was measured enzymatically (2300L STAT, Yellow Springs Instruments), and concentrations were corrected for exercise-induced changes in plasma volume according to Dill and Costill (32).

Complete blood counts (CBC) and immunophenotyping. Whole blood (2 mL) treated with EDTA was analyzed for total leukocytes, neutrophils, lymphocytes, monocytes, hemoglobin and hematocrit with an

automated Coulter counter by the clinical hematology group at McMaster University. An additional 2 mL of EDTA-treated whole blood was used to determine lymphocyte subsets. Ten μL of monoclonal antibody, directly conjugated with FITC (Anti-CD3) or PE (anti-CD16/CD56), were mixed with 100 μL of blood and used to numerate total T cells ($CD3^+$), natural killer (NK) cells ($CD3^-CD16^+CD56^+$) and NK T cells ($CD3^+CD16^+CD56^+$). All reagents were purchased from Becton Dickinson and samples were stained as per the manufacturer's instructions within 6 h of collection. Briefly, mixed samples were vortexed and incubated for 20 min at room temperature (RT) in the dark. After adding 2 mL of FACSLysing solution in order to lyse red blood cells, samples were vortexed and incubated a further 10 min at RT. Samples were centrifuged (300 g for 5 min at RT), washed with 2 mL phosphate buffered saline (PBS) containing 0.1% Na azide, centrifuged (200 g for 5 min at RT) and fixed with 0.5 mL PBS (1% paraformaldehyde). Samples were stored at 3°C for no more than 48 h before analysis by a FACScan flow cytometer and CELLQuest software (Becton Dickinson). The lymphocyte population was gated using forward-scatter vs. side-scatter characteristics and 10 000 events were counted per sample. Cell concentrations of each lymphocyte subset were calculated by multiplying the percentage of cells with appropriate fluorescence with the absolute number of lymphocytes obtained from the

CBC. Whole blood cell concentrations were corrected for exercise-induced changes in blood volume according to Dill and Costill (32).

Cytokine analysis. Whole blood (2 mL) treated with EDTA was centrifuged at 2000 g for 10 min and the plasma was stored at –70°C until analyzed. IL-6 and TNF-α were determined in duplicate with ELISA kits purchased from ENDOGEN (Woburn, USA). The sensitivity of these kits, as reported by the manufacturer is <1 pg/mL for IL-6 and <2 pg/mL for TNF-α. In our experience, some samples from the boys (9/54 for IL-6 and 4/54 for TNF-α) and from the men (7/54 for IL-6 and 10/54 for TNF-α) were below the detection level of the respective kit and were, therefore, set to the lowest positive number on the standard curve derived from the plate on which the sample was determined. The intra-assay coefficient of variation (CV) averaged 11.2% (range, 0.25-29%) for IL-6 and 5.5% (range, 0.16-26.3%) for TNF-α. The inter-assay CV for both cytokines averaged 6.5% (range, 0.22-14.9%). All concentrations were adjusted for exercise-induced changes in plasma volume (as above).

Statistical analyses. Data are presented as means ± SEM. Group differences in physical and fitness characteristics were analyzed by independent t-tests. A three-way mixed-factorial ANOVA with one between factor (group) and two within factors (trial and time) was used to analyze cardio-respiratory variables, glucose and immune cell proportions and counts. IL-6 and TNF-α concentrations were not normally distributed

and, therefore, log transformed before submitted to a three-way mixed factorial ANOVA (as above). Graphical presentation of IL-6 and TNF-α is based on concentrations before transformation. Where appropriate, a Tukey's HSD post-hoc test for unequal sample size was used to determine significance among means. STATISTICA for Windows 5.0 (StatSoft, OK) software was used for all analyses with the threshold for statistical significance set at $p \leq 0.05$.

3.4. RESULTS

HR and $\dot{V}O_2$. During exercise, HR, averaged across trials, was not different between boys (158 ± 3 beat/min) and men (157 ± 4 beat/min), representing 80.9 ± 1.2 and 81.0 ± 1.2% of maximal HR, respectively. The average HR for both groups was slightly, but significantly, lower (p=0.02) during FW-T (156 ± 2 beat/min) than CHO-T (160 ± 2 beat/min). To account for differences in body size, $\dot{V}O_2$ measured during exercise was expressed relative to BW (i.e. $mL \cdot kg^{-1} \cdot min^{-1}$). Oxygen uptake was not different between boys and men (p=0.97) or between FW-T and CHO-T (p=0.64) averaging 31.2 ± 1.1 and 31.4 ± 1.3 $mL \cdot kg^{-1} \cdot min^{-1}$, respectively, in the boys and 32.1 ± 1.0 and 31.9 ± 1.0 $mL \cdot kg^{-1} \cdot min^{-1}$, respectively, in the men. When $\dot{V}O_2$, averaged across trials, was expressed as a percentage of $\dot{V}O_{2max}$, boys (69.3 ± 1.5 %) had a slightly lower (p=0.01) value than men (73.3 ± 1.0%).

Plasma glucose. Plasma glucose concentration [glucose] at rest (Figure 1), averaged across trials, was slightly, but not significantly, lower ($p=0.07$) in the boys than in the men. Compared with CHO-T, post-exercise plasma [glucose] in both boys and men was lower ($p<0.001$) in FW-T. Plasma [glucose] immediately after and 60 min after exercise was not significantly different between groups in either trial.

CBC. Immediately after exercise, leukocyte counts were lower in boys than in men in both trials ($p<0.05$), with no effect of CHO ingestion (Table 2). During recovery in FW-T, leukocytes remained higher than resting values for both groups, but were lower ($p<0.001$) in boys compared with men. At 1 h of recovery, leukocytes were lower ($p<0.001$) during CHO-T, compared with FW-T in both groups. Lymphocytes increased after exercise ($p<0.001$) during FW-T to a similar extent in both groups (Table 2). During recovery in both trials, lymphocytes were lower than resting values in men, but not in boys ($p<0.001$). CHO ingestion had no significant effect on lymphocytes after exercise or during recovery. The pattern of change in monocytes was similar among groups and trials immediately after, and 1 h after exercise (Table 2). At all time points during FW-T and CHO-T, neutrophil concentrations (Table 2) were significantly higher in the men than in the boys ($p=0.01$). In both groups, exercise induced a significant ($p<0.001$) increase in neutrophils above resting values during FW-T. During CHO-T, exercise also increased neutrophil levels above

resting in the men, but not in the boys. At 1 h after exercise in FW-T, neutrophils continued to increase in men ($p<0.05$) but remained constant in boys, compared to post-exercise values. Compared with FW-T, neutrophils recovered close to resting values in CHO-T for both groups ($p<0.001$).

Lymphocyte subsets. During both trials, the proportion of T cells remained constant in boys, but decreased in men immediately after exercise ($p<0.05$, Table 3). The proportion of NK cells increased with exercise ($p<0.01$) during FW-T in both groups, but to a smaller extent in boys than in men ($p<0.001$, Table 3). CHO ingestion attenuated the increase in NK cell proportions in boys but not in men ($p<0.05$). By 1 h after exercise, NK cell proportions were not different from resting values in both groups and trials. The proportion of NK T cells did not increase significantly in boys in either trial (Table 3). For men, the proportion of NK T cells increased above resting levels immediately after exercise during FW-T ($p<0.05$), but not during CHO-T. By 1 h of recovery in both trials, the proportions of NK T cells were not different from rest in either group. Figure 2 shows the concentrations of T, NK and NK T cells before, immediately after and 1 h after exercise. Exercise caused an increase in T cell counts above resting values ($p<0.001$) in both groups during FW-T, but not during CHO-T. Comparable to the lymphocyte response, men had a significant ($p=0.04$) suppression of T cells during recovery, whereas

boys recovered to resting values. NK cells significantly ($p<0.001$) increased above resting values in boys during FW-T, but not during CHO-T. In contrast, NK cells increased with exercise ($p<0.001$) during FW-T and CHO-T to the same extent in men. NK cells were lower ($p<0.05$) in boys than in men immediately after exercise in both trials, but recovered to a similar extent between groups and trials. NK T cell counts did not significantly change with exercise in boys, but increased above resting values with exercise ($p<0.05$) during both trials in men. NK T cells were significantly ($p=0.01$) lower in boys than in men immediately after exercise during FW-T, only. By 1 h after exercise in both trials, NK T cells had recovered to resting values in both groups. The NK T cell population, regardless of group or trial, responded much less to exercise than the NK cell population.

Cytokines. Sufficient plasma for cytokine determination was available for 9 boys and 9 men only. This subset of subjects did not differ from the main group in any physical or exercise variable presented above. CHO intake had no effect on the cytokine response to exercise in either group, and the data from both beverage trials were therefore pooled. Resting concentrations of IL-6 (Fig. 3A) were significantly ($p<0.05$) higher in the boys compared with the men. Exercise caused a significant ($p<0.05$) increase in IL-6 in the men, but not in the boys. In the men, IL-6 remained elevated ($p<0.001$) during recovery compared with pre-exercise values. In

the boys, there was less than a 1 pg/mL change in IL-6 over time. Although TNF-α levels (Fig. 3B) were higher in the boys than in the men at each time point, these differences were not statistically significant. There was no significant effect of exercise on TNF-α levels in either group.

3.5. DISCUSSION

This study was designed to determine age-associated differences in the immune response to strenuous exercise with and without CHO intake, and is the first, to our knowledge, that directly compared children and adults under the same experimental conditions. Our data provide several novel findings with respect to the immediate response to exercise and the recovery of the immune system following exercise and can be summarized as follows 1) the magnitude of the NK and NK T cell responses to exercise is considerably smaller in boys than in men, 2) boys do not experience a post-exercise suppression of lymphocytes or T cells typically observed in adults, 3) the magnitude of the IL-6 response to exercise is smaller in boys than in men, and 4) CHO supplementation attenuated the immediate exercise-induced increase in some immune counts in boys, but not in men.

It is well recognized that NK cells are mobilized during exercise in response to an increase in epinephrine (5), due to a high surface density of ß-adrenergic receptors (33). Indeed we chose to measure NK cells because they are the most responsive type of cell to exercise (6).

Although we did not measure epinephrine, the smaller NK cell response in the boys would be consistent with a smaller epinephrine response. Indirect evidence (30) suggests that children may have a lower epinephrine response to sustained exercise then adults, but we are unaware of any direct age comparison during prolonged exercise. Alternatively, the smaller NK cell response could be due to a lower density of ß-adrenergic receptors on this cell type in boys, compared with men. This possibility is supported by previous work reporting a positive correlation between age and lymphocyte ß-adrenergic receptor density (34). Regardless of the mechanism(s), the attenuated NK cell response in the boys, compared with the men, is a novel finding and requires further investigation.

Another novel aspect of the present study is the NK T cell response to exercise in children. In adults, resting concentrations of NK T cells ($CD3^{+}CD16^{+}CD56^{+}$) cells seem to constitute a relatively small (<6%) proportion of peripheral blood lymphocytes (35,36). The proportion of NK T cells measured in the present study (~10%) is slightly higher than in these previous studies, but had a distinct response to exercise, which was considerably smaller than that recorded for NK cells in both age groups. Interestingly, Søndergaard et al. (37) reported a smaller increase in NK T cells ($CD3^{+}CD16^{+}CD56^{+}$), compared with NK cells ($CD3^{-}CD16^{+}CD56^{+}$), in response to epinephrine infusion in healthy young adults, which suggests that the NK T cell population may also have a distinct response to

exercise, a hypothesis that our results corroborate. Furthermore, the magnitude of the NK T cell response was also considerably smaller in the boys than in the men regardless of trial.

Taken together, these results suggest that, compared with adults, some components of the innate immune system in children are less responsive to physiological stress. However, the functional significance of this finding remains to be determined. NK cells are an important first line of defense against viruses and tumor growth (38,39). NK T cells, although considered components of the innate immune system, appear to facilitate and direct adaptive immune responses (40,41). NK and NK T cells may, therefore, play important roles in the proposed relationship between improved resistance to infection (e.g. respiratory infections) and regular physical activity (42). However, more research is clearly needed to describe the relationship between physical activity level and immune function in growing children.

Another main finding in the present study was the lack of post-exercise suppression in the lymphocyte and T cell populations in the boys. This apparent faster recovery of lymphoid cells supports our original hypothesis and is consistent with the notion that recovery from exercise tends to be faster in children, compared with adults. Post-exercise lymphopenia is a classical response to high intensity ($\geq$ 75% $\dot{V}O_{2max}$) exercise in adults (43) and is likely mediated by a prolonged elevation of

cortisol (6). The lack of a post-exercise suppression in lymphoid cells implies that boys do not experience an “open window” period as proposed for adult men. However, interpretation of an absent post-exercise lymphopenia in the boys is complicated by a lack of multiple sampling points during recovery. It is possible that we simply missed any suppression either before or after the 60 min sampling time. Although we cannot completely discount this possibility, it is noteworthy that Shore and Shephard (16) did not find suppression of lymphocytes or T cells in their children 30 min after a 30-min bout of exercise performed at an intensity corresponding to their ventilatory threshold. Even if suppression occurred earlier (i.e., at 30 min post-exercise) in the current study, the finding that lymphocytes had returned to resting levels in the boys, but not in the men, by 60 min post-exercise adds to the notion that children recover from exercise more quickly than adults, and our data add to this phenomenon in terms of the immune system. Another limitation in our study is that we did not measure immune function *per se*, but rather changes in the number and proportion of various immune cells, thereby restricting conclusions pertaining to clinical significance. There has been considerable debate over whether exercise alters, for example, intrinsic NK cell cytotoxicity (44) and lymphocyte proliferation (45) or whether these changes in immune function simply reflect alterations in cell numbers. Unfortunately, we cannot add to this debate in a meaningful way with the

present results, but studies are now underway in our laboratory to pursue this issue.

As regards cytokines, it is well established that strenuous exercise is a powerful stimulus to increase levels of various cytokines in the peripheral circulation (9,46). The magnitude of increase in IL-6 following exercise is typically intensity-dependent (47). In contrast, TNF-α generally increases only with high intensity, long duration exercise involving a large muscle mass (48). Therefore, it was not surprising that following exercise in the current study IL-6 increased without changes in TNF-α levels in the men (Fig. 3), since this pattern has been shown previously in adult men (49). In the present study, absolute changes in the concentration of IL-6 (~0.6 pg/mL) and TNF-α (~2.3 pg/mL) following exercise were small in the boys and did not achieve statistical significance. Scheet et al. (50) and Tirakitsoontorn et al. (51) reported similarly small absolute changes for IL-6 (~1.55 pg/mL and ~1.0 pg/mL, respectively) and TNF-α (~0.4 pg/mL and ~0.3 pg/mL, respectively) following exercise in healthy children, which did reach statistical significance. An obvious question, therefore, is whether small changes in IL-6 and TNF-α, which may or may not reach statistical significance, are of biological significance.

Scheet et al. (50) proposed that exercise-induced increases in the inflammatory cytokines IL-6 and TNF-α may inhibit growth mediators (e.g., insulin-like growth factor-1 (IGF-1)) resulting in a catabolic environment

such as is seen in other catabolic states (e.g., trauma, burns and sepsis). In their study, a "real-life" soccer practice of moderate intensity with rest periods was used as the exercise stimulus, with apparently no control over fluid intake or previous diet and exercise. In contrast, we used an exercise model of high intensity, prolonged cycling to impart significant stress on the immune system. With this approach, and under well-controlled study conditions, we still found only minor changes in IL-6 and TNF-α. It would therefore appear that young children are relatively resistant to a major inflammatory response to acute exercise and, thus, a catabolic state. However, since we did not measure circulating growth mediators (e.g., IGF-1), we cannot completely dismiss a catabolic response (e.g., a decrease in IGF-1 levels).

To our knowledge, this is the first study to investigate the effects of CHO intake on changes in immune cell counts following exercise in young (i.e., pre and early pubertal) children. Although exercise performed without CHO intake caused significant increases in the concentration of neutrophils, lymphocytes, T cells and NK cells in the boys, there was no change in the concentration of these cells from resting values following exercise in the trial with CHO intake. Interestingly in the men, only the proportion of NK T cells and T cell counts did not change from resting values during CHO-T. During recovery, leukocyte and neutrophil counts were lower during CHO-T than FW-T in both groups. The apparent age-

related difference in sensitivity of immune cells to CHO intake may be related to an age-related difference in the sensitivity of the stress hormone (e.g., epinephrine and cortisol) response to CHO intake during exercise. Taken together, our results imply that, with acute CHO supplementation, overall physiological stress is diminished, and studies are now underway in our laboratory to clarify possible growth-related changes in hormone-immune interactions during exercise in healthy children. The potential clinical significance of nutritional strategies such as CHO intake during exercise on immune function in children recovering from disease, for example, awaits further study.

In summary, 60 min of exercise, performed at a similar relative intensity, caused significantly smaller increases in total leukocytes, NK cells, NK T cells and IL-6 in pre- and early pubertal boys than in young adult men. The well-described post-exercise lymphopenia following high intensity exercise, which occurs in adults, was not identified in our boys. Exercise-induced changes in some elements of the immune system were more sensitive to CHO ingestion in the boys than in the men. Our results provide, for the first time, strong evidence that the magnitude and direction of exercise-induced changes in cellular components of the human immune system may be influenced by the age or maturity status of an individual. As such, this preliminary information should be considered when

interpreting "normal" leukocyte kinetics in response to exercise in children with, or recovering from, an immune-related disease.

3.6. ACKNOWLEDGMENTS

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Table 3.1 *Subject characteristics*

	Boys (n=12)	Men (n=10)
Age, yr	9.8 ± 0.1	22.1 ± 0.5*
Height, m	1.42 ± 0.03	1.77 ± 0.02*
Body weight, kg	35.1 ± 1.8	82.6 ± 2.0*
Body fat, %	15.9 ± 1.7	16.9 ± 1.1
$\dot{V}O_{2max}$, $mL \cdot kg^{-1} \cdot min^{-1}$	45.2 ± 1.2	43.8 ± 1.6
HR_{max}, beat/min	197 ± 2	194 ± 3

Values are means ± SEM. $\dot{V}O_{2max}$, maximal O_2 uptake; HR_{max}, maximal heart rate. *Significant difference between boys and men, p<0.05.

Table 3.2 *Total number of leukocytes, neutrophils, lymphocytes, and monocytes before, immediately after and 1 h after exercise in flavored water and carbohydrate trials for boys and men*

	FW-T			CHO-T		
	Pre	Post	Recovery	Pre	Post	Recovery
Leukocytes						
Boys	5.48 ± 0.36	7.34 ± 0.48*‡	6.63 ± 0.54*‡	5.42 ± 0.25*	6.57 ± 0.34*‡	5.52 ± 0.27†§
Men	6.35 ± 0.64	8.79 ± 0.72‡	8.43 ± 0.80‡	6.34 ± 0.46	8.51 ± 0.64‡	6.27 ± 0.52†§
Neutrophils						
Boys	2.48 ± 0.34*	3.49 ± 0.41*‡	3.59 ± 0.53*‡	2.43 ± 0.19*	3.01 ± 0.25*	2.53 ± 0.18*†
Men	3.67 ± 0.61	5.00 ± 0.71‡	6.01 ± 0.75‡§	3.48 ± 0.44	4.77 ± 0.57‡	3.98 ± 0.46†
Lymphocytes						
Boys	2.07 ± 0.12	2.71 ± 0.17‡	2.15 ± 0.09*§	2.06 ± 0.14	2.36 ± 0.19	2.08 ± 0.15*
Men	1.83 ± 0.12	2.63 ± 0.19‡	1.53 ± 0.10‡§	1.97 ± 0.13	2.51 ± 0.25‡	1.46 ± 0.14‡§
Monocytes						
Boys	0.37 ± 0.03	0.53 ± 0.07‡	0.43 ±0.04	0.36 ± 0.02	0.50 ± 0.04‡	0.42 ± 0.04
Men	0.50 ± 0.04	0.74 ± 0.05‡	0.58 ±-0.05§	0.51 ± 0.04	0.68 ± 0.06‡	0.53 ± 0.04§

Values are means ± SEM in 10^9 cells/L. FW-T, flavored water trial; CHO-T, carbohydrate trial. Pre, before exercise; Post, immediately after exercise; Recovery, 60 min after exercise. *Significantly different from men, $p<0.05$. †Significantly different from FW-T, $p<0.05$. ‡Significantly different from Pre, $p<0.05$. §Significantly different from Post, $p<0.05$.

Table 3.3 *Relative proportions of lymphocyte subsets before, immediately after and 1 h after exercise in flavored water and carbohydrate trials for boys and men*

	FW-T			CHO-T		
	Pre	Post	Recovery	Pre	Post	Recovery
T cells						
Boys	70.4 ± 1.4	67.4 ± 2.0	68.1 ± 1.7	69.0 ± 1.4	68.6 ± 2.2*	68.5 ± 1.4
Men	69.9 ± 2.7	63.4 ± 2.8‡	66.5 ± 4.3	69.1 ± 2.9	63.3 ± 3.2‡	70.0 ± 3.1§
NK cells						
Boys	9.9 ± 0.9	15.6 ± 1.8*‡	10.2 ± 1.4§	10.2 ± 0.9	12.5 ± 1.7*†	8.0 ± 0.9§
Men	9.9 ± 2.1	20.3 ± 2.1‡	7.7 ± 1.0§	7.7 ± 0.9	17.8 ± 1.7‡	6.7 ± 0.7§
NK T cells						
Boys	10.0 ± 1.8	10.2 ± 1.6*	9.1 ± 1.7	10.9 ± 2.6	12.9 ± 3.1	8.7 ± 1.3
Men	10.4 ± 2.2	16.3 ± 3.6‡	10.4 ± 1.8§	7.4 ± 1.2	12.8 ± 3.0	9.6 ± 2.8

Values are means ± SEM given in percentage. FW-T, flavored water trial; CHO-T, carbohydrate trial. Pre, before exercise; Post, immediately after exercise; Recovery, 60 min after exercise; *Significantly different from men, $p<0.05$. †Significantly different from FW-T, $p<0.05$. ‡Significantly different from Pre, $p<0.05$. §Significantly different from Post, $p<0.05$.

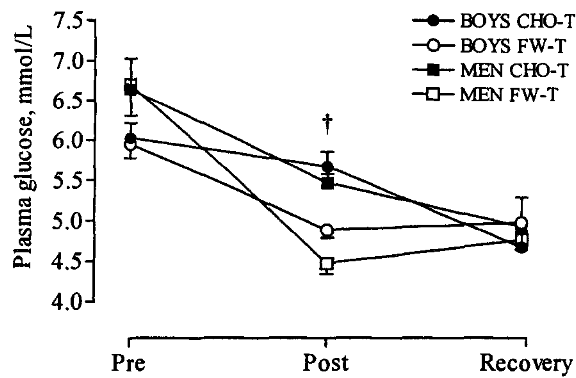

Figure 3.1 Plasma glucose concentration before, immediately after and 1 h after exercise in flavored water and carbohydrate trials for boys and men. Values are mean ± SEM. Pre, before exercise; Post, immediately after exercise; Recovery, 60 min after exercise. †Significant difference between FW-T and CHO-T for boys and men, $p<0.05$.

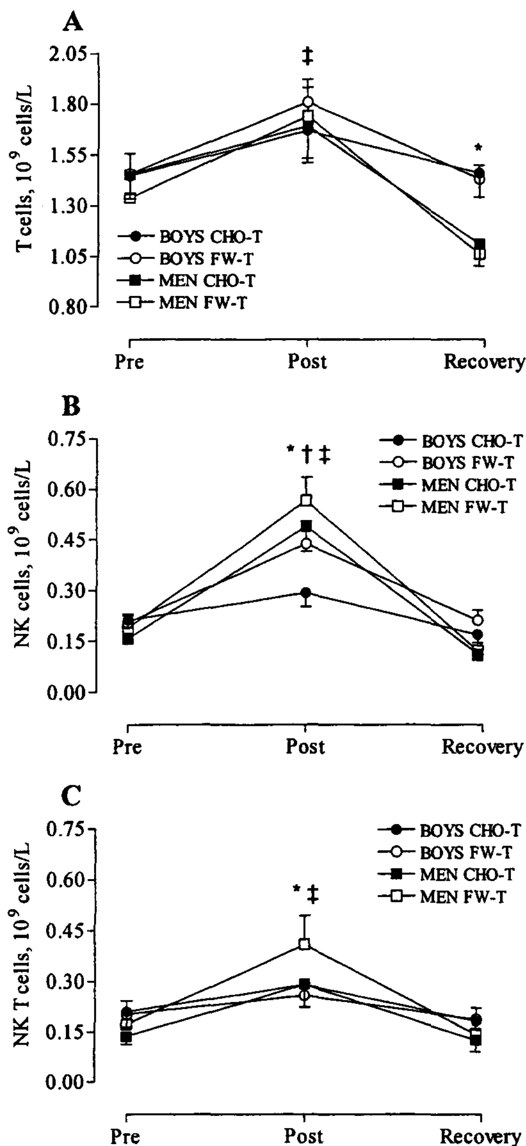

Figure 3.2 Blood concentrations of T cells **(A)**, NK cells **(B)**, and NK T cells **(C)** before, immediately after and 1 h after exercise in flavored water and carbohydrate trials for boys and men. Values are means ± SEM. Pre, before exercise; Post, immediately after exercise; Recovery, 60 min after exercise. *Significant difference between boys and men, $p<0.05$. †Significant difference between FW-T and CHO-T, $p<0.05$. ‡Significantly different than Pre, $p<0.05$. See RESULTS for details of comparisons.

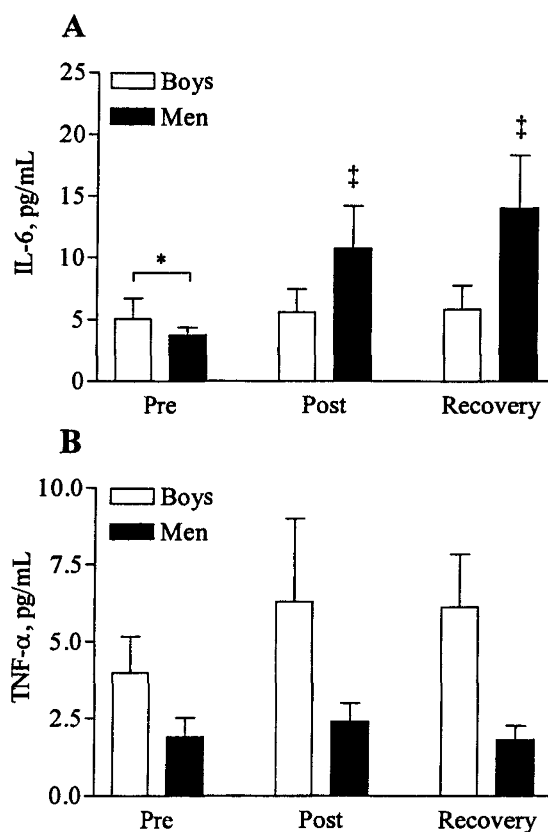

Figure 3.3 Plasma IL-6 (**A**) and TNF-α (**B**) concentrations before, immediately after and 1 h after exercise in flavored water and carbohydrate trials for boys (n=9) and men (n=9). Values are mean ± SEM. Pre, before exercise; Post, immediately after exercise; Recovery, 60 min after exercise. *Significant difference between boys and men, $p<0.05$; ‡Significantly different than Pre, $p<0.05$.

CHAPTER 4: PUBERTY EFFECTS ON NATURAL KILLER CELL RESPONSES TO EXERCISE AND CARBOHYDRATE INTAKE IN HEALTHY BOYS

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4.1. ABSTRACT

Previous research has demonstrated that young animals and humans experience smaller perturbations in natural killer (NK) cells in response to physiological stress. We therefore hypothesized that the effects of strenuous exercise and carbohydrate (CHO) intake on NK cells, previously reported in children, would be influenced by puberty. Twenty 12-yr-old boys, distinguished as pre-pubertal (Tanner (T) 1, n=7), early-pubertal (T2, n=7) or pubertal (T3-5, n=6), cycled for 60 min @ 70% $\dot{V}O_{2max}$ while drinking 6% CHO (CT) or flavoured water (WT). Blood was collected at rest, during (30 and 60 min) and following (30 and 60 min) exercise to identify NK cells as $CD3^{-}CD56^{dim}$ or $CD3^{-}CD56^{bright}$. CD69 expression on $CD3^{-}CD56^{+}$ cells was also determined. A puberty, CHO and exercise interaction was found for the proportion, but not number, of $CD56^{dim}$ cells

($p = 0.06$). CD56^{dim} cell counts were lower in CT *versus* WT ($p < 0.001$). Responses of CD56^{bright} proportions ($p = 0.007$) and counts ($p = 0.03$) depended on pubertal status, but not CHO. The CD56^{bright}:CD56^{dim} ratio remained stable during exercise, but during recovery was higher in T1 and T3-5 *versus* T2 ($p = 0.08$) and in CT *versus* WT ($p = 0.04$). During recovery, CD3^{-}CD56^{+} cells expressed higher levels of CD69 ($p = 0.01$), with no change in the proportion of CD69^{+} cells. These results confirm the influence of puberty on the distribution of NK cell subsets in response to exercise and CHO intake. Increased CD69 expression suggests that NK cells increase activation status during recovery from physiological stress.

4.2. INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes with potent cytolytic activity (1) and play important innate roles in anti-viral (2) and anti-cancer (3) defences. The traditional phenotype of NK cells, based on cell surface markers, is the co-expression of the Fcγ receptor III (CD16) and an isoform of the human neural cell adhesion molecule (CD56) and lack of expression of the T cell receptor polymeric complex, CD3 (1). As early as 1986, however, the existence of two unique and functionally distinct NK cell populations, based on the expression intensity of CD56, was noted (4). CD3^{-}CD56^{dim} cells, which express high levels of CD16, are more cytolytic than CD3^{-}CD56^{bright} cells, which express low or no levels of

CD16 (4). Moreover, mounting evidence suggests that the CD56^{bright} subset, which comprises ~10% of NK cells, may be of particular relevance in the early events of immune challenge by co-ordinating "cross-talk" between innate and adaptive arms of immunity (5). Little is known, however, regarding the responses of CD56^{dim} and CD56^{bright} cells to various forms of physiological stress such as high-intensity exercise.

In adults (6) and in children (7-10), NK cells (i.e., CD3^{-}CD16^{+}CD56^{+}) are the most responsive cell type to exercise, and increases in NK cell counts are attenuated in adults (11) and in children (8) by ingesting a carbohydrate (CHO) drink during exercise. It has also been demonstrated that NK cells are less responsive to physiological stress in pre-pubertal *versus* mature rats (12), and we have shown that the NK cell response to strenuous exercise is lower in pre- and early-pubertal boys *versus* men and more sensitive to CHO intake in the boys (8). The majority of exercise studies, however, have not distinguished between CD56^{dim} and CD56^{bright} subset responses, and we are aware of no study reporting responses of these distinct subsets to acute exercise and CHO intake.

Therefore, the primary purpose of this study was to determine the influence of puberty on the NK cell response to exercise and CHO intake, by recruiting healthy boys at the same chronological age, but different biological ages (i.e., pubertal status). We hypothesized that the NK cell

response to exercise would be greater with progressing physical maturity and that exercise would be an effective means to assess how the CD56^{dim} and CD56^{bright} subsets respond to physiological stress. Based on our previous findings that NK cell responses to exercise were more sensitive to CHO intake in pre- and early-pubertal boys *versus* men (8), we further hypothesized that CHO effects on NK cells would be more pronounced in pre-pubertal boys.

4.3. METHODS

Subjects. Twenty 12-year-old boys volunteered for this study approved by the McMaster University Research Ethics Review Board. Pubertal status of the boys was self-assessed based on pubic hair development according to Tanner (13). Boys were classified as pre-pubertal (Tanner stage 1, n=7), early-pubertal (Tanner stage 2, n=7), or pubertal (Tanner stages 3-5, n=6). Self-assessment of pubertal status according to pubic hair development has been shown to be valid and reproducible among boys (14). We also measured serum testosterone levels (see below) as an objective indication of pubertal status. Table 1 provides subject characteristics. All subjects were healthy, recreationally active and not taking medication. After the purpose, procedures and risks of the study were explained, the boys agreed verbally to participate and their parent then signed a written informed consent.

Preliminary session. An initial visit was conducted in order to measure body height (SECA 216 Accu-Hite Stadiometer, Creative Health Products, Plymouth, MI, U.S.A.), body mass (BM; BWB-800, Tanita, Tokyo, Japan), percent body fat (bio-electric impedance-101A, RJL Systems, Clinton Twp., MI, U.S.A) and to determine Tanner staging. Maximal O_2 uptake ($\dot{V}O_{2max}$) was also determined on a cycle ergometer (Ergomedic 818E, Monark, Sweden) using a progressive, continuous exercise test modified from Bar-Or and Rowland (15). Subjects began cycling at 30 Watts, with pedaling rate constant at 60 rpm, and work rate was increased by 30 Watts every 2 minutes. A test was determined maximal when pedaling rate dropped below 50 rpm for 3 seconds, despite strong encouragement and the respiratory exchange ratio was greater than 1.1. The highest 30-sec $\dot{V}O_2$ was taken as $\dot{V}O_{2max}$.

Experimental sessions. All subjects recorded their nutrient intake and physical activity for two days prior to their first experimental session, and these were then repeated on the two days prior to their next session. To avoid effects of circadian rhythm on immune measures, subjects arrived to the laboratory at either 0730h or 0830h on the day of testing. After ~10 min of supine rest, an indwelling venous catheter (Becton Dickinson, NJ) was placed in either an arm or a hand. After a further 10 min of supine rest, a resting, pre-exercise blood sample was drawn. Subjects then consumed a small standardized breakfast with their first

drink (12 mL/kg BM) to standardize pre-exercise nutrition. Volumes of 4 mL/kg BM were subsequently consumed at 15-min intervals throughout exercise and at 20 min into recovery. This CHO feeding schedule maintains body hydration, increase blood glucose levels, and influences NK cell responses to exercise in children (8). Thus, in one trial (CT) subjects consumed a 6% CHO-electrolyte solution (4% sucrose, 2% glucose, ~18 mmol/L Na^+, ~3 mmol/L K^+) and in another trial (WT), water (identical in flavour, sweetness and electrolyte concentration, but without CHO) for a total of 40 mL/kg BM. Forty minutes after the resting blood sample, subjects began cycling (Monark) at a power output equivalent to 70% of their predetermined $\dot{V}O_{2max}$. Exercise consisted of two 30-min bouts separated by a 5-7 min rest period. Additional blood samples were collected after 30 and 60 min of exercise and at 30 and 60 min of recovery. Blood samples were drawn while subjects remained seated on the cycle ergometer or quietly in the laboratory. The two experimental trials were conducted 1-2 weeks apart in a double-blind and counterbalanced fashion.

Glucose analysis. Whole blood treated with EDTA was centrifuged at 2000 g for 10 min and the plasma was stored at –50°C until analyzed. Plasma glucose was measured enzymatically (2300L STAT, Yellow Springs Instruments, Ohio, U.S.A.), and concentrations were corrected for

exercise-induced changes in plasma volume (see below). In our hands, the intra- and inter-assay CVs for this assay are < 1.5%.

Cortisol, growth hormone and testosterone analyses. Whole blood sampled at rest and at 60 min of exercise was allowed to clot and centrifuged at 2000 g for 10 min. The serum was stored at –70°C until analyzed in duplicate for cortisol and growth hormone (GH) using commercially available RIA kits (Diagnostic Products Corporation, CA). In our hands, the intra- and inter-assay CVs, respectively, are ≤ 2.5 and 8% for cortisol and ≤ 5 and 12% for GH, and post-exercise concentrations were corrected for exercise-induced changes in plasma volume (see below). Only resting serum samples from both experimental sessions were assayed in duplicate for total testosterone using a commercially available RIA kit (DPC, CA). In our hands, the intra- and inter-assay CVs, respectively, for this assay are ≤ 2 and 7%. The average resting testosterone value from both sessions was then considered as an objective estimate of pubertal status.

Catecholamine analysis. Whole blood collected at rest and at 60 min of exercise was treated with EGTA and reduced glutathione, centrifuged at 2000 g for 10 min and the plasma was stored at –70°C until analyzed for epinephrine (EPI) and norepinephrine (NEPI). Plasma catecholamines were analyzed by high performance liquid chromatography with electrochemical detection as previously described

(16). Recovery rates of catecholamines from plasma ranged between 80-85% and the intra-class correlation of this procedure is 0.96, representing very high reliability. All post-exercise concentrations were corrected for exercise-induced changes in plasma volume (see below).

Lymphocytes and NK cell subsets. Total lymphocyte counts were determined in whole blood treated with EDTA using an automated Coulter counter. Hemoglobin and hematocrit were also assessed in these samples to calculate changes in blood and plasma volume according to Dill and Costill (17) and all immune cell counts were corrected for exercise-induced changes in blood volume. EDTA-treated whole blood was used to determine NK cell subsets by two- and three-color immunophenotyping. Monoclonal antibodies directly conjugated with PerCP (CD3) or PE (CD56) were mixed (10 μL each) with 100 μL of whole blood. An additional 100 μL of blood were mixed with 10 μL each of CD3 (PerCP), CD56 (FITC) and CD69 (PE). CD69 was used as a marker of activation status of circulating NK cells. Mixed samples were briefly vortexed and incubated for 20 min at room temperature (RT) in the dark. After adding 2 mL of BD Pharm Lyse™ solution to lyse red blood cells, samples were vortexed and incubated a further 10 min at RT. Samples were centrifuged (300 g for 5 min at RT), washed with 2 mL of BD Pharmingen™ stain buffer, centrifuged (200 g for 5 min at RT) and fixed with 0.5 mL of BD Cytofix™ buffer. Samples were stored at 3°C for no more than 48 h before

run on a FACScan flow cytometer (Becton Dickinson, Mississauga, Canada) with CELLQuest software. A total of 10 000 events were collected in the lymphocyte gate based on forward (FSC)- *versus* side-scatter (SSC) characteristics. Figure 1 outlines the gating procedures for the proportions of NK cell subsets, which were analyzed "off-line" with WinMDI 2.8 software (Joseph Trotter, The Scripps Research Institute, CA). The lymphocyte population was gated using FSC *versus* SSC characteristics and a dot plot of CD3 and CD56 fluorescence was created from events within this lymphocyte gate. The expression of CD69 was determined in a similar fashion by creating a dot plot of CD3 and CD56 created from events within the lymphocyte gate. A histogram of CD69 fluorescence was then created from events within the gated $CD3^-CD56^+$ cell population. Because the expression of CD69 was relatively low, no attempt was made to distinguish CD69 expression on $CD56^{dim}$ and $CD56^{bright}$ cells. Cell counts of each NK cell subset were calculated by multiplying the percentage of cells with appropriate fluorescence with the absolute lymphocyte count. Blood for CD69 analysis was collected at rest, after 60 min of exercise and after 60 min of recovery only. The proportion of $CD3^-CD56^+$ cells expressing CD69 and the median fluorescence intensity (MFI) of $CD69^+$ cells were determined. To facilitate inter-subject comparisons, post-exercise and recovery CD69 MFIs were expressed as a percentage of the pre-exercise value.

Statistical analyses. Data are presented as means ± SEM, unless stated otherwise. Group differences in physical and fitness characteristics were analyzed by one-way ANOVA. Three-way mixed-factorial ANOVAs with one between factor (pubertal group) and two within factors (trial and time) were used to analyze glucose, hormone, and immune cell proportions and counts. Where appropriate, a Tukey's post-hoc test was used to determine significance among means. Pearson correlations were performed to determine associations between hormone concentrations and NK cell subsets. STATISTICA 5.0 (StatSoft, Tulsa, OK) was used for ANOVAs, and GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) was used for correlation analyses. The threshold for statistical significance was set at $p \leq 0.05$.

4.4. RESULTS

Plasma glucose (Figure 2). Glucose concentrations at rest were similar between groups and trials. Post-exercise glucose was higher in CT *versus* WT in all groups (trial × time, $p < 0.001$) and remained higher at 30 min of recovery.

Catecholamines, cortisol and GH (Table 2). EPI increased with exercise (time effect, $p < 0.001$), with no influence of pubertal group or trial. There was a group × trial × time interaction ($p = 0.03$) for NEPI. NEPI increased with exercise in T1 and T2 in both CT and WT, whereas T3-5

increased NEPI levels only in CT. Cortisol decreased over time (time effect, $p < 0.001$) with no influence of pubertal group or trial. There were group × time ($p = 0.02$) and trial × time interactions ($p < 0.001$) for GH. T3-5 and T1 boys increased GH levels with exercise, but T2 boys did not, and post-exercise GH levels were lower in CT *versus* WT.

Lymphocytes (Table 3). Circulating lymphocyte counts increased with exercise for all groups in both trials (time effect, $p < 0.001$). The increase in WT, however, tended to be greater than in CT (trial × time interaction, $p = 0.08$). In both trials, lymphocyte counts remained below resting levels at 30 and 60 min of recovery.

NK cell subsets. Consistent with the literature, the $CD56^{dim}$ subset, pooled across pubertal groups and trials, comprised 89 ± 1% of total $CD3^{-}CD56^{+}$ cells under resting conditions. However, the $CD56^{dim}$ subset comprised a slightly greater proportion of total $CD3^{-}CD56^{+}$ cells in boys at T2 (93 ± 1%), compared with boys at T1 (86 ± 2%) and T3-5 (87 ± 2%) (group effect, $p = 0.01$).

A strong trend for a 3-way interaction was found for $CD56^{dim}$ cells as a proportion of lymphocytes (group × trial × time interaction, $p = 0.06$, Figure 3). Differences in cell proportions between WT and CT at the same time point were evident in T1 by 30 min of exercise whereas in T3-5 inter-trial differences were only evident by 60 min of exercise and not at all in T2. In both trials, cell proportions had returned to resting levels by 60 min

in T1, whereas in T2 and T3-5 values at 30 and 60 min of recovery remained below resting levels in CT.

Although there was no 3-way interaction for CD56^{dim} cell counts (group × trial × time interaction, $p = 0.23$), a significant effect of CHO intake was found (trial × time interaction, $p < 0.001$, Figure 3). Values at 30 and 60 min of exercise but not at 30 or 60 min of recovery were lower in CT *versus* WT. In WT, values at 30 and 60 min of recovery were not different than rest, whereas in CT the 30 and 60 min recovery values were below resting levels.

CHO intake did not influence exercise-induced changes in CD56^{bright} cells, but there was a significant group × time interaction for both their proportion and number ($p = 0.007$ and $p = 0.03$, respectively, Figure 4). With respect to cell proportions, values for both T1 and T2 did not change over time, whereas T3-5 had higher values at 60 min of exercise *versus* rest. With respect to cell counts, values for T2 did not change over time. Only at 60 min of exercise were values significantly higher than at rest in T1, whereas T3-5 demonstrated higher values at 30 and 60 min of exercise *versus* rest. In all pubertal groups, recovery of CD56^{bright} cell counts was complete by 30 min.

To highlight the influence of exercise on the relationship between the CD56^{bright} and CD56^{dim} subsets, the ratio of CD56^{bright} to CD56^{dim} cells was calculated (Figure 5). This ratio remained stable during exercise in all

groups, but tended to increase during recovery more in T1 and T3-5 than in T2 (group × time interaction, $p = 0.08$). This ratio was also higher during recovery in CT *versus* WT (trial × time interaction, $p = 0.04$).

$CD69^+$ cells (Figure 6). CD69 levels were undetectable in two of the 20 subjects (both T2) and data for the remaining 18 boys are reported. There was no effect of group, exercise, or CHO intake on the proportion of $CD3^-CD56^+$ cells expressing CD69. However, the number of circulating $CD3^-CD56^+CD69^+$ cells increased with exercise and returned to resting levels by 60 min of recovery (time effect, $p < 0.001$), with no effect of pubertal group or CHO intake. The MFI of $CD69^+$ cells, as a percentage of pre-exercise values, was not influenced by pubertal group or CHO intake, but was greater at 60 min of recovery, compared with after 60 min of exercise and rest (time effect, $p = 0.01$). The absolute MFI of $CD69^+$ cells at rest, after 60 min of exercise and after 60 min of recovery were 16.3 ± 0.8, 16.9 ± 0.8, and 19.6 ± 1.0 fluorescence intensity units, respectively (time effect, $p < 0.001$).

Correlations. When data from all subjects were considered, resting testosterone levels did not correlate with any of the NK cell-related responses to exercise with or without CHO intake. However, GH concentrations at 60 min of exercise in both trials correlated with the proportion of $CD56^{dim}$ ($r = 0.49$, $p = 0.002$) and $CD56^{bright}$ cells ($r = 0.31$, $p = 0.055$) at the same time point.

4.5. DISCUSSION

This study investigated the influence of puberty on NK cell responses to exercise and CHO intake in healthy boys. The novelty of this work is the distinction between responses of $CD56^{dim}$ and $CD56^{bright}$ subsets in boys at the same chronological age, but varying biological ages (i.e., pubertal status). The main findings are that: 1) boys at more advanced stages of physical maturity demonstrated larger increases in the proportion of $CD56^{dim}$ and $CD56^{bright}$ cells; 2) the $CD56^{bright}$:$CD56^{dim}$ ratio during recovery was lowest in early-pubertal boys and highest with CHO intake; 3) CHO intake attenuated exercise-induced increases in the $CD56^{dim}$ subset, but not in the $CD56^{bright}$ subset; and 4) circulating $CD3^{-}CD56^{+}$ cells during recovery, compared with rest, expressed higher levels of the activation marker, CD69.

NK cells are the most responsive cell type to exercise due to their high surface density of β_2-adrenergic receptors (18). Thus, NK cells are rapidly mobilized into the peripheral circulation by a catecholamine-induced down-regulation of adhesion molecule expression (19). However, in the current study, boys at the most advanced stages of puberty (T3-5) demonstrated the greatest increase in the proportion of $CD56^{dim}$ during WT (Figure 3) in the absence of differences in EPI responses and with lower NEPI responses compared with the other pubertal groups (Table 2). T3-5 boys also had the greatest increases in the proportion of $CD56^{bright}$

cells (Figure 4). This maturity-related effect is consistent with our previous findings in 9- and 10-yr-old boys who exhibited a smaller increase in $CD3^{-}CD16^{+}CD56^{+}$ cell proportion, compared with men (8). Boas et al. (20) also found that increases in $CD3^{-}CD16^{+}CD56^{+}$ cell counts following 30-s of "all-out" cycling were smallest in pre-pubertal and largest in post-pubertal boys. Smaller NK cell response in pre- and early-pubertal individuals without differences in catecholamine responses could be due to a lower density of β-adrenergic receptors on NK cells; a possibility supported by a previously reported positive correlation between age and lymphocyte β-adrenergic receptor density (21). However, since boys in the current study were at the same chronological age, expression and/or activity of β-adrenergic receptors may also vary with pubertal status. Indeed, NK cells of prepubescent rats, compared with mature animals, are relatively resistant to β-adrenergic stimulation (12), but to our knowledge similar findings in humans are lacking.

Post-exercise concentrations of GH were positively correlated with the proportions of $CD56^{dim}$ and $CD56^{bright}$ cells at the same time point. Noteworthy is that GH induces T-cell migration *in vitro* (22) and that children express lower levels of GH receptors on $CD2^{+}$ lymphocytes (i.e., T and NK cells) than do adults (23). Whether GH receptor expression and/or activity on NK cells vary with puberty *per se* is unknown. A combination of smaller GH responses to exercise and lower GH receptor

expression and/or activity on NK cells may, therefore, contribute to smaller exercise-induced changes in their relative proportion in pre- and early-pubertal children.

In contrast to the relative proportion, the absolute number of $CD56^{dim}$ cells responding to exercise did not vary statistically by pubertal group due to a slightly greater increase in total lymphocyte counts in pre- and early-pubertal boys *versus* pubertal boys (Table 3). Thus, smaller perturbations in the distribution of $CD56^{dim}$ cells in pre- and early-pubertal boys were offset by a more pronounced overall lymphocytosis. However, $CD56^{bright}$ cell counts retained a graded pubertal effect (Figure 4). Given that the $CD56^{dim}$ subset is the more cytolytic NK cell subset (4), an overall greater lymphocytosis would serve to maintain cytolytic activity in the face of physiological stress. The differential findings of cell proportions and counts for the $CD56^{dim}$ subset also highlight the need to report both these aspects of cell responses to exercise.

Suppression of NK cell function during recovery from high-intensity exercise has been termed the “open window”; a period of time when the host may be at increased susceptibility to infection (24). A recent study (25) tracked changes in $CD56^{dim}$ and $CD56^{bright}$ cells over one month of competitive sports training in healthy women and found that when NK cell cytolytic activity was lowest $CD56^{bright}$ cell counts were highest and $CD56^{dim}$ cells remained unchanged (i.e., when $CD56^{bright}$:$CD56^{dim}$ ratio

was highest). In the current study, the CD56^{bright}:CD56^{dim} ratio remained unchanged during exercise, but was highest during recovery (Figure 5). Thus, the current and previous findings support the hypothesis that reduced NK cell function during recovery from high-intensity acute exercise (24) and periods of training (25) may be due to disproportionate changes in NK cell subsets. Our findings are, however, limited because we did not measure NK cell function *per se*. We therefore suggest that future exercise studies that include assays of NK cell cytolytic activity should account for the distribution of CD56^{dim} and CD56^{bright} cells to examine this possibility.

In adults, CHO intake during prolonged exercise attenuates increases in NK cell counts (26-28). CHO intake also attenuates the rise in the proportion and number of NK cells during shorter duration exercise in 9- and 10-yr-old boys (8). Nieman proposed that CHO-mediated effects on NK cell redistribution are due to a blunted stress hormone (e.g., EPI and cortisol) response mediated by maintained or increased blood glucose concentrations (11). In the current study, CHO intake attenuated the increase in the relative proportion of CD56^{dim} cells earlier in the pre-pubertal boys, compared with more physically mature boys (Figure 3), in the absence of higher plasma glucose concentrations (Figure 2). Moreover, when plasma glucose levels during exercise were higher and CD56^{dim} proportions lower in CT *versus* WT, EPI and cortisol levels were

not different between trials. Taken together, these findings question the proposed roles of blood glucose levels *per se* and changes in stress hormones as mediators of the redistribution of NK cells observed with exercise and CHO intake, at least in children. Furthermore, the $CD56^{bright}$ subset was resistant to the effects of CHO intake suggesting that factors mediating CHO effects on NK cell responses to exercise are specific to the $CD56^{dim}$ subset. Thus, previous studies reporting CHO effects on traditional $CD3^{-}CD16^{+}CD56^{+}$ NK cells may have been confounded by not distinguishing between $CD56^{+}$ subsets.

Another novel finding in this study is the increased CD69 expression on circulating $CD3^{-}CD56^{+}$ cells during recovery from exercise. CD69 is a sensitive marker of lymphoid activation (30) and correlates with cytolytic activity of NK cells (31). Previous work in adults reported that exercise with and without CHO intake increased the number of $CD69^{+}$ NK cells (29), but the current study is the first, to our knowledge, that reports the same effect in children. Although the $CD3^{-}CD56^{+}CD69^{+}$ cell count returned to pre-exercise levels by 60 min of recovery and there were no changes in the proportion of $CD3^{-}CD56^{+}$ cells positive for the CD69 antigen, circulating $CD3^{-}CD56^{+}$ cells at this time expressed higher levels of CD69 than at rest or post-exercise. The increase in CD69 expression was a consistent finding in 15 of 18 subjects, but the biological significance of an average 22% increase in CD69 on NK cells is unknown.

However, even a modest increase in CD69 expression may serve to "prime" NK cells to antagonize tumor growth and metastasis (32).

In summary, the results of this study highlight maturity-related differences in the NK cell response to high-intensity exercise and the differential response of $CD56^{dim}$ and $CD56^{bright}$ NK cells to exercise and CHO intake in healthy boys. Physical maturity also mediated the effects of CHO intake on the redistribution of NK cell subsets during and following exercise. Finally, recovery from high-intensity exercise was associated with a modest increase in the expression of CD69 on $CD3^{-}CD56^{+}$ cells. Although the biological significance of these findings requires further investigation, NK cells are an important first line of defence against tumor growth, and the unique immunoregulatory properties of the $CD56^{dim}$ and $CD56^{bright}$ subsets mark them as candidates for immunotherapy of cancer (1). Whether the redistribution of $CD56^{dim}$ and $CD56^{bright}$ cells in response to exercise could be of therapeutic benefit in children recovering from cancer (33), for example, remains to be determined.

4.6. ACKNOWLEDGMENTS

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Table 4.1. *Subject characteristics*

	T1	T2	T3-5
n	7	7	6
Height, m	1.48 ± 0.03*†	1.57 ± 0.06	1.58 ± 0.10
Body weight, kg	41.0 ± 7.0	46.6 ± 9.9	44.4 ± 9.8
Body fat, %	17.3 ± 8.6	20.3 ± 4.2	13.5 ± 3.8
Testosterone, nmol/L	3.0 ± 3.2*	2.8 ± 3.3*	10.0 ± 9.1
$\dot{V}O_{2max}$, L/min	1.84 ± 0.16	2.05 ± 0.35	2.15 ± 0.46
$\dot{V}O_{2max}$, $mL \cdot kgBM^{-1} \cdot min^{-1}$	46 ± 8	45 ± 6	48 ± 4
HR_{max}, beats/min	197 ± 6	196 ± 8	203 ± 10

Values are means ± SD. T1, Tanner stage 1 (pre-pubertal); T2, Tanner stage 2 (early-pubertal); T3-5, Tanner stages 3-5 (pubertal); *n*, number of subjects; $\dot{V}O_{2max}$, maximal O_2 uptake; BM, body mass; HR_{max}, maximal heart rate. *Significantly different from T3-5, $p < 0.05$. †Significantly different from T2, $p < 0.05$.

Table 4.2. *Stress hormone concentrations before, during and after exercise in carbohydrate and water trials in boys of different pubertal stages.*

	CT			WT		
	Pre	Post	Recovery	Pre	Post	Recovery
			Epinephrine, pg/mL[(1)]			
T1	41 ± 8	111 ± 29	ND	40 ± 10	156 ± 32	ND
T2	27 ± 7	127 ± 24	ND	22 ± 5	115 ± 28	ND
T3-5	26 ± 7	152 ± 36	ND	33 ± 10	134 ± 32	ND
			Norepinephrine, pg/mL[(1,4)]			
T1	294 ± 14	907 ± 137‡	ND	291 ± 35	870 ± 131‡	ND
T2	234 ± 36	630 ± 117	ND	218 ± 41	725 ± 142‡	ND
T3-5	208 ± 36	960 ± 221‡	ND	287 ± 63	389 ± 67§	ND
			Cortisol, nmol/L[(2)]			
T1	551 ± 51	392 ± 73	285 ± 33	553 ± 86	416 ± 90	300 ± 61
T2	563 ± 82	370 ± 57	256 ± 41	604 ± 66	351 ± 78	316 ± 59
T3-5	523 ± 61	404 ± 55	270 ± 24	584 ± 43	312 ± 31	285 ± 17
			Growth Hormone, ng/mL[(1,3,5)]			
T1	1.0 ± 0.5	4.1 ± 1.0	ND	1.0 ± 0.4	6.5 ± 0.9	ND
T2	0.6 ± 0.1	2.0 ± 0.4	ND	0.6 ± 0.1	3.8 ± 0.9	ND
T3-5	0.7 ± 0.1	7.0 ± 2.7	ND	0.7 ± 0.1	11.4 ± 2.2	ND

Values are means ± SEM. CT, carbohydrate trial; WT, water trial; Pre, 40 min before exercise; Post, after 60 min of exercise; Recovery, 60 min after exercise; ND, not determined. [1]Main effect for time (Post > Pre), $p < 0.05$. [2]Main effect for time (Post < Pre; Recovery < Pre), $p < 0.05$. [3]Main effect for trial (CT < WT), $p < 0.05$. [4]Interaction effects: ‡significantly different from Pre within same trial; §significant difference between CT and WT, $p < 0.05$. [5]Interaction effects: Post > Pre in T1 and T3-5, but not in T2, $p < 0.05$; T1 and T2 < T3-5 at Post, $p < 0.05$.

Table 4.3. *Total lymphocyte counts before, during and after exercise in carbohydrate and water trials in boys of different pubertal stages.*

	CT					WT				
Exercise time, min	–40	30	60	+30	+60	–40	30	60	+30	+60
T1	2.1 ± 0.1	2.6 ± 0.2	2.4 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	2.1 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	1.7 ± 0.0	1.7 ± 0.1
T2	2.2 ± 0.1	2.6 ± 0.2	2.6 ± 0.2	1.9 ± 0.2	2.0 ± 0.2	2.2 ± 0.1	2.7 ± 0.3	2.8 ± 0.4	1.8 ± 0.2	1.9 ± 0.2
T3-5	1.8 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	1.5 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	2.5 ± 0.3	2.5 ± 0.2	1.8 ± 0.1	1.7 ± 0.1

Values are means ± SEM in cells · 10^9/L. Main effect time (30 and 60 > –40; +30 and +60 < –40), $p < 0.05$. Trial × time interaction (CT < WT at 60), $p = 0.08$.

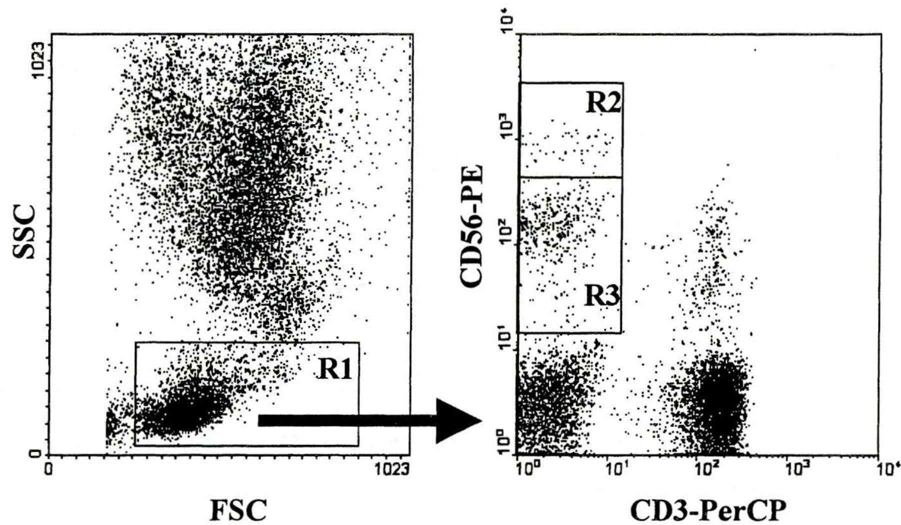

Figure 4.1. Flow cytometric analysis of $CD56^{+}$ natural killer cells in whole blood. *Left panel:* Gated lymphocyte population (R1) based on forward- (FSC) *versus* side-scatter characteristics. *Right panel:* $CD3^{-}CD56^{bright}$ (R2) and $CD3^{-}CD56^{dim}$ (R3) cell populations derived from events within the lymphocyte gate.

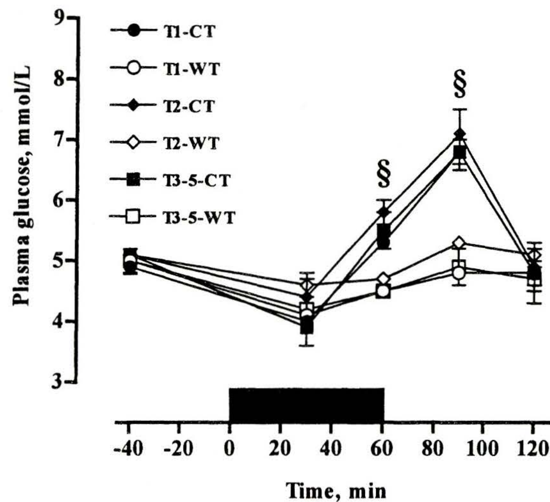

Figure 4.2. Plasma glucose concentrations before, during and after exercise in water and carbohydrate trials in boys of different pubertal status. Values are means ± SEM. Shaded box represents exercise disregarding 5-min rest. T1, Tanner stage 1; T2, Tanner stage 2; T3-5, Tanner stages 3-5; CT, carbohydrate trial (closed symbols); WT, water trial (open symbols). §Significant difference between CT and WT, $p < 0.05$.

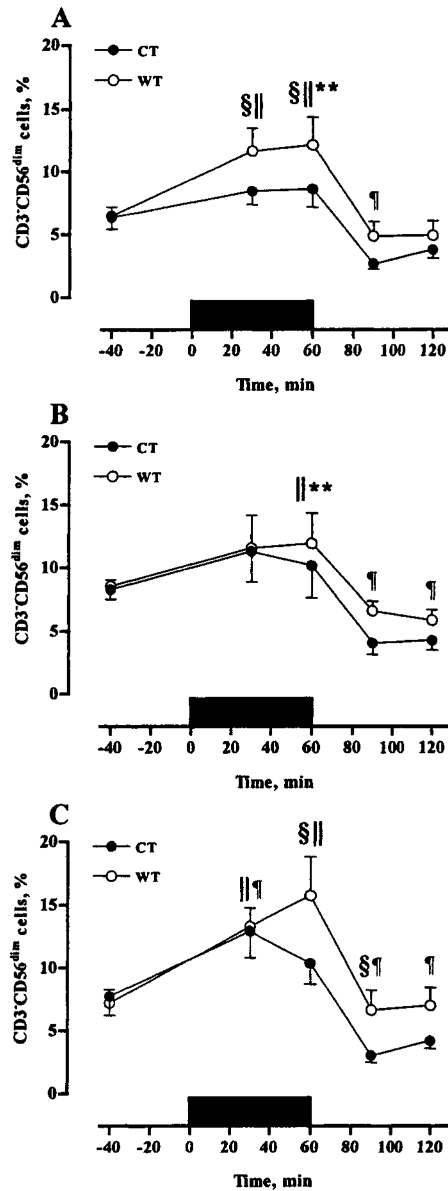

Figure 4.3. CD3⁻CD56^{dim} cell proportions before, during and after exercise in water and carbohydrate trials in pre-pubertal (**A**), early-pubertal (**B**) and pubertal (**C**) boys. Values are mean ± SEM. Shaded boxes represent exercise disregarding 5-min rest. §Significant difference between CT and WT, $p < 0.05$. ‖Significantly different from –40 min within WT, $p < 0.05$. ¶Significantly different from –40 min within CT, $p < 0.05$. **Significantly different than T3-5 in WT, $p < 0.05$.

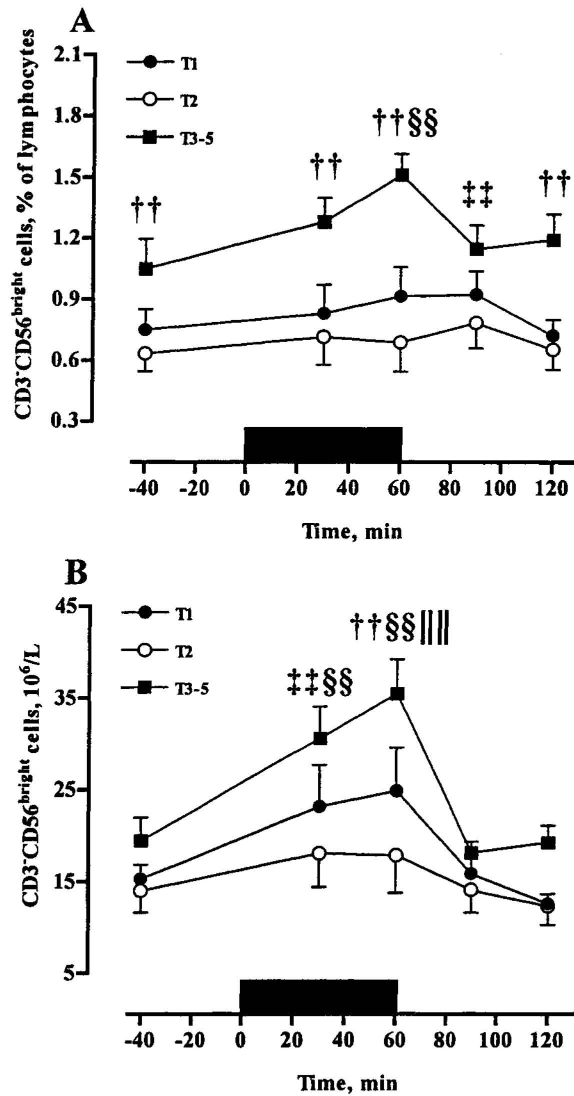

Figure 4.4. CD3⁻CD56^{bright} cell proportions (**A**) and counts (**B**) before, during and after exercise in boys of different pubertal status. Values are mean ± SEM. Shaded boxes represent exercise disregarding 5-min rest. ††T3-5 significantly different from T1 and T2, $p < 0.05$. ‡‡T3-5 significantly different from T2, $p < 0.05$. §§Significantly different from –40 min in T3-5, $p < 0.05$. || ||Significantly different from –40 min in T1 and T3-5, $p < 0.05$.

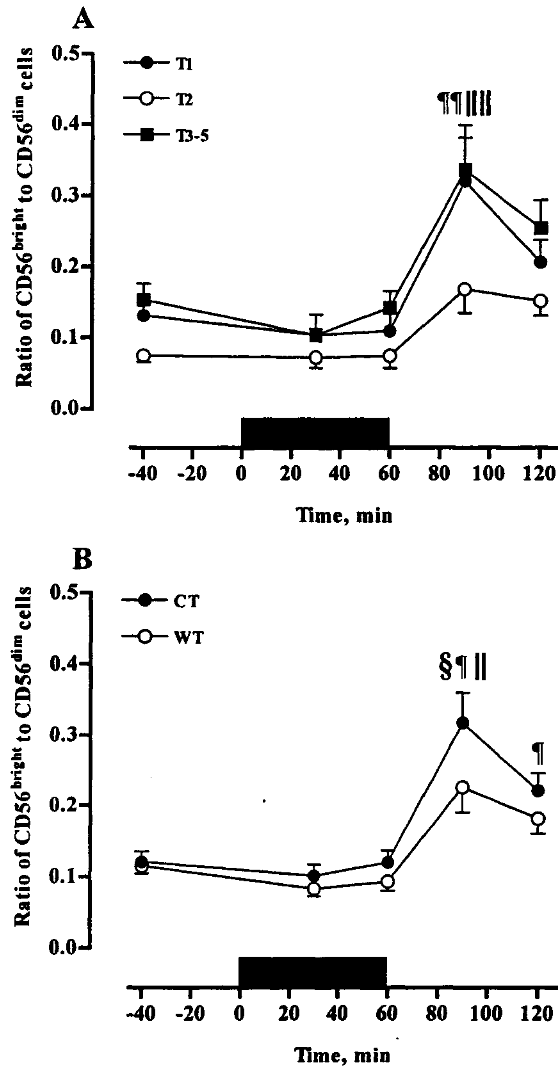

Figure 4.5. Ratio of CD3⁻CD56^{bright} cells to CD3⁻CD56^{dim} cells before, during and after exercise in boys of different pubertal status (**A**) and in carbohydrate and water trials (**B**). Values are mean ± SEM. Shaded boxes represent exercise disregarding 5-min rest. §Significant difference between CT and WT, $p < 0.05$. ||Significantly different from –40 min in WT, $p < 0.05$. ¶Significantly different from –40 min in CT, $p < 0.05$. || ||Significantly different from –40 min in T1 and T3-5, $p < 0.05$. ¶¶T1 and T3-5 significantly different from T2, $p < 0.05$.

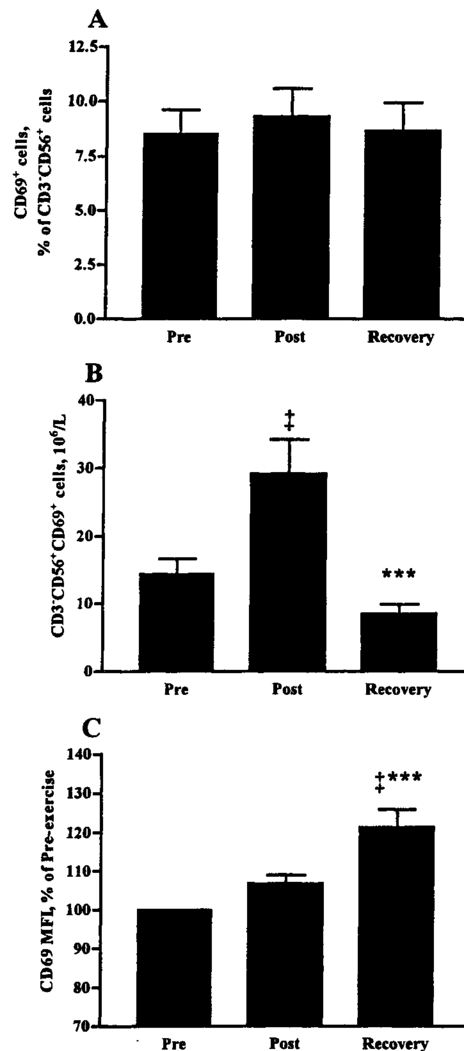

Figure 4.6. Expression of CD69 on circulating $CD3^-CD56^+$ cells: Proportion of $CD3^-CD56^+$ cells expressing CD69 (**A**); $CD3^-CD56^+CD69^+$ cell counts (**B**); and median fluorescence intensity (MFI) of CD69 on $CD3^-CD56^+$ cells as a percentage of pre-exercise values (**C**). Values are mean ± SEM. Pre, 40 min before exercise; Post, after 60 min of exercise; Recovery, 60 min after exercise. ‡Significantly different from Pre, $p < 0.05$. ***Significantly different from Post, $p < 0.05$.

CHAPTER 5: IMMUNOLOGICAL CHANGES IN RESPONSE TO EXERCISE: INFLUENCE OF AGE, PUBERTY AND GENDER

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5.1. ABSTRACT

PURPOSE: This study tested the hypothesis that exercise-induced perturbation and recovery of the immune system would vary with age, puberty and gender in healthy children and adolescents. **METHODS:** Twelve-yr-old girls (YG; n=14) and boys (YB; n=20) and 14-yr-old girls (OG; n=11) and boys (OB; n=13) cycled for 60 min @ 70% $\dot{V}O_{2max}$. Blood was collected before, at 30 and 60 min of exercise and at 30 and 60 min of recovery to measure total leukocytes, leukocyte and lymphocyte subsets, and cytokines. Age and pubertal (Tanner stage) effects within genders and gender effects within age and pubertal groups were determined. **RESULTS:** Exercise-induced increases in lymphocytes, $CD3^-CD16^+CD56^+$ counts, and IL-6 were ~83, ~90, and ~390% greater in OG *versus* YG ($p < 0.05$). Recovery leukocytosis and neutrophilia were

~56 and ~35% greater in OB *versus* YB ($p < 0.05$). Pubertal stage did not have a statistically significant influence on responses in girls, but the lowest pubertal stage consistently showed smaller changes in lymphocytes and $CD3^-CD16^+CD56^+$ counts. Recovery neutrophilia was ~120% greater in post-pubertal boys *versus* pre-pubertal or pubertal boys ($p < 0.05$). Responses of lymphocytes and $CD3^-CD16^+CD56^+$ counts, respectively, were ~120 and ~82% greater in OG *versus* OB ($p < 0.05$), with no differences between YG and YB. Exercise-induced increases in total leukocytes, lymphocytes, and $CD3^-CD16^+CD56^+$ counts were at least 35% greater in girls *versus* boys of similar pubertal status ($p < 0.05$). Regardless of age, puberty or gender, IL-8 levels were significantly higher during recovery *versus* rest ($p < 0.05$). **CONCLUSION:** These results highlight the need to control for age, puberty and gender when interpreting immunological responses to exercise in a pediatric population.

5.2. INTRODUCTION

Physical inactivity among North American children and adolescents is cause for concern with a persistent increase in the prevalence of childhood obesity (e.g., 29) and an increased occurrence of adult-like health conditions such as Type 2 diabetes mellitus (14). In this regard, the immune system is intimately linked to obesity and its associated complications (8), but to what extent the early onset of these adult-like

disorders during childhood is linked to immune development is unknown. Indeed, the immune system undergoes considerable development in both cell number and function throughout childhood (3) and adolescence (4). Moreover, physical activity is a potent stimulus to the immune system in both adults (20) and children (e.g., 11). That regular exercise during childhood may influence normal growth and development of the immune system, and thus etiology of disease, is of particular interest. However our understanding of even basic immunological changes in response to acute exercise during childhood and adolescence and of how these responses may vary with age, puberty and gender is deficient.

In adults, the effects of acute exercise on immune function have received considerable attention (20). Whereas strenuous, prolonged exercise often causes a post-exercise suppression of immune function, exercise of moderate intensity and duration tends to enhance several aspects of immunity (20). Similar exercise-induced perturbations to the immune system of children and adolescents, however, may carry health implications unique to the growing individual. We have recently reported that under identical exercise conditions, changes in some cellular and soluble components of the immune system were smaller in pre- and early-pubertal boys *versus* adult men (27). Further, recovery of immune perturbations following strenuous exercise was faster in the children than in the adults (27). Although our study was the first to compare children and

adults under identical, well-controlled experimental conditions, other investigators have reported immune changes following various forms of aerobic-type exercise in young boys and girls (6,11,21-23,28,30) and older adolescent boys and girls (6,15-17,19). No study, however, has systematically and simultaneously examined the influence of age, puberty and gender on immunological responses to exercise in a healthy pediatric population.

An improved understanding of how exercise impacts a child's immune system is of clinical importance. The safe prescription of exercise for children with, or recovering from, an immune-related disease requires the distinction between normal and abnormal exercise-induced responses. Therefore, we investigated the effects of age, puberty and gender on changes in various cellular and soluble components of the immune system in response to standardized high-intensity aerobic exercise. To minimize possible effects of previous antigenic experience on immune measures and confounding effects of age-associated factors on pubertal comparisons, children at two distinct chronological ages were tested. Based on our previous observations (27), we hypothesized that perturbations to, and recovery of, the immune system would be smaller and faster, respectively, in younger *versus* older children and in less mature *versus* more mature individuals. Given the reported gender differences in components of the immune system among children (3) and

adolescents (4), we further hypothesized that gender differences would exist in the immunological changes induced by exercise.

5.3. METHODS

Study design and Subjects. A total of 58 subjects volunteered to participate in this study approved by the McMaster University Research Ethics Review Board. We recruited healthy 12- and 14-yr-old boys (n=33) and girls (n=25) who were at various stages of puberty. All subjects performed a preliminary session and an experimental session. By design, we made separate age and pubertal comparisons within each gender and gender comparisons within age and pubertal groups. Pubertal status of each subject was determined by self-assessment of pubic hair development (boys) or breast development (girls) according to Tanner (25). Self-assessment of pubertal status according to development of pubic hair in boys and breasts in girls has been shown to be valid and reproducible (13). Table 1 provides subject characteristics according to age and pubertal status. All 14-yr-old girls maintained regular menstrual cycles and were not taking oral contraceptive therapy (OCT), whereas three 12-yr-old girls reported having had their first menses, but had not yet developed regular cycles. The 14-yr-old girls were tested in the mid-follicular phase of their menstrual cycle. For the 12-yr-old girls who had reached menarche, no attempt was made to test them at a particular time because of the sporadic nature of their cycles. All subjects were healthy

with no recent allergies or illness and none were taking medication. After the purpose, procedures and risks of the study were explained, the children agreed verbally to participate and a parent then signed a written informed consent.

Preliminary session. A preliminary visit was conducted to measure body height (SECA 216 Accu-Hite Stadiometer, Creative Health Products, Plymouth, MI, U.S.A.), body mass (BWB-800, Tanita, Tokyo, Japan), and percent body fat (bio-electric impedance-101A, RJL Systems, Clinton Twp., MI, U.S.A) and for determination of Tanner stage. Maximal O_2 uptake ($\dot{V}O_{2max}$) was determined on a cycle ergometer (Ergomedic 818E, Monark, Sweden) using a progressive, continuous exercise test. Subjects began cycling at either 30 or 60 Watts depending on age and estimated fitness level, with pedaling rate constant at 60 rpm. Work rate was increased by 30 Watts every 2 minutes for all subjects. A test was determined maximal when pedaling rate dropped below 50 rpm for 3 seconds, despite strong encouragement and the respiratory exchange ratio was greater than 1.1. Other maximal criteria related to heart rate (HR) and $\dot{V}O_2$ were not used because equations of age-predicted maximum HR are not accurate during childhood and a plateau in $\dot{V}O_2$, despite increasing exercise intensity, is not consistently observed in children. During the test, HR was continuously monitored with a Polar HR monitor (Polar A1, Polar Electro, Kempele, Finland) and subjects breathed

through a Hans Rudolph valve with an appropriately-sized mouthpiece. Expired air was collected continuously and analyzed for O_2 (Beckman O_2 analyzer OM-11, Beckman Inc., CA, U.S.A.) and carbon dioxide (HP47210A capnometer, Hewlett Packard, CA, U.S.A.) with analyzers connected to a Vista PC interface with Turbofit software (VacuMed, Ventura, CA, U.S.A.) on a personal computer. The highest 30-sec $\dot{V}O_2$ was taken as the $\dot{V}O_{2max}$.

Experimental session. Subjects were instructed to maintain their habitual diets, but to avoid "fast-food"-type meals, and to avoid excessive physical activity for the two days immediately prior to their experimental trial. All subjects complied with these instructions. To minimize circadian effects on immune measures, every experimental session was conducted in the morning with subjects arriving to the laboratory at either 0730h or 0830h in at least a 10-h fasted state. Upon arrival, subjects voided their bladder, were weighed in the nude using an electronic scale (Tanita), and then rested supine for ~10 min after which time an indwelling venous catheter (Becton Dickinson, NJ) was placed in either an arm or a hand. After a further 10 min of supine rest, a resting, pre-exercise blood sample was drawn. Subjects then consumed a small standardized breakfast, which served to standardize pre-exercise nutrition. Throughout the session, subjects were given flavored water to drink at a rate to maintain body hydration (27). Forty minutes after the resting blood sample, subjects

began cycling (Monark) at a power output equivalent to 70% of their pre-determined $\dot{V}O_{2max}$, with the target intensity achieved in the first 5 min by analysis of expired gas (Beckman and Hewlett Packard). Exercise consisted of two 30-min bouts separated by a 5-7 min rest period. Additional expired gas samples were collected at steady state from minutes 11-15 and 26-30 of each exercise bout to insure the proper work intensity, with the power output adjusted accordingly. Additional blood samples were collected after 30 and 60 min of exercise and at 30 and 60 min of recovery. Blood samples were drawn while subjects remained seated on the cycle ergometer or quietly in the laboratory. The catheter was kept patent by flushing with ~1.5 mL of sterile saline (0.9% NaCl) after each blood sampling. Consequently, the first 2 mL of blood at each sampling time was discarded. During the recovery period, subjects sat quietly and were allowed to empty their bladder if necessary.

Total leukocytes and leukocyte Subsets. Whole blood treated with EDTA was analyzed for total leukocytes, neutrophils, lymphocytes, and monocytes using an automated Coulter counter at the McMaster University Medical Centre Core Laboratory. Hemoglobin and hematocrit were also assessed in these samples to calculate changes in blood and plasma volume according to Dill and Costill (10) and all immune cell concentrations were corrected for exercise-induced changes in blood volume.

Lymphocyte Subsets. EDTA-treated whole blood was used to determine lymphocyte subsets by direct immunofluorescence and flow cytometry. The following mouse anti-human monoclonal antibodies (Mab) and fluorochrome conjugates were used: CD3-PerCP, CD3-FITC, CD4-PerCP, CD8-PerCP, CD16-FITC, CD19-FITC, and CD56-PE. Lymphocyte subsets were classified as total T cells ($CD3^+$), T_{helper} cells ($CD3^+CD4^+$), $T_{cytotoxic}$ cells ($CD3^+CD8^{bright}$), B cells ($CD3^-CD19^+$) and natural killer (NK) cells ($CD3^-CD16^+CD56^+$). All reagents were purchased from BD Biosciences and samples were stained as per the manufacturer's instructions within 6 h of collection. Well-mixed whole blood (100 µL) was added to 12 × 75-mm Falcon tubes containing an appropriate cocktail of Mab (10 µL each). Samples were vortexed and incubated for 20 min at room temperature (RT) in the dark. After adding 2 mL of BD Pharm Lyse™ solution to lyse red blood cells, samples were vortexed and incubated a further 10 min at RT. Samples were centrifuged (300 g for 5 min at RT), washed with 2 mL of BD Pharmingen™ stain buffer, centrifuged (200 g for 5 min at RT) and fixed with 0.5 mL of BD Cytofix™ buffer. Samples were stored at 3°C for no more than 48 h before run on a FACScan flow cytometer (Becton Dickinson, Mississauga, Canada) with CELLQuest software. The lymphocyte population was gated using forward-scatter vs. side-scatter characteristics and 10 000 events per lymphocyte gate were collected. Analyses of lymphocyte subsets were

performed "off-line" with WinMDI 2.8 software (Joseph Trotter, The Scripps Research Institute, CA, U.S.A.). Cell counts of each lymphocyte subset were calculated by multiplying the percentage of cells with appropriate fluorescence by the absolute number of lymphocytes. Due to logistical restraints, $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{bright}$ cells were not determined in blood samples drawn at 30 min of exercise or at 30 min of recovery, whereas $CD3^{-}CD19^{+}$ cells were not determined at 30 min of recovery.

Cytokines. Whole blood treated with EDTA was centrifuged at 2 000 g for 10 min and the plasma was stored at –50°C until analyzed. ELISA kits (R&D Systems, Minneapolis, MN) were used to determine plasma concentrations of IL-6 (Cat. No. HS600B), IL-8 (Cat. No. D8000C), and TNF-α (Cat. No. HSTA00C) in duplicate. The sensitivities of these kits, as reported by the manufacturer, are 0.039 pg/mL for IL-6, 3.5 pg/mL for IL-8, and 0.12 pg/mL for TNF-α. In our hands, the intra- and inter-assay CVs, respectively, are ≤ 5% and 9% for IL-6, ≤ 4% and 10% for IL-8, and ≤ 4% and 12% for TNF-α. Cytokine were determined in blood sample collected at rest, after 60 min of exercise and after 60 min of recovery. All post-exercise cytokine concentrations were adjusted for changes in plasma volume (as above).

Statistical analyses. Data are presented as means ± SEM, unless stated otherwise. To determine group differences in physical and fitness

characteristics one-way ANOVAs were used. Separate two-way ANOVAs with one between factor (group) and one within factor (time) were used to analyze immune cell proportions and counts and cytokine concentrations for age, puberty and gender comparisons. Where appropriate, a Tukey's post-hoc test was used to determine significance among means. STATISTICA 5.0 (StatSoft, Tulsa, OK) was used for ANOVAs. In all cases, the threshold for statistical significance was set at $p \leq 0.05$.

5.4. RESULTS

All subjects completed the exercise testing with no differences between groups in the relative exercise intensity, which averaged 68.0 ± 0.7% of $\dot{V}O_{2max}$ or 29.2 ± 0.4 mL·kg body mass^{-1}·min^{-1}.

Age effects. Table 2 provides counts of total leukocytes and leukocyte subsets in 12-yr-old girls (YG) and boys (YB) and 14-yr-old girls (OG) and boys (OB) for age and pubertal comparisons. Total leukocytes and lymphocytes, but not neutrophils or monocytes, were greater or tended to be greater in OG *versus* YG at 30 and 60 min of exercise (Interaction effect, $p = 0.08$ and $p < 0.001$, respectively). In both OG and YG, total leukocytes remained elevated during the recovery period, but lymphocytes returned to resting levels by 30 min. Neutrophils were consistently higher in OG *versus* YG throughout the session (Main effect age, $p = 0.03$). Total leukocyte and leukocyte subsets during exercise

were not significantly different between YB and OB, but at 60 min of recovery total leukocytes ($p = 0.01$) and neutrophils ($p = 0.05$) were higher in OB *versus* YB.

Table 3 provides proportions and counts of lymphocyte subsets in YG, YB, OG and OB for age and pubertal comparisons. Exercise caused reductions in the proportion of total $CD3^+$ and $CD3^+CD4^+$ cells that were greater or tended to be greater in OG *versus* YG (Interaction effect, $p = 0.08$ and $p = 0.02$, respectively). Cell proportions in OG and YG were not significantly different from resting levels at 60 min of recovery. In contrast, the proportion of $CD3^+CD8^{bright}$ cells did not change with exercise OG or YG, but tended to be higher in OG *versus* YG at 60 min of recovery (Interaction effect, $p = 0.06$). There were no age differences within boys for responses of these cell proportions during exercise or into recovery.

$CD3^+CD4^+$ and $CD3^+CD8^{bright}$ cell counts increased with exercise (Main effect time, $p < 0.001$ for both) similarly between YG and OG and between YB and OB. Values returned to resting levels by 60 min of recovery in all age groups. In contrast, the increase in total $CD3^+$ cell counts was greater in OG *versus* YG at 30, but not at 60 min of exercise (Interaction effect, $p = 0.003$), and recovery of $CD3^+$ cell counts was complete by 30 min in OG and YG. Changes in total $CD3^+$ cell counts during and following exercise were not significantly different between YB

and OB; however total $CD3^+$ cell counts in boys remained below resting levels at 30 and 60 min of recovery.

Although the proportion of $CD3^-CD19^+$ cells decreased with exercise (Main effect time, $p < 0.001$), there were no age differences within girls or boys. By 60 min of recovery, $CD3^-CD19^+$ proportions were not significantly different from resting levels in OG and OB, but were higher than rest in YG and YB (Main effect time, $p < 0.001$). In contrast to cell proportions, exercise did not affect $CD3^-CD19^+$ cell counts, but values at 60 min of recovery were higher than at rest in YG and YB (Main effect time, $p < 0.02$), but not in OG and OB.

The proportion of $CD3^-CD16^+CD56^+$ cells increased with exercise (Main effect time, $p < 0.001$), but there were no age differences within girls or boys. The exercise-induced increase in $CD3^-CD16^+CD56^+$ cell counts was greater in OG *versus* YG (Interaction effect, $p = 0.002$), but not in YB *versus* OB. In all age groups, $CD3^-CD16^+CD56^+$ cell counts returned to resting levels by 30 min of recovery.

Table 4 provides concentrations of IL-6, TNF-α and IL-8 in YG, YB, OG and OB for age and pubertal comparisons. Exercise did not influence TNF-α levels in YG or OG, whereas concentrations gradually increased over time in OB, but remained stable in YB (Interaction effect, $p = 0.07$). Within girls, IL-6 increased significantly in OG during and following exercise, but did not change in YG (Interaction effect, $p = 0.002$). In both

age groups of boys, IL-6 increased to the same extent during exercise and recovery. Regardless of age, IL-8 remained stable immediately after exercise, but was significantly higher at 60 min of recovery *versus* rest (Main effect time, $p < 0.001$).

Puberty effects in girls. As there were only four girls classified at Tanner stage 2 (T2G), statistical comparisons were only made between girls at Tanner stage 3 (T3G) and girls at Tanner stages 4 and 5 (T4-5G). In general, there were no differences between T3G and T4-5G for exercise-induced changes in, or recovery of, immune variables, with few exceptions. Exercise decreased the proportion of $CD3^{+}CD8^{bright}$ cells in T4-5G, whereas it remained stable in T3G (Interaction effect, $p = 0.03$), but there was no difference between these pubertal groups at 60 min of recovery.

The exercise-induced increase in IL-8 in T3G tended to be greater than in T4-5G (Interaction effect, $p = 0.08$). However, at 60 min of recovery both pubertal groups demonstrated higher IL-8 levels compared to rest (Main effect time, $p < 0.001$).

Although we did not include T2G data in the statistical comparisons, it is noteworthy that the magnitude of change in both proportion and number of total $CD3^{+}$ and $CD3^{-}CD16^{+}CD56^{+}$ cells and in total lymphocytes in T2G was consistently less than in T3G and T4-5G (see Figures 3 and 4).

Puberty effects in boys. Exercise-induced increases in total leukocytes and neutrophils were similar across pubertal stages in boys, but the recovery leukocytosis and neutrophilia were consistently greater in boys at Tanner stage 5 (T5B) compared to boys at Tanner stage 1 (T1B), Tanner stage 2 (T2B) and Tanner stages 3 and 4 (T3-4B) (Interaction effect, $p = 0.03$ and $p = 0.058$ for leukocytosis and neutrophilia, respectively) (Figure 1). No other immune cell changes were significantly different across Tanner stages in boys.

Similar to age effects on TNF-α in boys, TNF-α levels decreased slightly over time in T1B, but gradually increased in T5B (Interaction effect, $p = 0.03$) (Figure 2).

Gender effects in age groups. Exercise-induced increases in total leukocytes and lymphocytes, but not neutrophils or monocytes, were greater in OG *versus* OB (Interaction effect, $p = 0.053$ and $p < 0.01$, respectively). In contrast, there were no gender differences between YG and YB for changes in these cells.

Gender differences between OG and OB and between YG and YB were found for the proportion of total $CD3^+$ cells (Interaction effect, $p = 0.03$ for both comparisons). The decrease in $CD3^+$ proportion was similar between OG and OB at 30 and 60 min of exercise, but OG had a greater rebound at 30 min of recovery compared with OB. The decrease in $CD3^+$ proportion at 30 and 60 min of exercise was greater in YB *versus* YG, with

full recovery in YB by 30 min. In contrast, only a gender difference between YG and YB remained for the responses of total CD3$^+$ cell counts (Interaction effect, $p = 0.02$), due to lower counts at 30 and 60 min of recovery compared with resting values in YB, but not in YG.

No gender differences were found in the responses of the proportion or number of CD3$^-$CD19$^+$, CD3$^+$CD4$^+$, or CD3$^+$CD8^{bright} cells between OG and OB or between YG and YB.

Although there were no gender differences in the responses of CD3$^-$CD16$^+$CD56$^+$ cell proportions to exercise within age groups, when CD3$^-$CD16$^+$CD56$^+$ cell counts were considered, gender differences between OG and OB were found at 30 and 60 min of exercise (Interaction effect, $p = 0.03$), but not between YG and YB.

The only cytokine-related gender difference within age groups was for IL-6 which increased in YB, but remained stable in YG (Interaction effect, $p = 0.002$). In contrast, the exercise-induced increase in IL-6 (Main effect time, $p < 0.001$) was similar between OG and OB.

Gender effects in pubertal groups. Gender differences in total leukocyte responses were found between T3G and T3-4B (Interaction effect, $p = 0.04$) and between T4-5G and T3-4B (Interaction effect, $p = 0.04$) at 30 and 60 min of exercise, but not during recovery (Figure 3). In contrast, gender differences between T4-5G and T5B (Interaction effect, $p = 0.03$) were apparent only at 30 and 60 min of recovery. Gender

differences in lymphocyte responses were also found between T3G and T3-4B (Interaction effect, $p = 0.047$) and between T4-5G and T3-4B (Interaction effect, $p < 0.001$) at 30 and 60 min of exercise, but not between T4-5G and T5B (Figure 4). Lymphocyte counts in both genders had returned to resting levels by 30 min of recovery.

Exercise caused decreases in total $CD3^+$ proportions in T3G and T4-5G but not in T3-4B (Interaction effect, $p = 0.06$ and $p = 0.001$, respectively). In T3-4B, the proportion of $CD3^+$ cells did not fluctuate significantly from resting levels at any time point. A significant interaction was also found between T4-5G and T5B (Interaction effect, $p = 0.02$), due to T4-5G having a higher $CD3^+$ proportion at 30 min of recovery compared with T5B. When the total $CD3^+$ cell count was considered, only differences between T4-5G and T3-4B were found at 30 min of exercise (Interaction effect, $p = 0.06$).

Gender differences in $CD3^-CD16^+CD56^+$ cell proportions were found between T3G and T3-4B at 30 min of exercise (Interaction effect, $p = 0.03$) and between T4-5G and T3-4B at 30 and 60 min of exercise (Interaction effect, $p = 0.049$). These gender differences between pubertal groups in cell proportions were also found for $CD3^-CD16^+CD56^+$ cell counts (Figure 5). Responses of $CD3^-CD16^+CD56^+$ cell proportions and counts were not significantly different between T4-5G and T5B.

The only cytokine-related gender difference within pubertal groups was for IL-8, which increased over time in T3G, but did not change in T3-4B (Interaction effect, $p = 0.05$).

5.5. DISCUSSION

An improved understanding of normal perturbations to the immune system in healthy children and adolescents in response to physiological stress carries significant health and clinical implications. To date, however, there has been a clear lack of definitive investigations on this issue. Therefore, we designed this study to provide a comprehensive analysis of immunological changes in response to exercise in children and adolescents. Our results are descriptive in nature, but provide several novel findings with respect to the effects of age, puberty and gender on exercise-induced changes in cellular and soluble components of the immune system.

Age effects. We found that age-related differences in immunological responses to exercise during childhood were more pronounced in girls than in boys. Although the literature contains studies of immunological responses to exercise in 10- to 12-yr-old (11) and 14- to 16-yr-old (17) girls, to our knowledge, this study is the first to compare girls at different ages under identical experimental conditions. Notwithstanding significant methodological differences in previous studies (e.g., laboratory (11) *versus* field (17)), their results suggested that age

differences existed. For example, exercise increased total leukocytes by ~84% in older girls (17), but only by ~32% in younger girls (11). Likewise, the increase in NK cells (i.e., $CD3^{-}CD16^{+}CD56^{+}$), the most responsive cell type to exercise (20), was ~154% in older girls (17) and ~125% in the younger girls (11). In our girls, age differences amounted to increases of ~36 and ~56% in YG and OG, respectively, for total leukocytes (Table 2) and increases of ~150 and ~245% in YG and OG, respectively, for NK cells (Table 3). In addition, our results highlight the importance of reporting both relative proportions and absolute counts of immune cells. For example, greater decreases in the proportion of $CD3^{+}CD4^{+}$ cells in OG *versus* YG were offset by a greater overall lymphocytosis in the older girls. Thus, the exercise-induced increase in the absolute number of $CD3^{+}CD4^{+}$ cells was similar between OG and YG. In this regard, it is noteworthy that previous studies in children (6,11,21,23) have reported cell counts only.

Cytokine changes during and following exercise were also different between YG and OG in the current study. Specifically, IL-6 increased during exercise and into recovery in OG, but did not fluctuate from resting levels in YG (Table 4), an age-related finding identical to our previous report comparing boys with men (27). IL-6 is an important cytokine with significant anti-inflammatory and metabolic roles in adults (12). That IL-6 increased with exercise in adolescent girls is consistent with previous literature on this age group (17), but the literature is void of studies

describing cytokine changes with exercise in groups of younger girls. Unfortunately, previous studies that have included young girls (22,28) have pooled their results with those of boys making it impossible to discern responses of each gender. Importantly, we followed the cytokine response into the recovery period and that IL-6 continued to increase into recovery in OG suggests that age-related differences may be more related to inflammatory events than to metabolic regulation. Indeed, older girls also had a more pronounced neutrophilia during recovery from exercise, compared with younger girls (Table 2), consistent with a greater inflammatory response. Previous work in females has shown that, following exercise-induced muscle damage, adult women with regular menstrual cycles and not taking OCT significantly increased serum levels of creatine kinase (CK), an indirect marker of muscle damage, whereas pre-menarcheal girls showed no increase (2). It may be, therefore, that our younger girls demonstrated smaller inflammatory-related responses because they experienced less trauma to the contracting skeletal muscle during the cycling task.

With respect to our boys, the age-related difference in recovery leukocytosis and neutrophilia (Table 2) is also consistent with our previous observations in 9- and 10-yr-old boys, compared with men (27). We also found that TNF-α levels gradually increased into recovery in OB, but remained stable over time in YB, but in contrast to the girls, changes in IL-

6 over time were similar between YB and OB (Table 4). These results, in part, suggest that exercise may have induced a greater inflammatory response in the older *versus* younger boys, a similar observation to that found in females discussed above. Previous work in males has also shown that boys, compared with men, respond to exercise designed to induce muscle damage with smaller increases in CK (24). Regardless of why recovery kinetics of immune changes were faster in younger girls and boys, this observation is consistent with the notion that children generally recover more quickly following strenuous exercise.

Other immune-related responses to exercise in boys were not different between ages. For example, NK cells increased by ~133% in YB and by ~177% in OB. The magnitude of these changes, however, is lower than previously reported in 14- to 18-yr-old boys (~238%) following a wrestling practice (15) and in 20- to 25-yr-old men (~200%) following identical exercise as employed in the current study (27), but higher than in 9- and 10-yr-old boys (~110%) also using the current exercise protocol (27). Therefore, the available data support the notion of age differences in NK cell responses to exercise in males, but the gap between age groups in the current study may have been too narrow to significantly highlight possible differences.

Puberty effects. The findings of this study show that when the physical maturity of children and adolescents is considered, many of the

immunological responses to exercise are more similar than dissimilar among pubertal groups. However, the recovery of total leukocytes and neutrophils following exercise (Figure 1) and changes in TNF-α levels (Figure 2) maintained maturity-related effects in the boys. In addition, the lack of exercise effect on the proportion of $CD3^{+}CD8^{bright}$ cells in T3 *versus* T4-5 girls is consistent with less perturbation in the less mature girls. Our findings in the girls are somewhat limited because we only had appropriate numbers of subjects at two different pubertal stages for statistical comparisons. Notwithstanding the low number of subjects at Tanner stage 2 (n=4), these girls consistently demonstrated much smaller exercise-induced increases in total lymphocyte and NK cell counts (Figures 3 and 4). Therefore, future work needs to pursue possible pubertal effects on immune changes in larger numbers of girls representing more stages of puberty.

Gender effects. One of the most novel findings in this study was the significant gender difference in exercise-induced changes in various immune cells in adolescents, but not in younger children. Gender differences in adolescents were most notable in the overall lymphocyte and more specifically the NK cell response to exercise. Importantly, there were no gender differences in the proportion of NK cells, only the NK cell count, due to a greater overall lymphocytosis in the female adolescents, which further argues in favor of reporting both proportions and numbers of

cells. Previous work from Cooper and colleagues reported increases in NK cells of ~238% following a wrestling practice in boys (15) and of ~154% following a water polo practice in girls (17). In contrast, increases in NK cells during exercise were ~245% in our girls and ~177% in our boys (Table 3). Furthermore, we have shown that overall lymphocytosis following 90 min of endurance exercise is ~38% greater in women not taking OCT *versus* men (26), and DeLanne et al. (9) reported a ~82% greater increase in lymphocytes in women *versus* men following 30 min of cycling. Taken together, it seems that gender differences in lymphoid responses to exercise are revealed under controlled experimental conditions and begin to manifest sometime during adolescence. That gender differences in various immune measures were observed in adolescents, but not in younger children, and age differences were observed for girls, but not for boys, suggests that factors related to the presence of female sex hormones may be an important determinant of exercise-induced immune changes. However, we have recently shown that exercise-induced changes in immune cells (total leukocytes, neutrophils and lymphocytes) in women not taking OCT are not different between the mid-follicular and mid-luteal phases of the menstrual cycle (26). Therefore, the presence of, and fluctuations in, sex hormones may not be overly important in exercise-induced perturbations to the immune system.

Another novel finding in this study was the IL-8 response to exercise in healthy children and adolescents. With few exceptions, IL-8 levels did not change after 60 min of exercise, but significantly increased to ~75% above resting values at 60 min of recovery. In adult studies, increases in systemic IL-8 levels require prolonged strenuous exercise (18). However, recent studies also demonstrate that contracting skeletal muscle working at a moderate intensity can increase IL-8 protein expression within muscle fibres (1). Given that IL-8 is a potent angiogenic factor (5), increased levels of this cytokine during recovery from exercise in children may reflect muscle adaptation, beneficial to growth.

Summary and Perspectives. The findings reported herein represent a comprehensive examination of age, puberty and gender effects on immunological changes in response to exercise in healthy children and adolescents. By design, we recruited children at two distinct chronological ages in order to minimize possible effects of previous antigenic experience on immune measures and confounding effects of age-associated factors on pubertal comparisons. The data show that exercise performed at the same relative intensity results in smaller overall perturbations to the immune system in young boys and girls compared with older adolescent boys and girls, with differences more pronounced in the girls. In general, younger individuals also experienced faster recovery of these perturbations. Unfortunately, our data cannot provide mechanisms for the

observed age-, puberty- and gender-related differences, and the clinical and biological significance of these findings remain to be determined. Recently, Cooper and colleagues (7) proposed that exercise-induced changes in immune and inflammatory mediators (e.g., cytokines) in children may have implications for overall growth and development. We find this an intriguing possibility, and suggest that one reason why young children are relatively resistant to major inflammatory responses during and following exercise may be to minimize disruption to other anabolic mediators (e.g., insulin-like growth factor-1), which are conducive to optimal adaptation and growth.

5.6. ACKNOWLEDGMENTS

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Table 5.1. *Subject characteristics of girls and boys according to age and pubertal status*

	n	Height, m	Weight, kg	Body fat, %	$\dot{V}O_{2max}$, $mL \cdot kgBM^{-1} \cdot min^{-1}$	$\dot{V}O_{2max}$, $mL \cdot kgFFM^{-1} \cdot min^{-1}$	HR_{max}, beats/min
				Age			
Girls							
12-yr-old	*14*	1.56 ± 0.07[a]	46.1 ± 7.5[a,b]	18.6 ± 6.7	41 ± 5[a]	51 ± 4[a]	195 ± 8
14-yr-old	*11*	1.63 ± 0.05[a,b]	58.3 ± 4.8[a,c]	22.9 ± 5.2[a]	40 ± 5[b,c]	52 ± 6	197 ± 7
Boys							
12-yr-old	*20*	1.54 ± 0.08[b]	44.0 ± 8.9[c,d]	17.7 ± 6.6	46 ± 6[a,b]	56 ± 6[a]	199 ± 8
14-yr-old	*13*	1.71 ± 0.07[a,b]	57.6 ± 6.6[b,d]	13.8 ± 6.6[a]	46 ± 6[c]	53 ± 6	199 ± 7
				Pubertal status[1]			
Girls							
T2	*4*	1.52 ± 0.10[a]	40.0 ± 5.8[a,c]	16.8 ± 7.3	42 ± 5	50 ± 3	191 ± 5
T3	*11*	1.58 ± 0.06[b]	49.1 ± 7.0[b,d]	19.6 ± 6.4	43 ± 5	53 ± 5	194 ± 7
T4-5	*10*	1.62 ± 0.05[c]	58.7 ± 4.7[b,c,e,f,g]	23.0 ± 5.7[a]	38 ± 4	49 ± 5	199 ± 7
Boys							
T1	*7*	1.48 ± 0.03[b,c,d]	41.0 ± 7.2[e,h]	17.9 ± 9.3	46 ± 8	55 ± 7	197 ± 6
T2	*7*	1.57 ± 0.06[e]	46.6 ± 9.9[f,i]	20.3 ± 4.2	45 ± 6	56 ± 6	196 ± 8
T3-4	*9*	1.61 ± 0.09[d]	47.6 ± 10.2[g,j]	15.4 ± 5.2	48 ± 5	57 ± 5	198 ± 7
T5	*10*	1.72 ± 0.07[a,b,c,d,e]	58.7 ± 5.8[a,d,h,i,j]	12.2 ± 6.5[a]	46 ± 6	52 ± 5	202 ± 8

Values are means ± SD. *n*, no. of subjects; $\dot{V}O_{2max}$, maximal O_2 uptake; BM, body mass; FFM, fat-free mass; HR_{max}, maximal heart rate; T, Tanner stage. [1]Based on self-assessment of breast (girls) and pubic hair (boys) development according to Tanner. For each variable within *Age* and *Pubertal status*, values with same letters are significantly different from each other, $p < 0.05$.

Table 5.2. *Total leukocyte, neutrophil, lymphocyte and monocyte counts at rest and during and following exercise in 12-yr-old and 14-yr-old boys and girls*

	Rest	Exercise		Recovery	
Time, min	-40	30	60	+30	+60
Leukocytes					
YG	5.0 ± 0.3	6.9 ± 0.4	6.8 ± 0.4	5.6 ± 0.3	6.4 ± 0.4
OG	5.5 ± 0.3	8.7 ± 0.7*‡	8.6 ± 0.7*‡	6.7 ± 0.5	7.5 ± 0.5
YB	4.9 ± 0.2	6.8 ± 0.3	7.3 ± 0.4	5.7 ± 0.4	6.5 ± 0.5
OB	5.5 ± 0.3	7.3 ± 0.5	7.6 ± 0.6	6.7 ± 0.8	8.0 ± 0.8†
Neutrophils					
YG	2.1 ± 0.2	3.2 ± 0.3	3.1 ± 0.3	2.8 ± 0.3	3.5 ± 0.4
OG	2.5 ± 0.3	4.1 ± 0.5	4.2 ± 0.5*	4.2 ± 0.4*	4.8 ± 0.5*
YB	2.0 ± 0.2	3.1 ± 0.3	3.6 ± 0.3	3.2 ± 0.4	4.0 ± 0.4
OB	2.5 ± 0.2	3.5 ± 0.3	3.9 ± 0.6	4.1 ± 0.7	5.2 ± 0.8†
Lymphocytes					
YG	2.1 ± 0.1	2.6 ± 0.1	2.7 ± 0.2	2.0 ± 0.1	2.1 ± 0.1
OG	2.1 ± 0.1	3.3 ± 0.2*‡	3.2 ± 0.3*‡	1.8 ± 0.1	1.8 ± 0.1
YB	2.0 ± 0.1	2.6 ± 0.2	2.7 ± 0.2	1.8 ± 0.1	1.8 ± 0.1
OB	2.0 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	1.8 ± 0.1	1.9 ± 0.1
Monocytes					
YG	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
OG	0.4 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
YB	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
OB	0.4 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.1

Values are means ± SEM given in cells·10^9/L. YG, 12-yr-old girls; OG, 14-yr-old girls; YB, 12-yr-old boys; OB, 14-yr-old boys. Main effect time ($p < 0.05$) for leukocytes (30 and 60 min Exercise > Rest; 60 min Recovery > Rest), neutrophils (30 and 60 min Exercise > Rest; 30 and 60 min Recovery > Rest), lymphocytes (30 and 60 min Exercise > Rest) and monocytes (30 and 60 min Exercise > Rest). Interaction effects: *Significantly different from YG, $p < 0.05$. †Significantly different from YB, $p < 0.05$. ‡Significantly different from OB, $p < 0.05$.

Table 5.3. *Proportions and counts of lymphocyte subsets at rest and during and following exercise in 12-yr-old and 14-yr-old boys and girls.*

	Cell proportions, %[1]					Cell counts, $10^9/L$[2]				
	Rest	Exercise		Recovery		Rest	Exercise		Recovery	
Time, min	-40	30	60	+30	+60	-40	30	60	+30	+60
Total $CD3^+$										
YG	72 ± 2	68 ± 2	68 ± 2	75 ± 2	72 ± 2	1.48 ± 0.09	1.79 ± 0.10	1.84 ± 0.18	1.50 ± 0.10	1.52 ± 0.09
OG	70 ± 2	64 ± 2	65 ± 2	74 ± 2‡	73 ± 2	1.52 ± 0.11	2.15 ± 0.15*	2.07 ± 0.21	1.35 ± 0.11	1.34 ± 0.11
YB	75 ± 1	70 ± 1	69 ± 2	72 ± 1	72 ± 2	1.52 ± 0.06	1.76 ± 0.06	1.80 ± 0.09	1.26 ± 0.05*	1.26 ± 0.06*
OB	72 ± 1	66 ± 1	67 ± 1	72 ± 2	71 ± 2	1.55 ± 0.06	1.80 ± 0.11	1.80 ± 0.12	1.33 ± 0.06	1.32 ± 0.07
$CD3^+CD4^+$										
YG	43 ± 1	ND	40 ± 2	ND	45 ± 1	0.88 ± 0.06	ND	1.10 ± 0.12	ND	0.95 ± 0.06
OG	43 ± 2	ND	34 ± 2*	ND	44 ± 1	0.93 ± 0.07	ND	1.06 ± 0.09	ND	0.80 ± 0.06
YB	38 ± 2	ND	34 ± 1	ND	39 ± 2	0.78 ± 0.05	ND	0.93 ± 0.07	ND	0.73 ± 0.05
OB	39 ± 2	ND	33 ± 2	ND	40 ± 1	0.84 ± 0.05	ND	0.89 ± 0.06	ND	0.77 ± 0.07
$CD3^+CD8^{bright}$										
YG	22 ± 1	ND	20 ± 1	ND	21 ± 1	0.46 ± 0.03	ND	0.55 ± 0.05	ND	0.45 ± 0.03
OG	23 ± 2	ND	21 ± 2	ND	24 ± 1*	0.49 ± 0.04	ND	0.65 ± 0.07	ND	0.43 ± 0.04
YB	23 ± 1	ND	21 ± 1	ND	22 ± 1	0.47 ± 0.03	ND	0.57 ± 0.05	ND	0.44 ± 0.06
OB	23 ± 1	ND	22 ± 1	ND	23 ± 2	0.48 ± 0.03	ND	0.56 ± 0.05	ND	0.43 ± 0.03
$CD3^-CD19^+$										
YG	9 ± 1	7 ± 1	8 ± 1	ND	10 ± 1	0.19 ± 0.03	0.18 ± 0.03	0.21 ± 0.03	ND	0.23 ± 0.03
OG	6 ± 1	5 ± 1	5 ± 1	ND	7 ± 1	0.14 ± 0.03	0.16 ± 0.02	0.15 ± 0.02	ND	0.14 ± 0.02
YB	8 ± 1	7 ± 1	7 ± 1	ND	9 ± 1	0.15 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	ND	0.18 ± 0.02
OB	7 ± 1	6 ± 1	6 ± 1	ND	9 ± 1	0.15 ± 0.03	0.14 ± 0.03	0.15 ± 0.03	ND	0.16 ± 0.03
$CD3^-CD16^+CD56^+$										
YG	7 ± 1	13 ± 1	12 ± 1	3 ± 0	5 ± 1	0.14 ± 0.02	0.36 ± 0.05	0.33 ± 0.05	0.07 ± 0.01	0.1 ± 0.01
OG	7 ± 1	16 ± 1	16 ± 1	5 ± 1	6 ± 1	0.16 ± 0.02	0.57 ± 0.08*‡	0.52 ± 0.07*‡	0.09 ± 0.02	0.12 ± 0.02
YB	7 ± 1	12 ± 1	13 ± 2	5 ± 1	6 ± 1	0.15 ± 0.01	0.33 ± 0.05	0.36 ± 0.06	0.10 ± 0.02	0.10 ± 0.02
OB	6 ± 1	13 ± 2	13 ± 2	4 ± 1	4 ± 1	0.13 ± 0.02	0.37 ± 0.07	0.34 ± 0.05	0.08 ± 0.01	0.08 ± 0.01

Values are means ± SEM. YG, 12-yr-old girls; OG, 14-yr-old girls; YB, 12-yr-old boys; OB, 14-yr-old boys; ND, not determined. [1]Main effect time (p < 0.05) for $CD3^+$ (30 and 60 min Exercise < Rest), $CD3^+CD4^+$ (60 min Exercise < Rest), $CD3^+CD8^+$ (60 min Exercise < Rest) $CD3^-CD19^+$ (30 and 60 min Exercise < Rest; 60 min Recovery > Rest in YB and YG only), $CD3^-CD16^+CD56^+$ (30 and 60 min Exercise > Rest). [2]Main effect time (p < 0.05) for $CD3^+$ (30 and 60 min Exercise > Rest; 30 and 60 min Recovery < Rest in YB and OB only), $CD3^+CD4^+$ (60 min Exercise > Rest), $CD3^+CD8^+$ (60 min Exercise > Rest) $CD3^-CD19^+$ (30 and 60 min Exercise > Rest in YG and YB only; 60 min Recovery > Rest in YG and YB only), $CD3^-CD16^+CD56^+$ (30 and 60 min Exercise > Rest). Interaction effects: *Significantly different from YG, $p < 0.05$. ‡Significantly different from OB, $p < 0.05$.

Table 5.4. *IL-6, TNF-α and IL-8 concentrations at rest and during and following exercise in 12-yr-old and 14-yr-old boys and girls.*

	Pre	Post	Recovery
IL-6			
YG	1.29 ± 0.25	2.02 ± 0.33	1.87 ± 0.28
OG	0.99 ± 0.12	2.28 ± 0.24	3.81 ± 0.88*
YB	1.13 ± 0.11	1.82 ± 0.16	2.50 ± 0.30*
OB	1.18 ± 0.18	2.43 ± 0.34	3.06 ± 0.54
TNF-α			
YG	0.87 ± 0.06	0.87 ± 0.07	1.02 ± 0.07
OG	0.94 ± 0.22	0.97 ± 0.26	1.01 ± 0.26
YB	0.99 ± 0.11‡	1.10 ± 0.14‡	0.98 ± 0.11
OB	0.56 ± 0.21	0.63 ± 0.23	0.82 ± 0.27
IL-8			
YG	4.0 ± 0.6	6.0 ± 1.1	8.7 ± 1.4
OG	4.5 ± 0.5	6.8 ± 1.5	10.6 ± 1.9
YB	5.9 ± 0.5	5.7 ± 0.5	8.5 ± 0.8
OB	5.4 ± 1.0	5.1 ± 0.7	8.3 ± 0.9

Values are means ± SEM given in pg/mL. YG, 12-yr-old girls; OG, 14-yr-old girls; YB, 12-yr-old boys; OB, 14-yr-old boys; Pre, 40 min before exercise; Post, after 60 min of exercise; Recovery, 60 min after exercise. Main effect time ($p < 0.05$) for IL-6 (Post > Rest; Recovery > Rest), TNF-α (Recovery > Rest in OG and OB only), IL-8 (Recovery > Rest) Interaction effects: *Significantly different from YG, $p < 0.05$. ‡Significantly different from OB, $p < 0.05$.

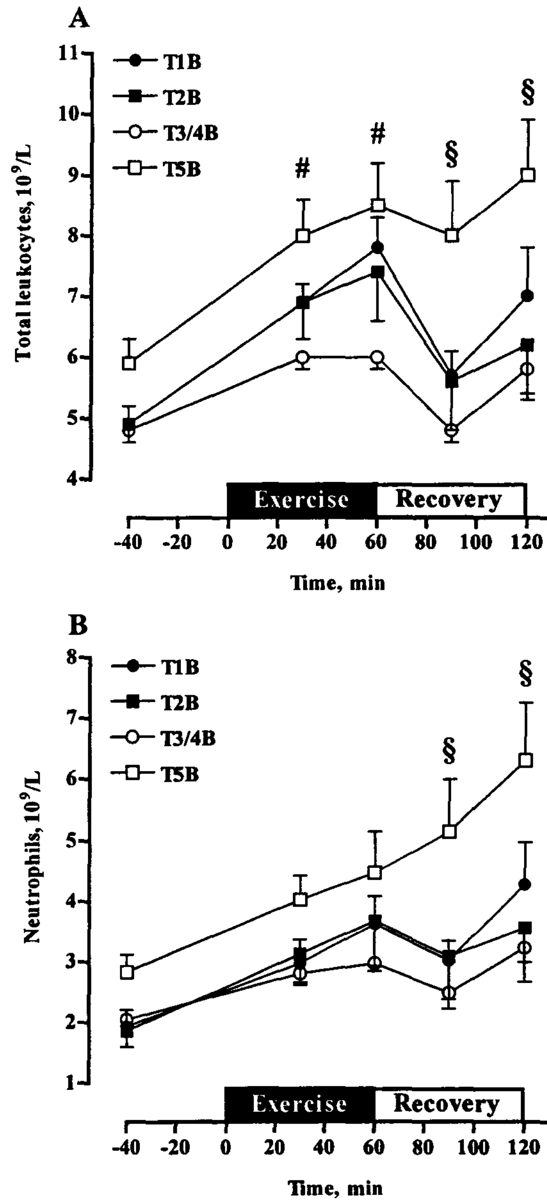

Figure 5.1. Total leukocyte (A) and neutrophil (B) counts before and during and after exercise (disregarding five min rest between exercise bouts) in boys at different pubertal stages. Values are mean ± SEM. T1B, boys at Tanner stage 1 (pre-pubertal); T2B, boys at Tanner stage 2; T3-4B, boys at Tanner stages 3 and 4; T5B, boys at Tanner stage 5 (post-pubertal). Interaction effects: #T5B significantly different from T3-4B, $p < 0.05$. §T5B significantly different from T1B, T2B and T3-4B, $p < 0.05$.

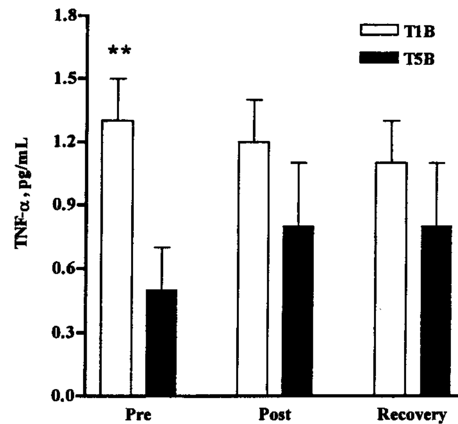

Figure 5.2. TNF-α concentrations before, immediately after and 60 min after exercise (disregarding five min rest between exercise bouts) in pre-pubertal and post-pubertal boys. Values are means ± SEM. T1B, boys at Tanner stage 1 (pre-pubertal); T5B, boys at Tanner stage 5 (post-pubertal); Pre, 40 min before exercise; Post, after 60 min of exercise; Recovery, 60 min after exercise. Interaction effect: **Significantly different from T5B, $p < 0.05$.

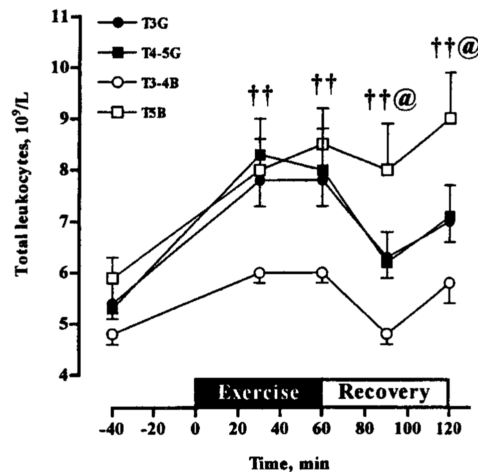

Figure 5.3. Total leukocyte counts before and during and after exercise (disregarding five min rest between exercise bouts) in girls and boys at different pubertal stages. Values are mean ± SEM. T3G, girls at Tanner stage 3; T4-5G, girls at Tanner stages 4 and 5; T3-4B, boys at Tanner stages 3 and 4; T5B, boys at Tanner stage 5. Interaction effects: ††T3-4B significantly different from T3G and T4-5G, $p < 0.05$; @T5B significantly different from T3G and T4-5G, $p < 0.05$.

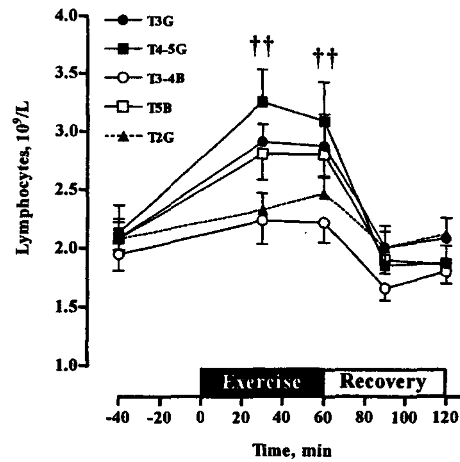

Figure 5.4. Lymphocyte counts before and during and after exercise (disregarding five min rest between exercise bouts) in girls and boys at different pubertal stages. Values are mean ± SEM. T3G, girls at Tanner stage 3; T4-5G, girls at Tanner stages 4 and 5; T3-4B, boys at Tanner stages 3 and 4; T5B, boys at Tanner stage 5; T2G, girls at Tanner stage 2 (broken line; for comparison purposes). Interaction effect: ††T3-4B significantly different from T3G and T4-5G, $p < 0.05$.

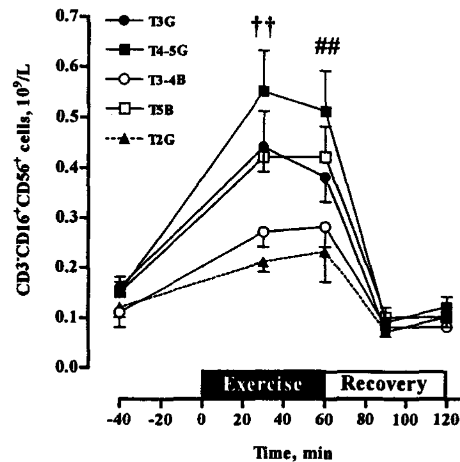

Figure 5.5. $CD3^-CD16^+CD56^+$ cell counts before and during and after exercise (disregarding five min rest between exercise bouts) in girls and boys at different pubertal stages. Values are mean ± SEM. T3G, girls at Tanner stage 3; T4-5G, girls at Tanner stages 4 and 5; T3-4B, boys at Tanner stages 3 and 4; T5B, boys at Tanner stage 5; T2G, girls at Tanner stage 2 (broken line; for comparison purposes). Interaction effects: ††T3-4B significantly different from T3G and T4-5G, $p < 0.05$; ##T3-4B significantly different from T4-5G, $p < 0.05$.

CHAPTER 6: INFLUENCE OF GENDER, MENSTRUAL PHASE, AND ORAL CONTRACEPTIVE USE ON IMMUNOLOGICAL CHANGES IN RESPONSE TO PROLONGED CYCLING

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6.1. ABSTRACT

This study determined the influence of gender, menstrual phase (MP), and oral contraceptive (OC) use on immunological changes in response to endurance exercise. Twelve women and 11 men similar in age, aerobic power and activity level cycled for 90 min @ 65% $\dot{V}O_{2max}$. Women were OC users (n = 6) or non-users (NOC) and cycled during the follicular (FOL) and the luteal (LUT) phases. Venous blood was collected before and after exercise to determine leukocyte counts, IL-6 concentrations, and cortisol. Higher resting levels of neutrophils (~1.5-fold) and cortisol (~2.5-fold) were found in OC *versus* NOC and men. Exercise-induced immune cell count and IL-6 changes were similar between men and NOC, except for a ~38% greater lymphocyte response in NOC *versus*

men ($P = 0.07$). Neutrophil, monocyte and lymphocyte responses to exercise during LUT in OC were greater than during FOL and also greater than the responses in men ($P \leq 0.003$). Changes in immune cell counts were consistently greater during LUT in OC *versus* NOC, regardless of MP, but only neutrophil responses reached statistical significance ($P = 0.01$). The exercise-induced change in IL-6 was ~80% greater in NOC *versus* OC during FOL ($P = 0.06$), but similar between these groups during LUT. Cortisol changes with exercise were not different between groups or MP. These results highlight the necessity to control for gender, and in particular OC use, when designing studies evaluating exercise and immunology.

6.2. INTRODUCTION

The influence of gender on physiological responses to exercise has received much attention in the last decade. The presence of, and fluctuations in, sex hormones appear to be important in regulating substrate utilization (52), muscle fatigue (22), temperature regulation (25) and endocrine responses (13) during exercise in humans. Gender differences in exercise responses have clear implications for understanding gender-specific adaptations to exercise for athletic performance and overall health. The impact of exercise on immune function has also received considerable attention in recent years (44), but

to what extent gender and fluctuations in sex hormones influence immunological responses to exercise is unclear.

Sex hormones play important roles in the immune system under non-exercise conditions, and several gender-related differences in immune function have been identified. For example, women tend to have greater responsiveness to immunization (35), higher serum concentrations of some immunoglobulins (Ig)(19), a higher absolute number of T helper lymphocytes (1), and a differential regulation of cytokine production (19,29). Moreover, compared with the follicular phase (FOL), the luteal phase (LUT) of the menstrual cycle is associated with increased concentrations of leukocyte and lymphocyte subsets (8,17), a greater capacity of immune cells to produce cytokines (8,17,27), greater plasma cytokine activity (29), but a variable effect on plasma cytokine levels (2,10,27). In contrast, other studies associate FOL with greater cytokine production from immune cells (29,47) and higher serum IL-6 levels (2). Considering these differences in resting immune function between men and women and between phases of the menstrual cycle, we were interested in possible gender and menstrual phase (MP) effects on immunological changes in response to exercise.

Notwithstanding a wealth of exercise immunology literature (32,44), relatively few studies have compared exercise-induced immune changes between men and women or between phases of the menstrual cycle. A

number of publications have reported that exercise-induced changes in cell counts and function (3,33,37-39,57) and plasma cytokine levels (33,56) were not different between men and women. However, it appears that these studies did not control for the menstrual status of the women at the time of testing. Most of these studies were also unclear on how subjects were matched or what, if any, dietary controls were implemented. In a recent paper (42), no differences in immune cell counts or cytokine levels were reported between 12 women and 84 men competing in a marathon. The average age of the entire subject pool was ~42 years, and there was no mention as to the age of the women, whether they maintained a regular menstrual cycle, at what phase of the menstrual cycle testing occurred, or if they used oral contraceptives (OC). In other studies, no effect of menstrual cycle was reported on cytokine responses to walking exercise in the cold (20) or on resting salivary IgA levels during a period of training (9). In contrast to studies reporting no differences, other investigations have reported gender differences in immune-related responses to cycling (14) and eccentric exercise (30,51). In at least one of these studies (51), the phase of the menstrual cycle in which women were tested and OC use was standardized. Thus, it appears that the effects of gender and MP on immunological responses to prolonged exercise in humans are unclear due to a lack of systematic, well-controlled investigations.

Therefore, this study was designed, in part, to clarify the influence of gender and MP on immunological changes in response to endurance exercise. We recruited women who were users of OC and non-users (NOC) to elucidate possible influences of OC use on immunological changes in response to exercise, in light of previous research documenting clear effects of OC use on the immune system (41,59) and associated hormones (e.g., cortisol (34)) under resting conditions.

6.3. METHODS

Subjects. Twelve healthy young women and 11 healthy young men volunteered to participate in this study approved by the McMaster University Research Ethics Review Board. All subjects provided written informed consent before participating. Six of the 12 women were OC users and all used a tri-phasic OC type of estrogen and progesterone; 4 used Tricyclen 28, 1 used Tricyclen 21, and 1 used Triphasil 28. Women in the NOC group maintained regular menstrual cycles. Physical and fitness characteristics of the three groups are given in Table 1. Age and aerobic power expressed relative to fat-free mass were not different between groups, and they engaged in similar amounts of self-assessed recreational activity as prospectively recorded in physical activity logs. All subjects were free of disease and infection and were not taking medications.

Study design. In a preliminary session, maximal aerobic power ($\dot{V}O_{2max}$) was assessed and anthropometric data collected for each subject. At least seven days after this initial visit, subjects completed an experimental session, which consisted of cycling for 90 min at 65% of their previously determined $\dot{V}O_{2max}$ with venous blood samples collected before (PRE) and immediately after (POST) exercise. Women completed one experimental session during FOL (day 8 ± 3) and one during LUT (day 20 ± 2). There were no differences between the OC and NOC groups as to what day of the menstrual cycle testing occurred. In addition, ovulation was confirmed in NOC women with an ovulation kit (Clear Plan Easy Ovulation Test, Novartis Canada, Mississauga, ON). To counterbalance the order in which women completed the experimental sessions, six started during FOL and six during LUT. This design required some women to complete all testing within one menstrual cycle and some required testing to carry-over into their next cycle.

Preliminary session. The preliminary session was primarily for the purpose of determining individual $\dot{V}O_{2max}$ values using a progressive, continuous protocol conducted on an electronically braked cycle ergometer (Lode, Netherlands) with a computerized open-circuit gas collection system (Moxus Modulator $\dot{V}O_2$ system with O_2 analyzer S-3A/I and CO_2 analyzer CD-3A, AEI Technologies Inc., IL). All subjects completed 2 min of cycling at 75 W followed by 2 min at 150 W and then 2

min at 200 W. Power output was then increased by 25 W increments every minute until pedaling rate could not be maintained over 60 rpm despite strong encouragement. $\dot{V}O_{2max}$ was taken as the highest 15-sec value during the test and confirmed by an apparent plateau in $\dot{V}O_2$ and/or an RER greater than 1.12. Power output corresponding to 65% of $\dot{V}O_{2max}$ was then estimated from the linear relationship between power output and $\dot{V}O_2$, and this work-rate was confirmed by measuring $\dot{V}O_2$ at the estimated work intensity for each subject approximately 30 min following the maximal test.

Experimental session. To avoid the possible influence of prior diet on the effects of exercise on immunological responses, all subjects completed prospective diet records for a minimum of two weekdays and one weekend day before the first trial, and the diet preceding the subsequent trial (for the women) was consistent for each subject. Subjects were also instructed to maintain their regular exercise routines throughout the study, but no strenuous exercise was allowed during the two days prior to each testing session.

Following an overnight fast (~10 hrs), subjects arrived to the laboratory at the same time early in the morning (either 07:00 or 09:00) to ensure that timing of exercise and blood collection was consistent across all subjects and all sessions. As this study was a small part of the subjects' participation in a larger experiment related to energy metabolism,

they were not allowed to eat until testing was completed, but water was provided to maintain body hydration during the testing day. After supine rest, fat-free mass was estimated by bioelectric impedance analysis (RJL Systems BIA-101A, Mt. Clemens, MI). A 20-gauge plastic catheter (Becton Dickinson, NJ) was then placed into the antecubital vein of the right arm for blood collection. Blood samples were drawn into one EDTA vacutainer, one heparin vacutainer and one vacutainer without anticoagulant (Becton Dickinson, NJ) at PRE and POST of the cycling task. To eliminate the possibility that *ex-vivo* IL-6 production and secretion from circulating immune cells contributed to plasma levels, the heparin vacutainer was pre-treated with brefeldin A (at a final concentration of 10 μg/ml whole blood) to prevent release of intracellular IL-6. Blood in the anticoagulant-free vacutainer was allowed to clot at room temperature (RT) and the heparinized vacutainer was placed on ice until both tubes were centrifuged at 1750g at 4°C for 10 min. Plasma and serum were then separated and stored at -50°C until analyzed.

Blood analyses. EDTA-treated blood samples were well-mixed and left at RT before delivered to the McMaster University Medical Centre Core Lab for subsequent analysis of total leukocytes, neutrophils, monocytes, and lymphocytes using an automated Coulter counter. This blood sample was also used to determine hemoglobin and hematocrit so that blood and plasma volume changes could be estimated according to

Dill and Costill (16). All immune cell counts were adjusted for exercise-induced changes in blood volume.

Determination of testosterone, estradiol, progesterone and cortisol was performed in duplicate on PRE serum samples using solid phase radioimmunoassays (Cat. No. TKTT1, TKE21, TKPG1, and TKCO1, respectively, from Diagnostic Products Corporation, CA). Cortisol was also determined on POST samples. The intra- and inter-assay CVs are $< 7\%$ for testosterone, $\leq 8\%$ for estradiol, $\leq 9\%$ for progesterone, and $\leq 8\%$ for cortisol. Post-exercise cortisol levels were adjusted for changes in plasma volume.

A commercially available "high sensitivity" ELISA kit (Cat. No. HS600B, R&D Systems, Minneapolis, MN) was used to measure plasma levels of IL-6. According to the manufacturer, the sensitivity of this assay is 0.039 pg/ml and in our hands the intra- and inter-assay CVs are $\leq 9\%$, respectively. Post-exercise IL-6 concentrations were adjusted for changes in plasma volume.

Statistical analyses. All values are given in mean ± SD. Subject characteristics (Table 1) were analyzed by one-way ANOVA. Given we were interested in the recruitment of immune components into the peripheral circulation, we calculated the delta change (Δ) in values (i.e., POST – PRE) before submitting to statistical analyses. To determine the effects of OC and MP on resting concentrations of hormone, immune cell

counts and IL-6 and the immune and hormone changes with exercise, women were analyzed separately from men with a 2-way repeated measures ANOVA (group × phase). The corresponding resting values and exercise-induced changes for the men were then compared with each of the women's values (i.e., NOC FOL and LUT, OC FOL and LUT) by a Student's independent t-test with a Bonferonni correction such that a P value ≤ 0.0125 was taken as significant and P values > 0.0125 and ≤ 0.05 were considered as trends. When ANOVAs were used, significance was set at $P \leq 0.05$, and where appropriate, a Tukey's honestly significant difference post-hoc test was applied to determine the significance among means. Resting concentrations of immune cell counts and IL-6 and their respective changes with exercise were tested for a normal distribution, which was confirmed. STATISTICA for Windows 5.0 (StatSoft, Tulsa, OK) software was used to perform ANOVAs and to test for normality. Microsoft Office Excel 2003 (Redmond, WA) software was used to perform t-tests. To assess the association between selected changes in immune parameters and resting sex hormone concentrations, Pearson correlations were calculated. In all cases, correlations were performed with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA) and considered significant when $P \leq 0.05$.

6.4. RESULTS

Hormones. Table 2 provides resting hormone concentrations for the three groups. Estradiol levels in men were higher than in OC during both FOL ($P = 0.018$) and LUT ($P = 0.002$), but lower than in NOC during LUT only ($P = 0.015$). Progesterone levels in men were higher, but not statistically different than OC during FOL ($P = 0.031$) and LUT ($P = 0.036$) and higher than NOC during FOL ($P = 0.017$), but lower than NOC during LUT ($P = 0.009$). Testosterone was higher in men than either group of women ($P < 0.001$ for all comparisons).

Estradiol levels were lower in OC compared with NOC (Main effect group, $P = 0.007$), but did not fluctuate significantly across MP (Main effect phase, $P = 0.38$). Progesterone levels were similar in OC across phases. In NOC, progesterone was higher during LUT than FOL ($P = 0.02$). Consequently, progesterone during LUT in NOC was greater than during LUT in OC ($P = 0.02$). Testosterone levels in OC were lower than NOC (Main effect group, $P = 0.01$).

Resting cortisol concentrations in OC were ~2.5-fold higher than in NOC (Main effect group, $P = 0.058$) and men ($P < 0.01$ for FOL and LUT comparisons). Only cortisol was measured following exercise, and the absolute change in concentration was similar between OC and NOC (308 ± 201 *versus* 430 ± 306 nmol/l, $P = 0.51$), FOL and LUT (485 ± 316 *versus*

277 ± 351 nmol/l, $P = 0.18$), and men and women (223 ± 317 *versus* 485 ± 316 nmol/l, $P = 0.24$).

Immune cell counts. Table 3 provides PRE and POST values of immune cell counts and IL-6 for the three groups. Compared with men, OC had higher, but not statistically different total leukocyte and neutrophil counts during FOL ($P = 0.022$ and $P = 0.027$, respectively) and LUT ($P = 0.029$ and $P = 0.05$, respectively). There were no differences between men and NOC in any of the measured immune cells at rest. The total leukocyte count was higher, but not statistically different in OC *versus* NOC (Main effect group, $P = 0.08$) whereas the lymphocyte count was higher during LUT than FOL (Main effect phase, $P = 0.03$).

Figure 1 provides the exercise-induced changes in immune cell counts for the three groups. The observed changes in total leukocytes, neutrophils, lymphocytes and monocytes in OC during LUT were greater than in men ($P \leq 0.003$ for all comparisons). Because there were no differences in immune cell responses between MP in NOC, these data were pooled before being compared with men. Total leukocyte, neutrophil and monocyte changes were not different between men and NOC, but the change in lymphocytes in NOC was greater, but not statistically different than in men ($P = 0.07$).

The exercise-induced change in total leukocytes in OC was greater during LUT than FOL ($P = 0.02$) and greater than in NOC during FOL ($P =$

0.005) and LUT ($P = 0.01$). A similar statistical pattern was evident for neutrophils. The change in monocytes in OC was greater during LUT compared with FOL ($P = 0.04$). Likewise the change in lymphocytes in OC during LUT was higher, but not statistically different than during FOL ($P = 0.08$).

IL-6. Resting IL-6 levels were not different between OC and NOC or MP, and women's values were not different from those of the men. The exercise-induced change in IL-6 (Figure 2) was not different between MP or between men and women. The change in IL-6 during FOL, however, was greater, but not statistically different in NOC *versus* OC ($P = 0.06$).

Correlations. Resting levels of sex hormones were not correlated with the exercise-induced increase in any immune variable ($P \geq 0.20$ for all correlations). In spite of significant % body fat differences between the groups it was not associated with any immunological measures.

6.5. DISCUSSION

This study determined the influence of gender, MP and OC use on immunological changes in response to exercise. The results indicate that OC use increases the magnitude of change in commonly reported aspects of the cellular immune system during LUT, but not FOL. The exercise-induced increase in lymphocytes, but not in total leukocytes, neutrophils or monocytes, averaged across MP, were greater in NOC *versus* men. In addition, the IL-6 response to exercise during FOL was greater in NOC

versus OC. Finally, our observations at rest confirm previous findings of elevated cortisol in OC users, compared with NOC and men.

Although investigations into immunological responses to exercise have grown exponentially in the last two decades, our understanding of differences or similarities in the responses among men and women from earlier studies has been weakened by several methodological limitations. We specifically designed our experiment to control for fitness and activity levels and diet of the subjects. Further, each subject exercised at an identical intensity, relative to their individual maximal capacity. With this experimental design, our comparison of men and women not using OC is consistent with previous reports by showing no significant effect of gender on changes in total leukocytes, neutrophils and monocytes in response to exercise (3,33,38,57). In contrast, our data show that the lymphocyte response in women not taking OC is greater than in men. This result is consistent with the findings of De Lanne et al. (14) who reported an ~82% greater lymphocyte increase in women *versus* men following 30 min of cycling. In the present study, the increase in lymphocytes was ~38% greater in NOC *versus* men.

The reasons for this apparent gender difference in the lymphocyte response to exercise are unclear, but since there was no difference in responses between MP, fluctuations in sex hormones may not be the primary mechanism of action. The lymphocyte response to exercise is

composed of cumulative changes in lymphocyte subsets, but is driven by natural killer (NK) cells which are the most responsive cell type to exercise (44) due to their high surface density of β_2-adrenergic receptors (28). Exercise-induced lymphocytosis is mediated by an exercise-induced increase in epinephrine (31,55). Paradoxically, epinephrine responses to exercise are generally smaller in women than in men (13,24), but women show greater lymphocyte β_2-adrenergic receptor density (58) and post-receptor activity (21,36) compared with men. Thus, the catecholamine-induced down-regulation of adhesion molecule expression (40) leading to lymphocyte mobilization during exercise may be greater in women than in men. Since NK cell activity of whole blood is less in women than in men (5,59), an overall greater lymphocytosis in response to exercise in women may be a compensatory mechanism to maintain functional immune status in the face of physiological stress.

Another main finding in this study is that the leukocytosis of exercise fluctuated across the menstrual cycle in OC, but not in NOC. Specifically, exercise-induced changes in total leukocytes, neutrophils, monocytes and lymphocytes during LUT were greater than during FOL in OC. These changes during LUT in OC were always greater than the exercise-induced changes in men. Similarly, the changes in all immune cell counts were consistently greater in OC during LUT than in NOC, regardless of MP. However, statistical significance was reached for the

changes in total leukocytes and neutrophils only. The greater neutrophil response in OC *versus* NOC and men may be due to a greater growth hormone (GH) response in the former group. It has been shown that GH infusion increases circulating neutrophil levels (26) and GH responses to exercise are greater in OC users (4,6). The high cortisol levels in OC may have also influenced neutrophil trafficking in response to exercise by inhibiting cell adhesion to the endothelium (12). However, cortisol levels can not explain why neutrophil changes were different between MP within OC since this hormone remained constant across MP. Alternatively, exercise-induced changes in epinephrine may also account for differences in neutrophil responses since epinephrine infusion also increases circulating neutrophil levels (55). However, the available literature does not support differences between OC users and non-users in epinephrine responses to exercise (6,43). Regardless of the mechanism(s) mediating the observed effects of OC use, it seems clear that the presence of synthetic hormones has a greater influence on exercise-induced changes in immune cell counts than normal fluctuations in endogenous sex hormones. It is also important to note that hormone replacement therapy (HRT) in post-menopausal women has only a minor impact on resting concentrations of immune cells (45). However, whether exercise-induced changes in immune cell counts are different in women taking *versus* not taking HRT is unclear.

Although IL-6 levels at rest were virtually identical among groups, the exercise-induced increase in IL-6 during FOL was ~80% greater in NOC *versus* OC. That resting IL-6 levels were not different between NOC and OC is consistent with a lack of HRT effect on serum IL-6 levels in post-menopausal women (45). During FOL, resting estradiol levels were greater in NOC *versus* OC, but were not correlated with the change in IL-6, and it is unlikely that exercise-induced increases in estradiol or progesterone were greater in NOC *versus* OC (7). Alternatively, changes in systemic IL-6 levels during exercise may be related to metabolic responses in so far as release of IL-6 from skeletal muscle is enhanced when muscle glycogen is low (48), potentially to induce an increase in liver glucose output (50). In our women, however, post-exercise glycogen content (15) and glucose rate of appearance (M.C. Devries, M.J. Hamadeh, and M.A. Tarnopolsky, unpublished observations) were not different between NOC and OC during FOL. Therefore, future work should examine whether OC use influences IL-6 release from contracting muscle (50) and/or uptake by the liver (18) during exercise.

In this study, the exercise-induced increase in neutrophils was greatest during LUT in OC when estradiol levels were the lowest compared with the other groups. Estrogen has been shown to attenuate inflammatory responses to endotoxin in humans (46) and may attenuate inflammatory responses to exercise (53). In ovariectomized rats, estrogen

supplementation reduces neutrophil infiltration into skeletal muscle (54). In the current study, we did not find a correlation between resting estradiol levels and changes in neutrophil counts. It may be, however, that the increase in estrogen due to exercise was less during LUT in OC users as has been shown previously (7). A lower estrogen response to exercise in OC may allow a greater inflammatory response to occur in the contracting muscle, leading to greater neutrophil mobilization. Although we have reported that higher circulating neutrophil counts occur concomitantly with greater muscle inflammation following eccentric exercise in men *versus* women (51), the impact of estrogen on these changes remains to be determined.

Our findings of elevated cortisol and neutrophil levels at rest in OC users are consistent with an anti-inflammatory "environment". Previous work has shown that cortisol levels are higher in OC users *versus* non-users and are also increased during healthy pregnancy (34). In addition, *in vitro* exposure to synthetic estrogen and progestin reduces leukocyte transmigration through endothelial cells (23). Thus, the higher resting neutrophil counts observed in OC users may be a result of higher circulating cortisol concentrations (12) or a direct effect of synthetic hormones inhibiting their exit from the peripheral circulation. In line with the ~36% overall greater neutrophil levels in OC *versus* NOC, Porter et al. (45) reported a smaller non-significant 23% higher resting neutrophil count

in post-menopausal women taking HRT *versus* those not taking HRT. Given the older age of Porter et al.'s subjects (~62 years), a possible interaction of age and synthetic sex hormone effects on the immune system could explain the smaller differences in neutrophil counts, and would be an important area for future research.

Recently (49), it was proposed that IL-6 stimulates cortisol release leading to a neutrocytosis because IL-6 infusion into healthy humans resulted in increased levels of these factors. In the current study, resting levels of cortisol and neutrophil counts were elevated in OC users in the absence of higher resting IL-6 concentrations. Likewise, the greater exercise-induced increase in neutrophils in OC was not accompanied by a greater IL-6 response, and IL-6 is not known to be elevated during healthy pregnancy (11). Our results, therefore, suggest that factors other than IL-6 may be more related to changes in neutrophils and cortisol. However, we did not measure IL-6, neutrophils or cortisol during recovery from exercise when concentrations of these factors may have continued to change.

In summary, the lymphocytosis of exercise is greater in OC users during LUT and in non-users compared with men. The presence of synthetic sex hormones influences leukocyte changes during endurance exercise. The effect of exercise on IL-6 is greater in NOC *versus* OC during FOL, but not LUT. Our observations at rest (e.g., increased neutrophils and cortisol) confirm an anti-inflammatory "environment" in OC

users. Collectively, these findings highlight the necessity to account for gender when designing studies to evaluate exercise and immunology. In particular, the effects of OC use on lymphocyte subsets and cytokine changes in response to exercise, and their recovery, should be further investigated.

6.6. ACKNOWLEDGMENTS

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Table 6.1 *Subject characteristics.*

Characteristic	Men	NOC	OC
n	11	6	6
Age, yr	21 ± 1	21 ± 2	22 ± 3
Height, m	1.8 ± 0.1	1.7 ± 0.0†	1.6 ± 0.0†
Weight, kg	77 ± 11	66 ± 10	58 ± 8†
Body fat, %	18 ± 4	31 ± 4†	25 ± 3†‡
$\dot{V}O_{2max}$, $ml \cdot kgBM^{-1} \cdot min^{-1}$	45 ± 5	37 ± 9*	40 ± 6
$\dot{V}O_{2max}$, $ml \cdot kgFFM^{-1} \cdot min^{-1}$	56 ± 6	54 ± 11	54 ± 9

Values are means ± SD. *n*, number of subjects. NOC, non-oral contraceptive users; OC, oral contraceptive users; $\dot{V}O_{2max}$, maximal aerobic power; BM, body mass; FFM, fat-free mass. *Significantly different than men, $P \leq 0.05$. †Significantly different than men, $P \leq 0.0125$. ‡Significantly different than NOC, $P \leq 0.05$.

Table 6.2 *Resting hormone concentrations in men and in women during two phases of the menstrual cycle.*

	Men	NOC		OC	
		FOL	LUT	FOL	LUT
Estradiol, pmol/l	134 ± 70	195 ± 188	361 ± 263[a]	42 ± 41[a,c]	14 ± 11[b,c]
Progesterone, nmol/l	2.9 ± 1.0	1.8 ± 0.4[a]	10.3 ± 8.3[b,e,g]	1.9 ± 0.6[a,c]	1.9 ± 0.6[a,c,e,f]
Testosterone, nmol/l	19.9 ± 3.7	1.2 ± 0.2[b]	1.0 ± 0.4[b]	0.7 ± 0.2[b,c]	0.7 ± 0.1[b,c]
Cortisol, nmol/l	570 ± 145	527 ± 433	535 ± 540	1364 ± 772[b,d]	1355 ± 818[b,d]

Values are means ± SD. NOC, non-oral contraceptive users; OC, oral contraceptive users; FOL, mid-follicular phase; LUT, mid-luteal phase. [a]Significantly different than men, $P \leq 0.05$. [b]Significantly different than men, $P \leq 0.0125$. [c]Main effect for group (OC < NOC), $P \leq 0.05$. [d]Main effect for group (OC > NOC), $P = 0.058$. [e]Main effect for phase (LUT > FOL), $P \leq 0.05$. [f]Interaction effect, significantly different than LUT in NOC, $P = 0.02$. [g]Interaction effect, significantly different than FOL within NOC, $P = 0.02$.

Table 6.3 *Immune cell counts and IL-6 at rest and immediately after exercise in men and in women during two phases of the menstrual cycle.*

	Men	NOC		OC	
		FOL	LUT	FOL	LUT
Leukocytes					
Pre	5.6 ± 1.5	5.9 ± 1.3	6.4 ± 1.0	8.4 ± 3.0[a,b]	7.6 ± 1.9[a,b]
Post	8.5 ± 2.3	8.7 ± 1.1	9.6 ± 1.2	11.9± 3.7	14.4 ± 0.8
Neutrophils					
Pre	3.2 ± 1.3	3.6 ± 1.4	4.1 ± 1.2	5.7 ± 3.0[a]	4.8 ± 1.9[a]
Post	5.0 ± 1.8	5.2 ± 1.0	5.8 ± 1.3	8.2 ± 3.5	9.6 ± 0.9
Monocytes					
Pre	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.2
Post	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	1.0 ± 0.2
Lymphocytes					
Pre	1.7 ± 0.3	1.5 ± 0.4	1.6 ± 0.4[c]	1.8 ± 0.3	2.0 ± 0.3[c]
Post	2.5 ± 0.4	2.6 ± 0.4	2.7 ± 0.4	2.7 ± 0.5	3.4 ± 0.7
IL-6					
Pre	2.0 ± 1.1	2.0 ± 0.8	2.0 ± 1.4	2.4 ± 1.5	2.0 ± 0.5
Post	6.7 ± 2.8	7.7 ± 2.4	6.3 ± 0.8	5.5 ± 2.1	6.8 ± 1.0

Values are means ± SD given in cells·10^9/L (immune cell counts) and pg/mL (IL-6). NOC, non-oral contraceptive users; OC, oral contraceptive users; FOL, mid-follicular phase; LUT, mid-luteal phase; Pre, pre-exercise; Post, immediately after exercise. [a]Significantly different than men, $P \leq 0.05$. [b]Main effect for group (OC > NOC), $P = 0.08$. [c]Main effect for phase (LUT > FOL), $P = 0.03$.

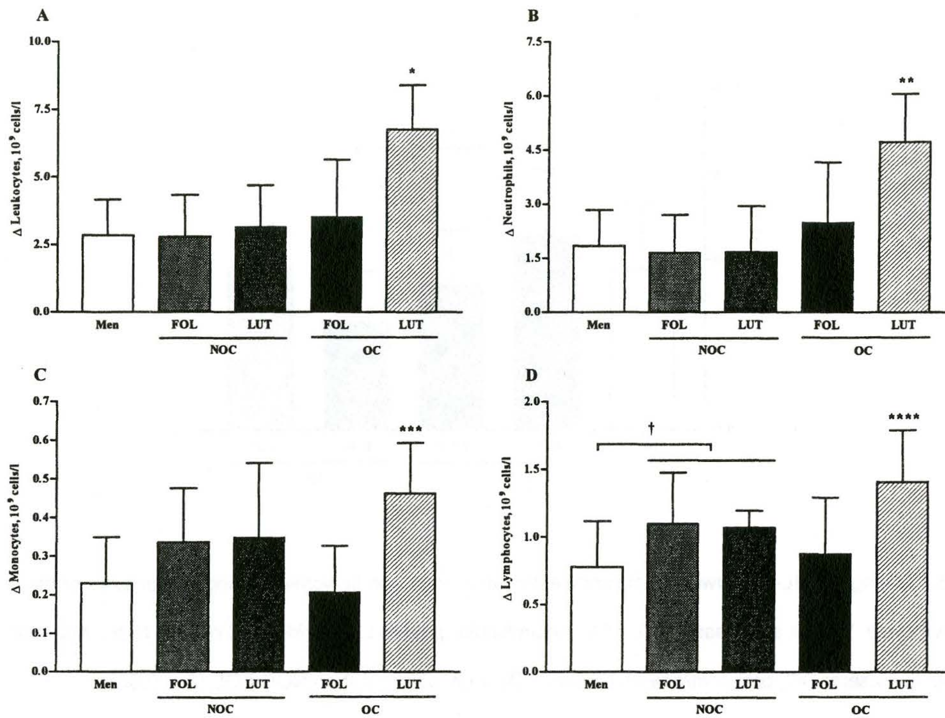

Figure 6.1 Changes in total leukocytes (A), neutrophils (B), monocytes (C), and lymphocytes (D) following endurance exercise in men and in women during two phases of the menstrual cycle. Values are mean ± SD. FOL, mid-follicular phase; LUT, mid-luteal phase; NOC, non-oral contraceptive users; OC, oral contraceptive users. *OC LUT different than OC FOL ($P = 0.02$), NOC LUT ($P = 0.01$), NOC FOL ($P = 0.005$), and men ($P < 0.001$). **OC LUT different than OC FOL ($P = 0.07$), NOC LUT ($P = 0.01$), NOC FOL ($P = 0.01$), and men ($P < 0.001$). ***OC LUT different than OC FOL ($P = 0.04$) and men ($P < 0.002$). ****OC LUT different than OC FOL ($P = 0.08$) and men ($P < 0.003$). †NOC, averaged across menstrual phases, different than men ($P = 0.07$).

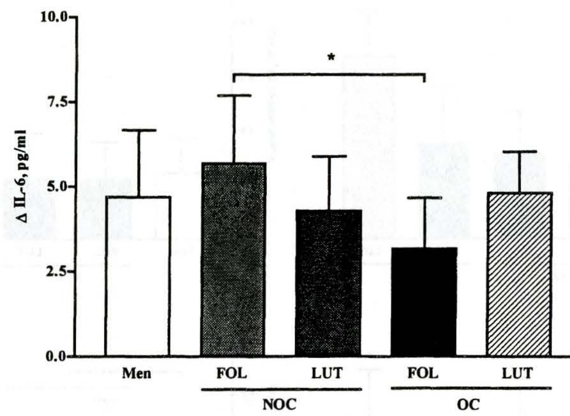

Figure 6.2 Changes in IL-6 following endurance exercise in men and in women during two phases of the menstrual cycle. Values are mean ± SD. FOL, mid-follicular phase; LUT, mid-luteal phase; NOC, non-oral contraceptive users; OC, oral contraceptive users. *OC FOL different than NOC FOL (P = 0.06).

CHAPTER 7: SUMMARY OF MAIN FINDINGS AND GENERAL DISCUSSION

This thesis was an attempt to improve our understanding of the impact of age, puberty, sex and CHO intake on immunological changes in response to exercise. The main findings of the studies conducted show that components of the innate immune system are less responsive to exercise (e.g., NK cells and IL-6) and display a faster recovery (e.g., neutrophils and IL-6) in younger children *versus* older individuals (Chapters 3 & 5). This relative resistance to innate immune perturbations during and following exercise is also evident at early *versus* late stages of puberty (Chapters 4 & 5). In addition, the studies demonstrate a sex difference in the lymphocyte and NK cell response to exercise in adolescents (Chapter 5) and in the lymphocyte response in adults (Chapter 6) in so far as these responses are greater in females *versus* males. Finally, compared to adults, children are more sensitive to the attenuating effects of CHO intake during exercise on neutrophils and NK cells (Chapter 3); the CHO effects on NK cells were also more pronounced in pre-pubertal boys *versus* early- and late-pubertal boys (Chapter 4). To facilitate interpretation of these findings and to emphasize overall effects of age and puberty in males (Chapters 3, 4, & 5) and sex-

related differences (Chapters 5 & 6) selected data from these chapters have been combined in graphical format and, where appropriate, supplemental data have been included (Appendices C-F).

7.1. Influence of AGE on Selected Immunological Changes in Response to Exercise

7.1.1. Neutrophils

The data indicate that although neutrophil mobilization during exercise without CHO intake is quite similar between children and adults (Chapter 3), between children of different ages (Chapter 5), and between genders (Chapters 5 & 6), the recovery neutrophilia is smallest in the youngest individuals, regardless of sex (Chapter 5 & Appendix C, Figure C.1).

The recovery neutrophilia observed in the older adolescents and adults is a "classical" response to high-intensity prolonged exercise (McCarthy & Dale, 1988), and is a likely result of increased cortisol levels achieved by the preceding exercise (McCarthy & Dale, 1988; Pedersen & Hoffman-Goetz, 2000). Increased cortisol levels inhibit cell adhesion to the endothelium (Cronstein *et al.*, 1992) and, consequently, recruitment of neutrophils from marginated pools. Exercise also causes the release of neutrophils from bone marrow (Suzuki *et al.*, 1999; Yamada *et al.*, 2002). Alternatively, recovery neutrophilia may reflect an inflammatory-related

response to exercise-induced muscle damage (Stupka *et al.*, 2000; Suzuki *et al.*, 1999), independent of hormone effects.

To investigate the possibility that cortisol influenced recovery neutrophilia in our subjects, a correlation was calculated between these factors. Given that cortisol exerts its effect on circulating immune cells with a certain time delay (Pedersen & Hoffman-Goetz, 2000), cortisol concentrations measured immediately after exercise were considered with the neutrophilia recorded 60 min later. In all age groups, except 9- and 10-yr-old boys and 12-yr-old girls, individuals with higher post-exercise cortisol levels demonstrated higher recovery neutrophil counts (Appendix C, Figure C.2). It is interesting to note, however, that cortisol levels in all age groups, except adult men, decreased over time (Appendix C, Figure C.3).

Although interpretation of these results is somewhat limited by a lack of functional assays, an increase in neutrophil counts is consistent with an inflammatory response in an exercise setting (Stupka *et al.*, 2000; Suzuki *et al.*, 1999). Unfortunately, few studies in children have followed neutrophil counts into recovery from exercise. Boas et al. (Boas *et al.*, 1996) found that neutrophils returned to resting levels by 60 min after a WanT in 8- to 17-yr-old boys. After 30 min of recovery from a 30-min aerobic cycling task, Shore and Shephard did not report a sustained neutrophilia in 10-yr-old boys and girls (Shore & Shephard, 1998). In 14-

to 18-yr-old adolescent males and females, however, Nieman et al. did report a significant neutrophilia 60 min after the end of a tennis practice (Nieman *et al.*, 2000). Thus, previous reports in children and adolescents, albeit with methodological differences, are consistent with the current findings. That recovery of neutrophils following exercise was faster in younger individuals is also consistent with the notion that children experience a faster physiological recovery from strenuous exercise than do adults (Baraldi *et al.*, 1991; Hebestreit *et al.*, 1993; Hebestreit *et al.*, 1996).

7.1.2. Natural killer cells

The data indicate that the magnitude of the NK cell response to exercise without CHO intake is smaller in young girls *versus* adolescent girls (Chapter 5) and in young boys *versus* adult men (Chapter 3), but consistent in boys from 9 to 14 years of age (Appendix D, Figure D.1).

NK cells are the most responsive cell type to exercise (Pedersen & Hoffman-Goetz, 2000) due to a high density of β_2-adrenergic receptors (Landmann, 1992) and their ability to acutely increase expression of β-adrenergic receptors (Maisel *et al.*, 1990). β_2-adrenergic stimulation results in NK cell detachment from endothelium (Benschop *et al.*, 1993b) likely mediated by down-regulation of adhesion molecules (Nagao *et al.*, 2000). Further, epinephrine (Kappel *et al.*, 1991) and norepinephrine (Kappel *et al.*, 1998) infusion into humans mimics the effects of exercise

on NK cells and chemical blockade of β-adrenergic receptors significantly attenuates stress-induced increases in NK cells (Klokker *et al.*, 1997). Taken together, these *in vitro* and *in vivo* observations strongly support a role for catecholamines, in particular epinephrine, in the NK cell response to exercise.

Given these observations, one might hypothesize that age-related differences in the NK cell response to exercise could be due to age-related differences in catecholamine responses. In our studies, this was not the case for either epinephrine or norepinephrine (Appendix D, Figure D.2 and D.3, respectively). Although 14-yr-old girls displayed higher post-exercise norepinephrine concentrations than 12-yr-old girls, this comparison did not reach statistical significance. Likewise, there were no significant correlations between exercise-induced changes in NK cells and either post-exercise epinephrine or norepinephrine levels (Appendix D, Figure D.4 and D.5, respectively). Thus, the data do not support a direct catecholamine-mediated effect on NK cells during high-intensity exercise in children and adolescents. However, because catecholamine concentrations were not determined in adult men, it cannot be discounted that epinephrine and/or norepinephrine responses to exercise may have been greater in the men *versus* the boys. Limited evidence from pediatric studies suggest that epinephrine and norepinephrine responses to

exercise may be smaller in children *versus* adults (Rowland, 1996; Rowland, 2005).

Consistent with the adult literature, NK cells are the most responsive cell type to exercise in children and adolescents (Boas *et al.*, 1996; Boas *et al.*, 2000b; Eliakim *et al.*, 1997; Nemet *et al.*, 2003; Nemet *et al.*, 2004; Perez *et al.*, 2001; Shore & Shephard, 1998; Wolach *et al.*, 1998). Studies from Boas and colleagues were the first to document age-related differences in NK cell responses to a WanT (Boas *et al.*, 1996) and a $\dot{V}O_{2max}$ test (Boas *et al.*, 2000a). Our findings confirm and extend these earlier observations by comparing the NK cell response to prolonged cycling under standardized experimental conditions between boys and men and between girls of different ages. Because multiple blood samples were collected during exercise, we also confirmed that changes in NK cells are relatively rapid in so far as concentrations at 60 min of exercise were no greater than at 30 min (Chapters 4 & 5). This finding is also consistent with the adult literature reporting that increases in NK cells after 60 or 90 min of constant-load exercise are no greater than after 30 min (Shek *et al.*, 1995). Our kinetic analysis of immune changes is an important methodological contribution to the pediatric literature. Additional studies are required to determine effects of exercise intensity.

7.1.3. Interleukin-6

Collectively, the data indicate that the youngest individuals, regardless of sex, experience the smallest changes in IL-6 either during (Chapter 5 and Appendix E, Figure E.1) or following (Chapter 5 and Appendix E, Figure E.2) exercise performed without CHO intake.

In recent years, IL-6 has become a "hot topic" in the exercise literature. Historically, interpretation of exercise-induced changes in systemic levels of IL-6 was made in context of immune-mediated host defences (Rhind *et al.*, 1995). However, due to novel observations on IL-6 production and release from contracting skeletal muscle (Steensberg *et al.*, 2000), it was later argued that increases in IL-6 levels during exercise may be related to regulation of glucose production (Pedersen *et al.*, 2001); a proposition previously raised by Gleeson (Gleeson, 2000).

Whether exercise-induced changes in IL-6 in children are also related to glucoregulation is unknown. To investigate this possibility, a correlation was performed between the respiratory exchange ratio (an indirect measure of whole body fat and CHO oxidation) measured just before the end of exercise and the change in IL-6 during exercise. No significant relationship was found ($r = -0.01$, $p = 0.93$; Appendix E, Figure E.3). Given that IL-6 production in contracting muscle is enhanced when glycogen content is low (Bishop *et al.*, 2001; Steensberg *et al.*, 2001), it is

likely that IL-6 is more related to muscle glycogen utilization than to an estimate of whole body CHO oxidation.

The anti-inflammatory properties of IL-6 have also been highlighted (Febbraio & Pedersen, 2002), which suggests that IL-6 production during exercise may also serve to attenuate inflammatory-related responses at the level of skeletal muscle (Ostrowski *et al.*, 1998), adipose (Lyngso *et al.*, 2002), and other tissues (Langberg *et al.*, 2002; Nybo *et al.*, 2002). Moreover, muscle-damaging exercise is associated with a continued increase in IL-6 levels during recovery, similar to recovery neutrophilia.

Most of the studies describing IL-6 responses to exercise in children and adolescents, with few exceptions (Nieman *et al.*, 2000; Perez Navero *et al.*, 1999), have been conducted by Cooper and colleagues (Nemet *et al.*, 2002; Nemet *et al.*, 2003; Scheett *et al.*, 1999; Tirakitsoontorn *et al.*, 2001). In context of the inflammatory-related properties of IL-6, these authors' working hypothesis related exercise-induced increases in IL-6 as antagonistic to anabolic mediators of growth (e.g., insulin-like growth factor-1 [IGF-1]), with the assumption that IL-6 acts as a pro-inflammatory cytokine serving a catabolic role. As eloquently argued by Febbraio and Pedersen (Febbraio & Pedersen, 2002), this assumption may not be correct. Given the anti-inflammatory properties of IL-6, smaller exercise-induced increases in IL-6 in young children as demonstrated by our studies (Chapter 5 & Appendix E, Figure

E.1) and previous pediatric studies (Chapter 1, Figure 1.6) may, therefore, reflect less pronounced inflammatory-related events (e.g., muscle damage). The IL-6 recovery data from our studies (Chapter 5, Appendix E, Figure E.2) further supports the notion that the degree of inflammatory-related responses in younger individuals is much less than in older individuals. As discussed in Chapter 5, there are, indeed, limited data in children suggesting that they are less susceptible to exercise-induced muscle damage, compared with adults (Arnett *et al.*, 2000; Soares *et al.*, 1996).

7.2. Influence of PUBERTY on Selected Immunological Changes in Response to Exercise

7.2.1. Neutrophils

Consistent with the effects of age, when subjects were grouped according to pubertal status, recovery neutrophilia was least in subjects at the earliest stages of puberty, regardless of sex (Chapter 5 & Appendix C, Figure C.4).

As previously discussed and demonstrated, cortisol is an important determinant of the magnitude of recovery neutrophilia. Given the lack of pubertal effect on recovery neutrophilia in boys at the same chronological age, it is noteworthy that cortisol changes with exercise were also not different among pubertal groups in these same boys (Chapter 4).

Only one previous study (Boas *et al.*, 1996) compared neutrophil recovery from exercise in boys of varying physical maturity and none have made similar comparisons among females. In the study by Boas et al. (1996), neutrophil counts returned to resting levels by one hour after a WanT in three separate pubertal groups. Lack of a recovery neutrophilia following very short duration exercise (e.g., 30 sec) is consistent with the notion that exercise duration is an important determinant in the magnitude of recovery neutrophilia (McCarthy & Dale, 1988; Pedersen & Hoffman-Goetz, 2000). In addition, it is unlikely that 30 sec of "all-out" cycling, involving primarily concentric contractions, would lead to appreciable muscle damage.

Given that recovery neutrophilia is associated with advancing age and physical maturity, and may be secondary to the degree of exercise-induced muscle damage, muscle mass may, therefore, be an important factor to consider. To investigate the possibility that inter-individual differences in muscle mass contributed to differences in the inflammatory-related response to exercise, a correlation was calculated between recovery neutrophilia and fat-free body mass (assessed by bioelectrical impedance analysis, see Chapter 3), an estimate of muscle mass. When subjects from Chapters 3 and 5 were considered, recovery neutrophilia was weakly, but significantly, correlated with fat-free mass ($r = 0.27$, $p = 0.02$, Appendix C, Figure C.5). It may be, therefore, that older or more

physically mature individuals possess the capacity to recruit more muscle mass during exercise, thus contributing, in part, to a greater inflammatory-related response.

7.2.2. *Natural killer cells*

The data indicate that boys at early to mid stages of puberty experience smaller increases in NK cells in response to exercise, compared with either pre- or post-pubertal boys (Appendix D, Figure D.6). Likewise, girls at early stages of puberty also experienced an attenuated NK cell response to exercise, compared with girls of more advanced physical development (Chapter 5).

It is unclear why boys at early to mid stages of physical maturity should experience a blunted NK cell response to endurance exercise. Following 30 sec of "all-out" cycling, Boas et al. (1996) reported a graded effect of puberty on changes in NK cells in so far as the magnitude of response was smallest in pre-pubertal boys, intermediate in peri-pubertal boys, and largest in post-pubertal boys. However, these pubertal categories reflected advancing chronological age (8-17 years). In our studies, changes in the relative proportions of NK cells in response to exercise were smallest in boys designated as early-pubertal (i.e., Tanner stage 2), even when chronological age was the same for all pubertal groups (Chapter 4). That girls at the earliest stages of breast development also had considerably smaller NK cell responses, compared

with girls of advanced breast development, despite the same chronological age (Appendix D, Figure D.7), is consistent with the male data. Collectively, these findings suggest that children are relatively resistant to major stress-induced perturbations in NK cells; a phenomenon that may be exasperated during puberty in boys and girls.

It has been demonstrated that prepubescent, compared with mature, rats are relatively resistant to adrenergic suppression of NK cells (Page & Ben Eliyahu, 2000), suggesting an inherent deficiency in the cellular response to mediators of stress. Whether this invulnerability to stress becomes more pronounced during puberty is unknown. In humans, β-adrenergic receptor density on lymphocytes increases with chronological age throughout childhood and adolescence (Galal, 1989; Reinhardt *et al.*, 1984), as does the isoprenaline-induced increase in intracellular levels of cyclic AMP (Galal, 1989). It is unknown, however, whether similar changes in receptor density and/or post-receptor activity are influenced by puberty *per se*. In adults, acute exercise increases expression of β-adrenergic receptors on NK cells (Maisel *et al.*, 1990), but similar data are unavailable in children. It is quite probable, however, that similar cellular changes do occur in exercising children, and if, or to what extent, these changes are further influenced by puberty would be interesting questions for future studies.

Alternatively, interactions between GH and NK cells may be altered during puberty. As described in Chapter 4, exercise-induced changes in NK cells were significantly correlated with post-exercise GH levels, and the GH response to exercise was largest in boys at mid to late (i.e., post) puberty. When the adolescent boys from Chapter 5 are also considered, GH responses are largest in post-pubertal (i.e., Tanner stage 5) boys (Appendix D, Figure D.8), and exercise-induced changes in NK cells remain positively correlated with post-exercise GH levels (Appendix D, Figure D.9). Although GH infused into adults does not significantly increase NK cell proportions or concentrations (Kappel *et al.*, 1993), interactions between GH and the immune system may be different in children (Bozzola *et al.*, 1982; Bozzola *et al.*, 1997). Possible differences in the effects of GH on NK cells in children may also be related to a lower expression of GH receptors on NK cells in children *versus* adults (Valerio *et al.*, 1997).

***7.2.3.** Interleukin-6*

The data indicate that, similar to the effects of age, IL-6 responses during (Appendix E, Figure E.4) and following (Appendix E, Figure E.5) exercise are smallest in pre-pubertal individuals and gradually increase with progressing physical maturity.

That the magnitude of the IL-6 response to exercise is associated with advancing chronological age and physical maturity suggests that this

cytokine may be important in global processes of growth. Indeed, transgenic mice expressing high levels of circulating IL-6 have reduced growth rates, which are partially reversed with administration of antibody to the IL-6 receptor (de Benedetti *et al.*, 1997). In children and adolescents with systemic juvenile rheumatoid arthritis, a condition characterized by stunted growth, IGF-1 levels are lower than normal and correlate negatively with IL-6 levels (de Benedetti *et al.*, 1997). In line with these observations, transgenic mice over-expressing IL-6 experience significant muscle atrophy due to increased proteolytic enzyme action, which can be reversed with administration of antibody to the IL-6 receptor (Tsujinaka *et al.*, 1996). On the other hand, IL-6 induces expression of vascular endothelial growth factor (Cohen *et al.*, 1996), a potent angiogenic factor. Moreover, IL-6 is constitutively expressed in skeletal muscle (Starkie *et al.*, 2001), and released during contraction (Steensberg *et al.*, 2000). Given these paradoxical roles of IL-6, it is tempting to speculate that the comparatively smaller exercise-induced increases in IL-6 in growing children are sufficient to serve an adaptive function, without compromising other anabolic mediators (e.g., IGF-1). A relatively faster recovery of IL-6 following exercise in the youngest individuals (Appendix E, Figure E.5) would also serve to minimize disruption to anabolic agents that are increased during recovery from exercise (e.g., IL-8, see Chapter 5).

7.3. Influence of SEX on Selected Immunological Changes in Response to Exercise

7.1.1. Lymphocytes

The data indicate that adolescent and adult females experience a greater overall lymphocytosis to exercise, compared with adolescent and adult males (Chapters 5 & 6, respectively). Interestingly, no sex difference was observed in younger children. For comparison purposes, the data from Chapters 5 and 6 have been combined for a graphical representation of lymphocyte responses (Appendix F, Figure F.1). Although exercise duration was 30 min longer in the study in Chapter 6, changes in lymphocytes after 90 min of exercise are no greater than after 60 min (Shek *et al.*, 1995).

Exercise-induced lymphocytosis is driven by a preferential recruitment of NK cells into the peripheral circulation, thereby increasing their relative proportion. Consequently, the relative proportion of total $CD3^{+}$ cells declines and that of $CD3^{-}CD19^{+}$ cells remains constant or decreases only slightly (see Chapter 5). Previous work in adults reported that women, compared with men, displayed a greater increase in total lymphocyte counts following standardized exercise (De Lanne *et al.*, 1960). In contrast, other studies have not found sex-related differences (Barriga *et al.*, 1993; Meksawan *et al.*, 2004; Moyna *et al.*, 1996; Venkatraman *et al.*, 1997). In these latter studies, it was unclear whether

menstrual cycle phase, activity level, dietary intake and use of OCT were controlled, which probably restricted the authors' ability to identify true differences.

Under non-exercise conditions, a number of lymphocyte-related functions are different between men and women and vary over the menstrual cycle (see Chapter 1, Section 1.2.4). Therefore, one might hypothesize that the presence of, and fluctuations in, reproductive hormones may influence lymphocyte changes in response to exercise. However, there was no difference in the lymphocyte response between the mid-follicular phase and the mid-luteal phase of the menstrual cycle in adult women not taking OCT (Chapter 6). Moreover, eight days of estrogen supplementation in healthy men, which increased plasma estradiol concentration, did not alter the lymphocyte response to exercise (Timmons *et al.*, 2003). Thus, sex differences in factors other than reproductive hormones must be important in the effects of exercise on lymphocytes in humans. Given that only the NK cell response to exercise displayed a significant sex difference among adolescents, factors related to this cell type may help explain sex differences in the overall lymphocytosis.

7.3.2. Natural killer cells

The data indicate that adolescent girls experience a greater NK cell response to exercise than adolescent boys (Chapter 5). Given the

previously described observations that adult females had similar lymphocyte responses between phases of the menstrual cycle and estrogen supplementation did not influence lymphocyte responses in men, fluctuations in sex hormones may not be the primary mechanism contributing to NK cell differences among adolescents.

As discussed above, catecholamines, in particular epinephrine, drive the NK cell response and overall lymphocytosis to exercise. Paradoxically, epinephrine responses to exercise are generally smaller in women than in men in some (Carter *et al.*, 2001; Davis *et al.*, 2000; Horton *et al.*, 1998), but not all (Mittendorfer *et al.*, 2002; Roepstorff *et al.*, 2002) studies. However in our subjects, adolescent males had greater overall epinephrine levels than females, which is consistent with the pediatric literature (Weise *et al.*, 2002), and the post-exercise epinephrine concentration was greater in boys *versus* girls (Appendix F, Figure F.2). In contrast, there was no gender difference in the norepinephrine response to exercise (Appendix F, Figure F.3). That post-exercise epinephrine levels were higher in males *versus* females is opposite to what might be anticipated given the larger NK cell response in the girls. One possibility to help reconcile this paradox may be at the receptor level.

It has been shown that, compared with men, women demonstrate greater lymphocyte β_2-adrenergic receptor density (Wheeldon *et al.*, 1994) and post-receptor activity (Halper *et al.*, 1984; Mills *et al.*, 1996). Thus, for

a given concentration of epinephrine, females may experience a greater down-regulation of adhesion molecule expression (Nagao *et al.*, 2000) and, therefore, greater NK cell mobilization. Whether similar sex differences in β-adrenergic receptor density and/or activity exist among adolescents is unknown. Moreover, it has been demonstrated that NK cell activity of whole blood is less in women than in men (Benschop *et al.*, 1993a; Yovel *et al.*, 2001). Therefore, greater stress-induced increases in NK cells in females may help offset possible deficiencies in NK cell function. However, a study in adolescents did not demonstrate sex differences in one aspect of resting NK cell function (Bartlett *et al.*, 1998). Clearly, more work is required to elucidate the mechanisms and biological significance of sex differences in NK cell responses to physical stress.

7.4. Influence of CHO INTAKE on Selected Immunological Changes in Response to Exercise

Prior to the studies conducted in this thesis, no placebo-controlled research had been performed on the effect of CHO intake on immunological changes in response to exercise in young children or adolescents. The data indicate that neutrophils and NK cells are more sensitive to CHO intake in children *versus* adults (Chapter 3) and that NK cells are more sensitive to CHO intake in pre-pubertal *versus* early- and late-pubertal boys (Chapter 4).

The mechanisms by which exogenous CHO affects circulating immune cells, and NK cells in particular, are unclear. Nieman (Nieman, 1998) proposed that maintained or increased blood glucose levels during exercise, due to CHO intake, results in a blunted stress hormone (e.g., epinephrine and cortisol) response. Consequently, there is a reduced hormonal effect on sequestered cell populations and, thus, less mobilization. Although in most immune studies CHO intake has not proven to influence catecholamine responses to exercise (Henson *et al.*, 1999; Nieman *et al.*, 1997), CHO intake does appear to lower the cortisol response (Bacurau *et al.*, 2002; Bishop *et al.*, 2002; Green *et al.*, 2003; Henson *et al.*, 1999; Nieman *et al.*, 1997). Moreover, it has been suggested that high-intensity exercise lasting longer than two hours is required before significant effects of CHO intake on changes in immunity are observed (Nieman, 2000).

In our adult group, CHO intake did not influence NK cell responses to 60 min of high-intensity exercise (Chapter 3), which is consistent with the above hypothesis that exercise must be more prolonged (i.e., >120 min) in order to observe an impact of CHO intake. However, this is clearly not the case in children. In addition to CHO-attenuating effects on NK cells after only 30 min of exercise, CHO intake did not influence epinephrine or cortisol responses to exercise (Chapter 4), which is in contrast to the proposed hormone-immune relationships in adults. The

GH response to exercise, however, was significantly attenuated with CHO intake, and exercise-induced changes in NK cells were significantly correlated with post-exercise GH concentrations.

In Chapter 4, the effects of CHO intake on NK cells were most notable in pre-pubertal boys and restricted to the $CD3^{-}CD56^{dim}$ subset. Moreover, plasma glucose levels did not fall to hypoglycemic levels during exercise without CHO intake, and attenuation of changes in NK cell proportions with CHO intake was evident in the absence of raised plasma glucose levels. These observations suggest that provision of exogenous CHO may have direct effects on NK cells, influencing their recruitment into the peripheral circulation.

Lymphocytes, similar to skeletal muscle cells, posses intracellular stores of glucose transporters that can be translocated to the plasma membrane to facilitate glucose uptake (Jacobs *et al.*, 1989). An acute bout of exercise increases glucose uptake by mononuclear immune cells (Bieger *et al.*, 1980). An increase in glucose uptake and utilization by immune cells, in particular NK cells, during exercise may affect intracellular pathways important in cell adhesion. Whether constitutive expression of glucose transporters and/or their translocation from intracellular stores in NK cells vary with age or pubertal status is unknown. However, insulin and contraction-stimulated glucose transport in skeletal muscle is greater in immature *versus* mature rats (Barnard *et al.*, 1992;

Gulve *et al.*, 1993) . The existence of similar maturity-related differences in glucose transport in NK cells might help explain the observed puberty-related differences in CHO effects on this cell population in boys.

7.5. Novelty of the Findings

The general objective of this thesis was to advance the state of knowledge regarding immunological changes in response to exercise performed with and without CHO intake during childhood, adolescence and young adulthood (see Chapter 1, Section 1.5.1.). To confirm that this objective was achieved, Table 7.1 summarizes the novel findings from the studies in this thesis and their contribution to the literature.

Table 7.1 *Summary of novel findings.*

Chapter	Novel Findings	Contribution to the Literature
3	Differences between children and adults in innate immune changes to, and recovery from, exercise	First study to directly compare children and adults; Children's immune system is less responsive to exercise; Children's immune system experiences faster recovery
	CHO intake attenuates immune changes to exercise in children	First study to determine effects of CHO intake on immune changes in children; Neutrophils and NK cells are more sensitive to CHO intake in boys *versus* men
4	Puberty influences NK cell responses to exercise *and* CHO intake in boys	First study to isolate the effects of puberty on NK cell responses to exercise; NK cells (proportions) are less responsive to exercise performed without CHO intake in pre- and early-pubertal boys; The sensitivity of NK cells (counts) to CHO intake is greatest in pre-pubertal boys
	NK cell subsets (i.e., 56^{dim} and 56^{bright}) are differentially affected by exercise *and* CHO intake	First study to identify alterations in 56^{dim} and 56^{bright} cells during and following prolonged cycling; The alteration in 56^{dim} and 56^{bright} cells during recovery from acute exercise is consistent with a reduction in NK cell cytotoxicity; First study to determine the effects of CHO intake on 56^{dim} and 56^{bright} responses to exercise; The attenuation of NK cells by CHO intake is due to attenuation of 56^{dim} and not 56^{bright} cells
5	Differences in immune changes to exercise between children and adolescents, between boys and girls, and between stages of puberty	First study to determine age effects on immune changes to exercise during childhood and adolescence; Age-related differences (older > younger) in cellular and soluble components of immune system are more pronounced in girls; First study to determine puberty effects on immune changes to exercise during childhood and adolescence; Puberty-related differences (more mature > less mature) in immune changes to exercise are consistent in boys and girls; First study to determine sex effects on immune changes to exercise during childhood and adolescence; Increases in total leukocytes, lymphocytes, and NK cells are more pronounced in girls *versus* boys
6	Differences in immune changes to exercise between men and women; Differences in immune changes to exercise between OC users and non-users	Increases in lymphocytes during prolonged cycling are more pronounced in women *versus* men; First study to compare OC users and non-users and men; Increases in IL-6 during exercise are more pronounced in OC non-users *versus* OC users only during the follicular phase of the menstrual cycle; OC use increases magnitude of immune changes to exercise compared with non-users and men; Inclusion of females in exercise and immune studies should control for menstrual phase and OC use

7.6. Future Research Directions

Throughout this thesis, discussion has revolved around observations indicating that young children are relatively resistant to major exercise-induced perturbations to their immune system. Given the antagonism between inflammation and growth, these findings suggest that the growing child may possess inherent mechanisms to alleviate inflammatory-related responses to physiological stress. Therefore, future research needs to clarify the orchestration between exercise, the immune system, and growth-related processes. Is there an optimal balance of exercise duration and intensity to promote anabolic-like, but limit catabolic-like responses? Although the biological significance of this question for healthy children is unclear, the answer is particularly germane for children with, or recovering from, an immune-related disease (e.g., acute lymphoblastic leukemia). Appropriate investigations on the interaction between exercise, the immune system and growth will also require longitudinal analyses.

Traditionally, exercise immunology researchers have been interested in the relationship between physical activity level and risk of infection or illness in adults. Similar investigations in children and adolescents are severely lacking. Consequently, this author believes that one of the most promising areas for future research will be to understand the relationship between physical activity and immune development in

early childhood (e.g., < 5 years of age). Given the emerging problems of physical inactivity and overweight, and the possible links between immune dysfunction and prevalence of adult-like health disorders during childhood, an improved understanding of the relationship between physical activity and immune health during the growing years is essential. Moreover, significant immune development occurs during the first years of life, a period of scheduled immunization for many children. Therefore, it will be important to elucidate the impact of physical activity level in young children on immune development, prevalence of infection and illness, and the adequacy of immunization.

Finally, new insights into immune-related changes during acute exercise (e.g., muscle-derived IL-6) are providing evolving interpretations of the role for the immune system in metabolic adjustments during exercise. Likewise, exercise affects the metabolic profile (e.g., increase glucose uptake and lactate production) of circulating immune cells. A fruitful area for future research, therefore, will be to determine the extent to which metabolic changes in circulating immune cells parallel those induced in skeletal muscle during exercise. In this regard, circulating immune cells may serve as a relatively non-invasive surrogate to skeletal muscle for the measurement of substrate utilization, enzyme activity, and gene expression induced by exercise, diet or other interventions.

7.7. References

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APPENDIX A: FLOW CYTOMETRY ANALYSIS OF LYMPHOCYTE SUBSETS

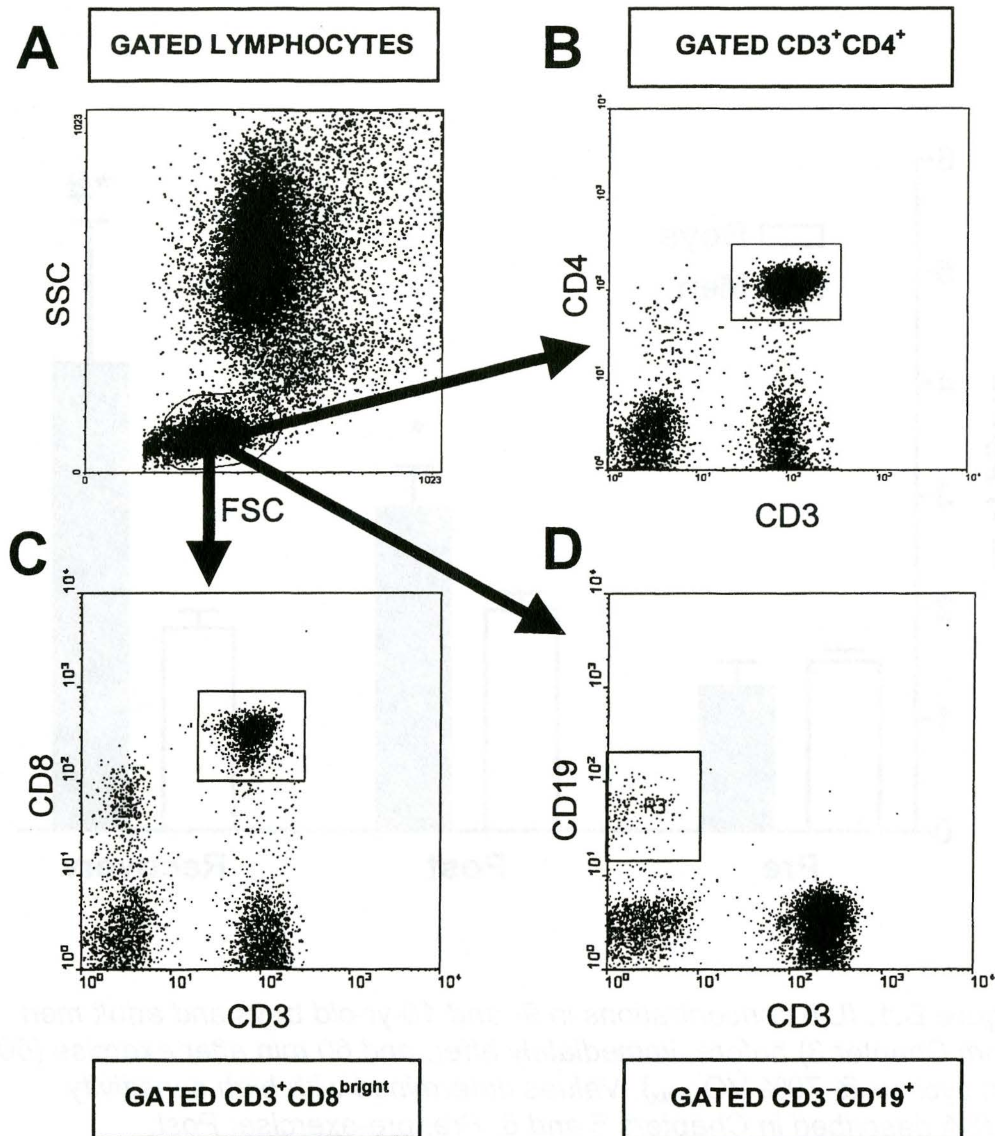

Figure A.1. *Example of flow cytometry gating procedure to identify lymphocyte subsets in whole blood. Lymphocytes are gated based on forward- and side-scatter light characteristics (**A**). Two colour dot-plots are created from lymphocyte gate to identify the relative expression of antigen on **B)** T_{helper} ($CD3^{+}CD4^{+}$), **C)** $T_{cytotoxic}$ ($CD3^{+}CD8^{bright}$), and **D)** B cells ($CD3^{-}CD19^{+}$).*

APPENDIX B: HIGH SENSITIVITY ELISA DETERMINATION OF IL-6

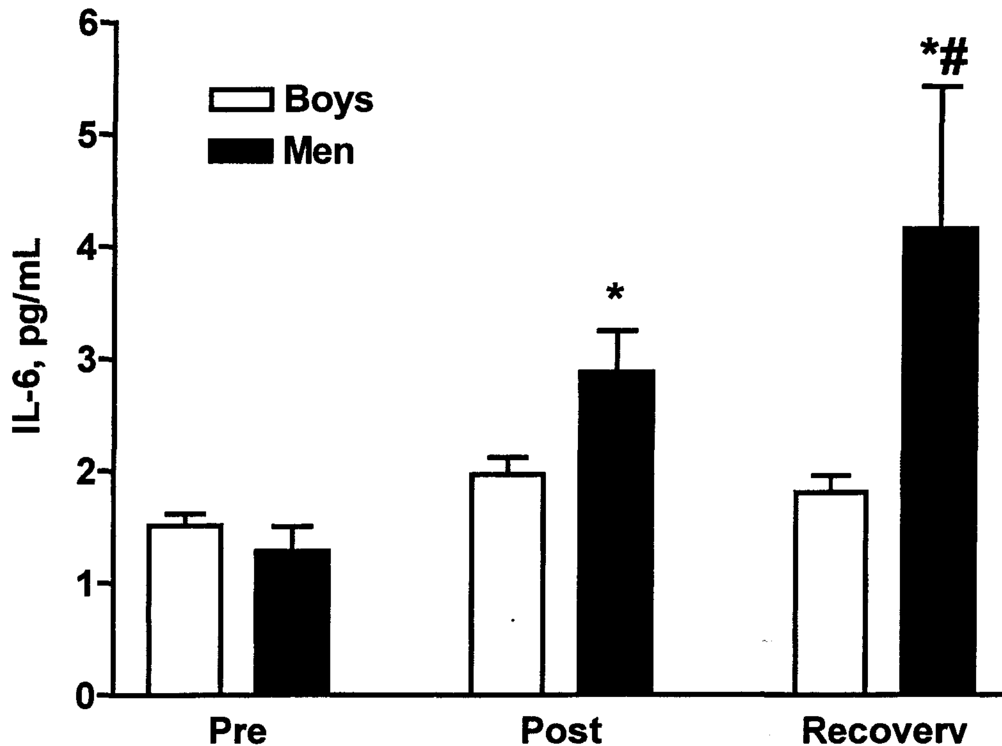

Figure B.1. *IL-6 concentrations in 9- and 10-yr-old boys and adult men (from Chapter 3) before, immediately after, and 60 min after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$). Values determined with high-sensitivity ELISA described in Chapters 5 and 6. Pre, pre-exercise; Post, immediately after exercise; Recovery, 60 min after end of exercise. Main effect group, p=0.09; Main effect time, p=0.008; Group × time interaction, p=0.039; *Significantly different from Pre; #Significant difference between boys and men.*

APPENDIX C: FACTORS ASSOCIATED WITH RECOVERY NEUTROPHILIA

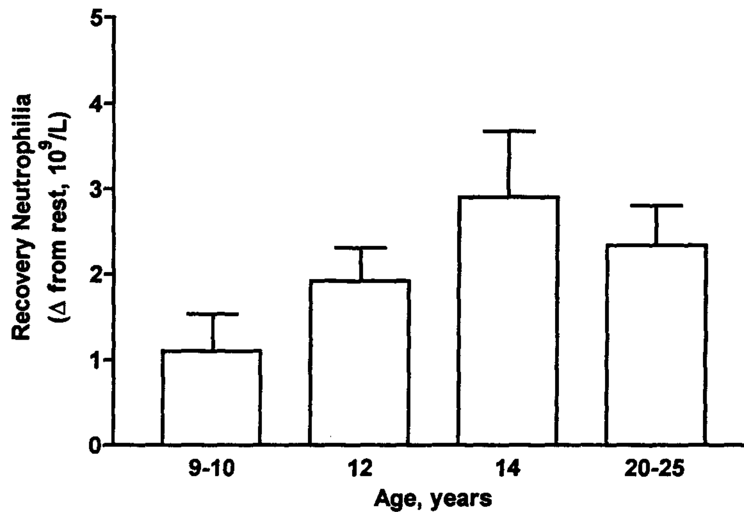

Figure C.1. *Recovery neutrophilia determined 60 min after exercise (60 min cycling @ 70% VO_{2max}) in males of different ages. Data redrawn from Chapters 3 and 5.*

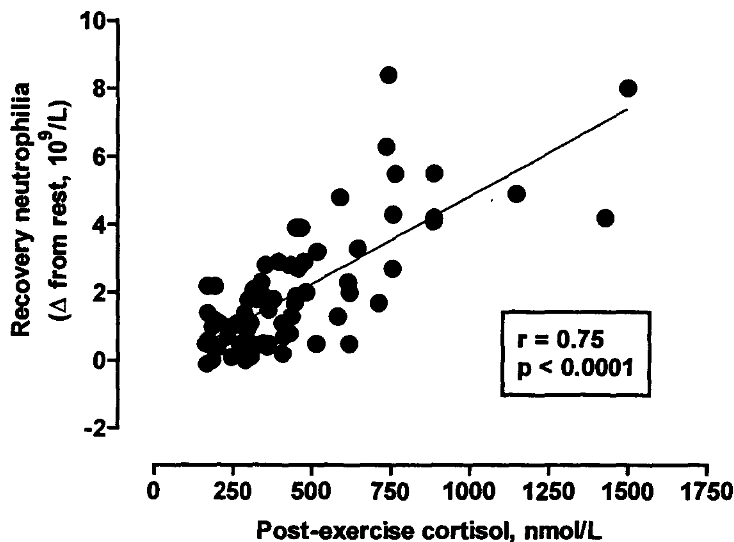

Figure C.2. *Relationship between recovery neutrophilia determined 60 min after exercise (60 min cycling @ 70% VO_{2max}) and post-exercise cortisol concentration in 9- and 10-, 12-, and 14-yr-old boys, 12- and 14-yr-old girls, and adult men. Data redrawn from Chapters 3 and 5 and unpublished observations.*

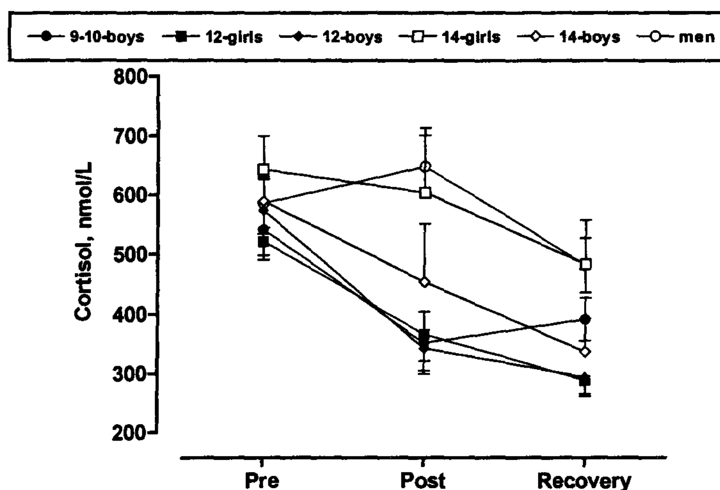

Figure C.3. *Cortisol concentrations before, immediately after and 60 min after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 9- and 10-yr-old boys (closed circles), 12-yr-old girls (closed squares), 12-yr-old boys (closed diamonds), 14-yr-old girls (open squares), 14-yr-old boys (open diamonds), and adult men (open circles). Pre, pre-exercise; Post, immediately after exercise; Recovery, 60 min after end of exercise. Data redrawn from Chapter 4 and unpublished observations.*

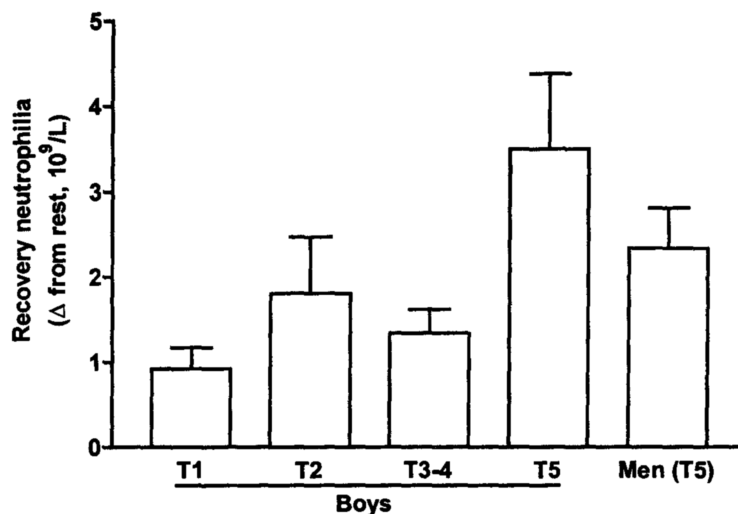

Figure C.4. *Recovery neutrophilia determined 60 min after exercise (60 min cycling @ 70% VO_{2max}) in males of different physical maturity. Data redrawn from Chapters 3 and 5.*

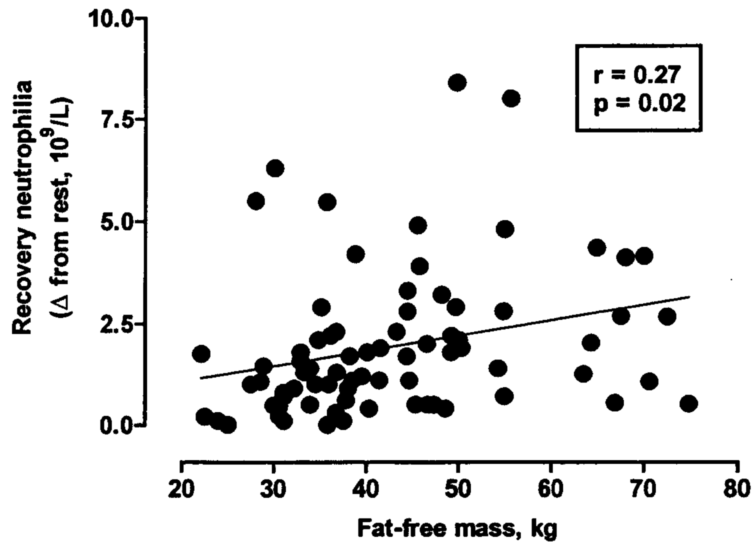

Figure C.5. *Relationship between recovery neutrophilia determined 60 min after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) and fat-free mass (estimate of muscle mass) in 9- and 10-, 12-, and 14-yr-old boys, 12- and 14-yr-old girls, and adult men. Data redrawn from Chapters 3 and 5 and unpublished observations.*

APPENDIX D: FACTORS ASSOCIATED WITH NK CELL RESPONSES

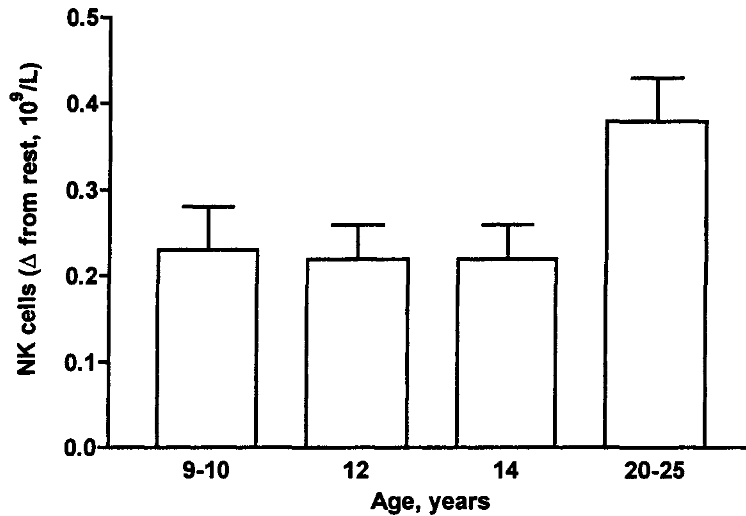

Figure D.1. *Magnitude of change in NK cells determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in males of different ages. Data redrawn from Chapters 3 and 5.*

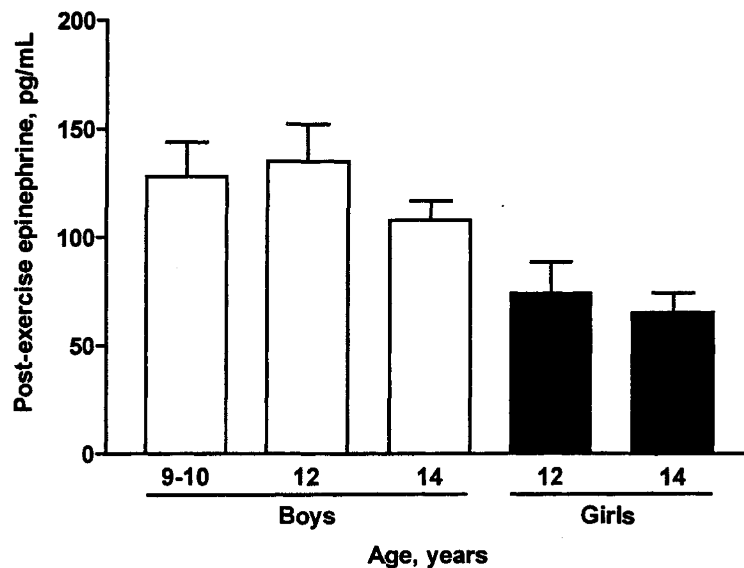

Figure D.2. *Epinephrine concentrations determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 9- and 10-, 12-, and 14-yr-old boys, 12- and 14-yr-old girls. Data redrawn from Chapter 4 and unpublished observations.*

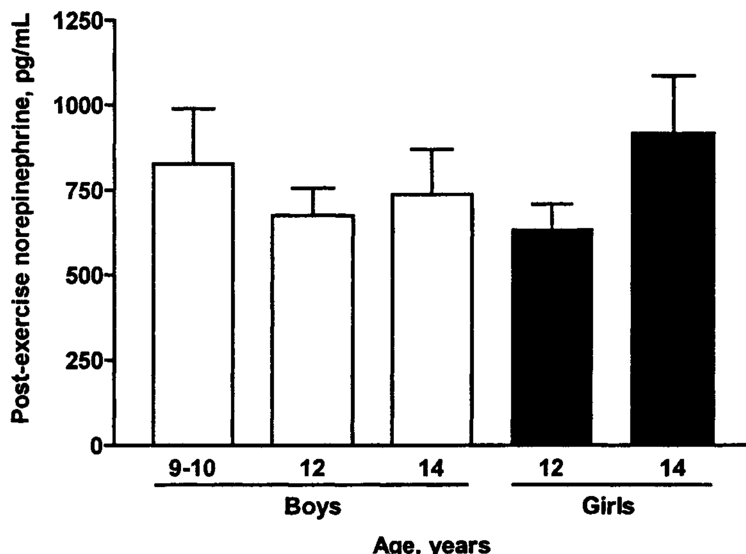

Figure D.3. *Norepinephrine concentrations determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 9- and 10-, 12-, and 14-yr-old boys, 12- and 14-yr-old girls. Data redrawn from Chapter 4 and unpublished observations.*

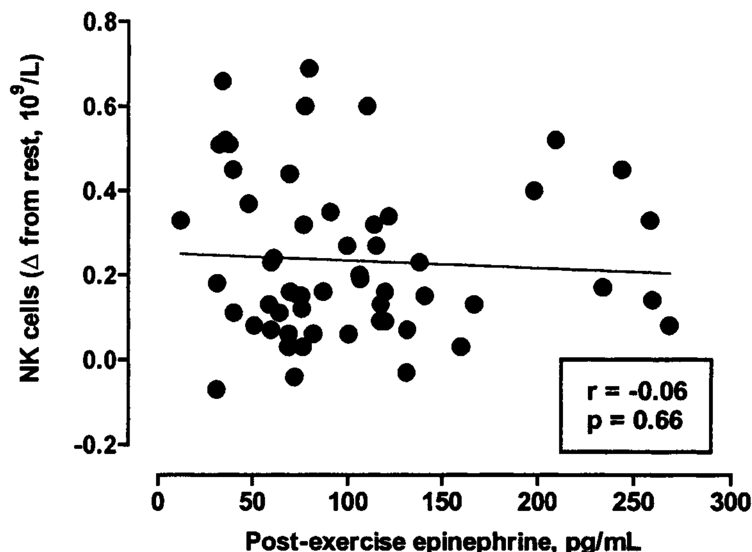

Figure D.4. *Relationship between magnitude of change in NK cells determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) and epinephrine concentration at the same time point in 9- and 10-, 12-, and 14-yr-old boys, 12- and 14-yr-old girls. Data redrawn from Chapters 3 and 5 and unpublished observations.*

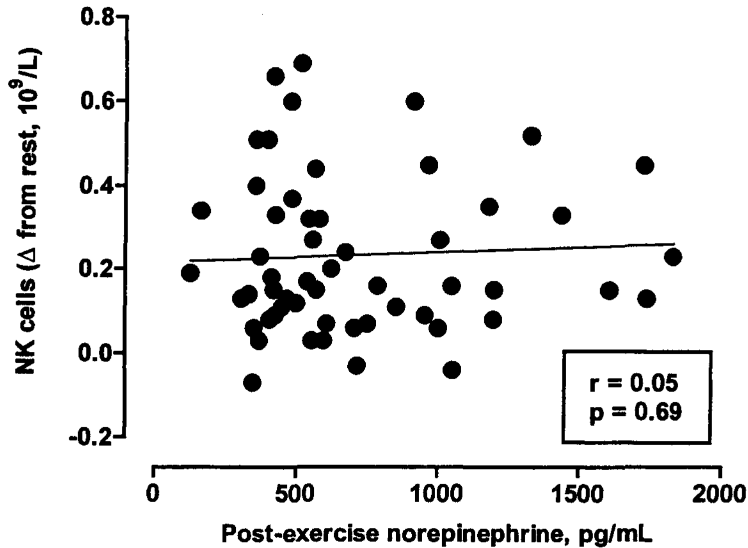

Figure D.5. *Relationship between magnitude of change in NK cells determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) and norepinephrine concentration at the same time point in 9- and 10-, 12-, and 14-yr-old boys, 12- and 14-yr-old girls. Data redrawn from Chapters 3 and 5 and unpublished observations.*

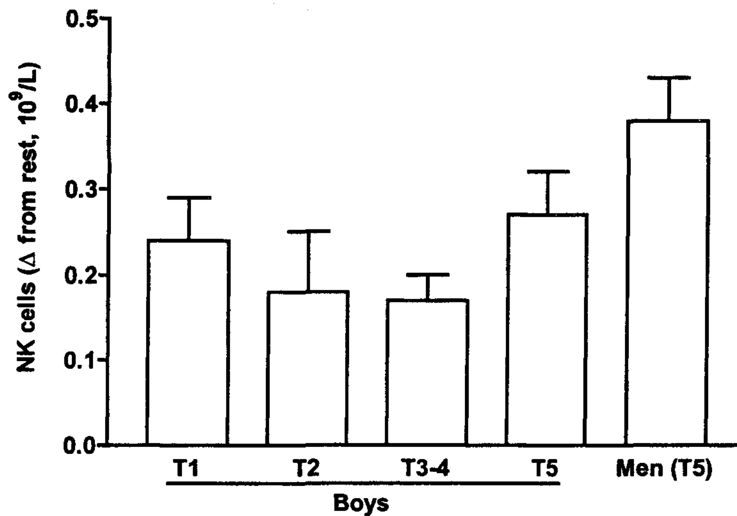

Figure D.6. *Magnitude of change in NK cells determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in response to 60 min exercise @ 70% in males of different physical maturity. Data redrawn from Chapters 3 and 5.*

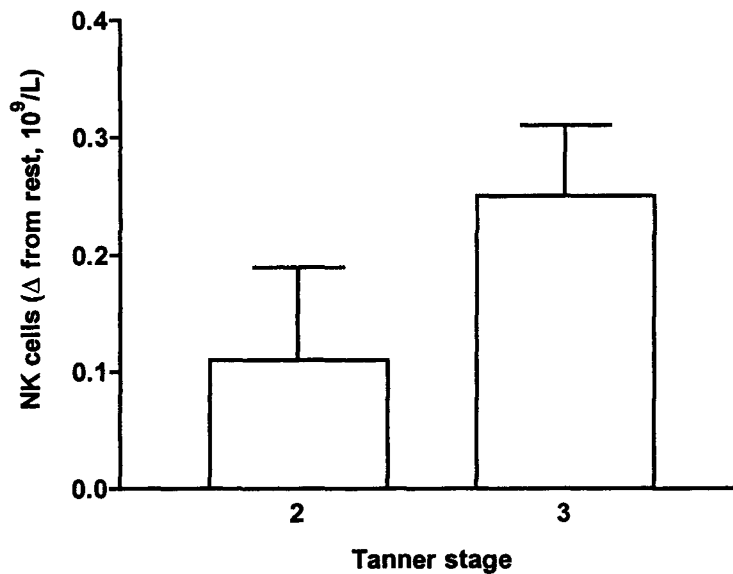

Figure D.7. *Magnitude of change in NK cells determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 12-yr-old girls of different physical maturity. Data redrawn from Chapter 5.*

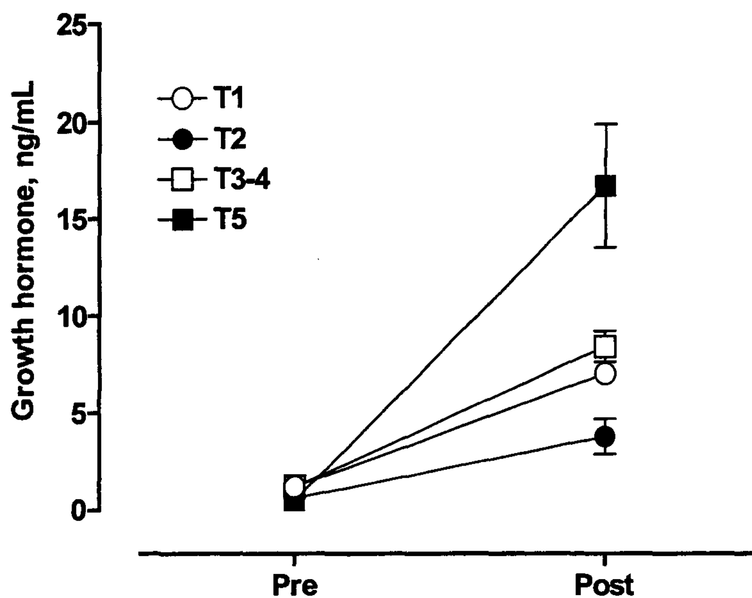

Figure D.8. *Growth hormone concentrations before and immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 12- and 14-yr-old boys of different physical maturity. Pre, pre-exercise; Post, immediately after exercise. Data redrawn from Chapter 4 and unpublished observations.*

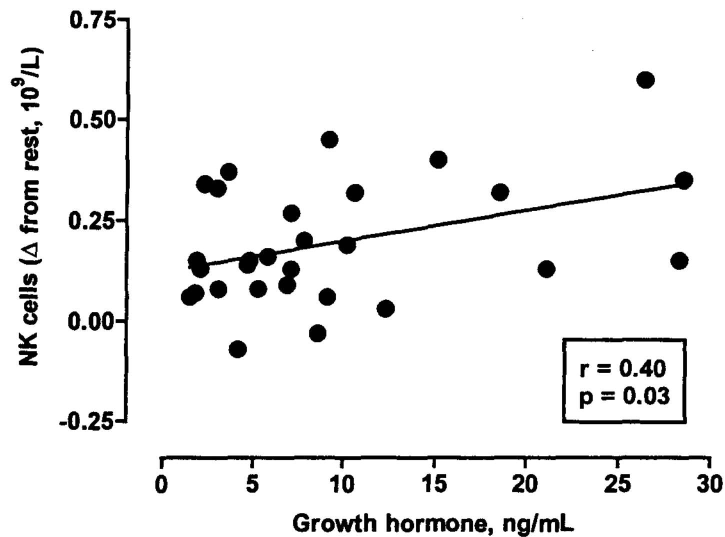

Figure D.9. *Relationship between magnitude of change in NK cells determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) and growth hormone concentrations at the same time point in 12- and 14-yr-old boys. Data redrawn from Chapters 4 and 5 and unpublished observations.*

APPENDIX E: FACTORS ASSOCIATED WITH IL-6 RESPONSES

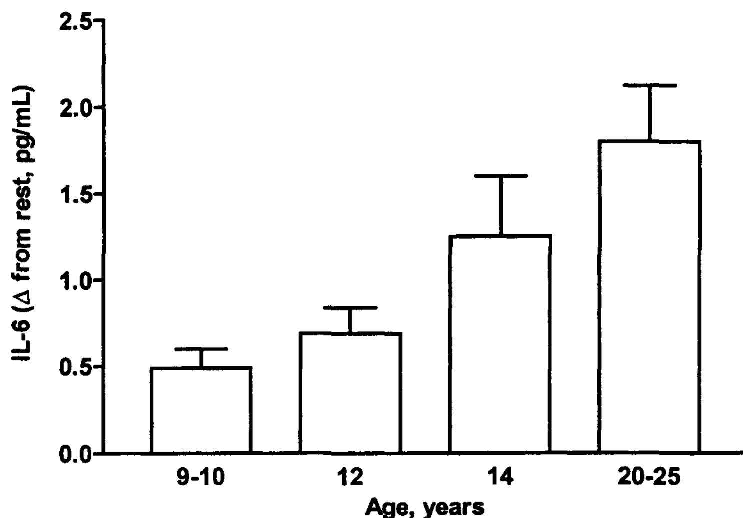

Figure E.1. *Magnitude of change in IL-6 determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in males of different ages. Data redrawn from Chapter 5 and unpublished observations.*

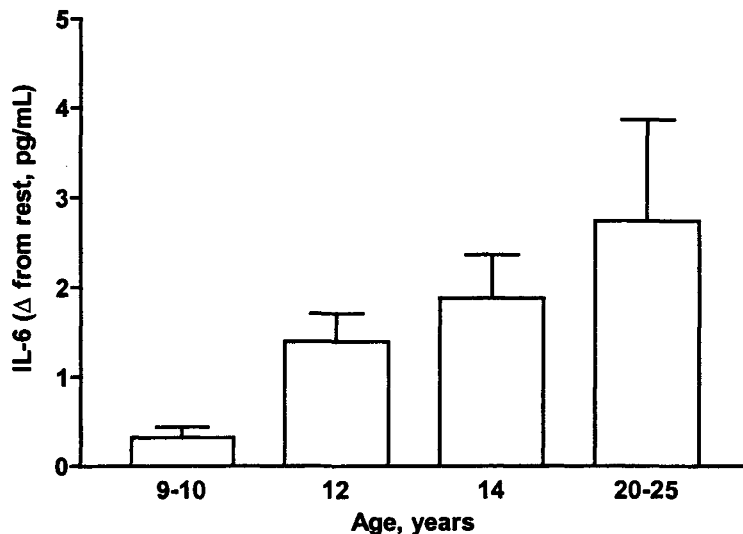

Figure E.2. *Magnitude of change in IL-6 determined 60 min following the end of exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in males of different ages. Data redrawn from Chapter 5 and unpublished observations.*

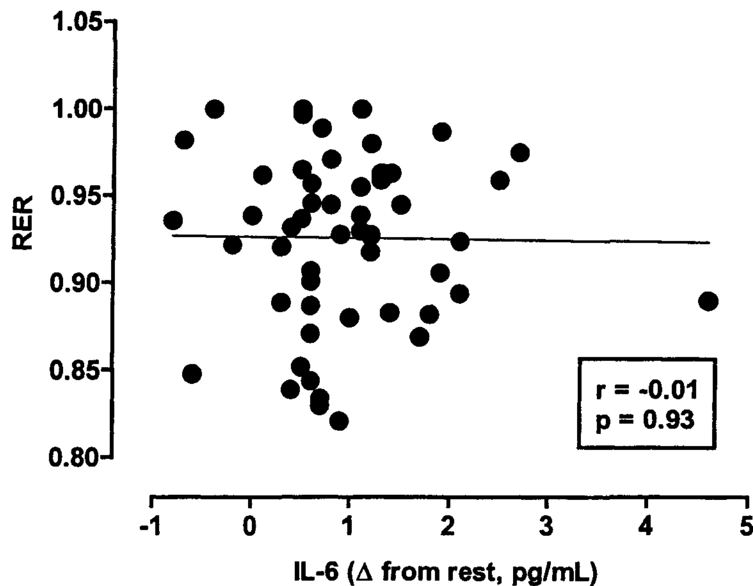

Figure E.3. *Relationship between respiratory exchange ratio and change in IL-6 determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 12- and 14-yr-old boys and 12- and 14-yr-old girls. Data redrawn from Chapter 5 and unpublished observations.*

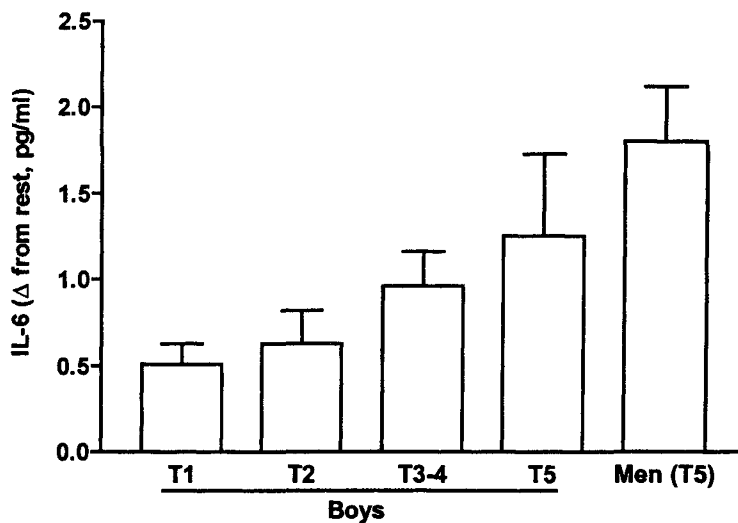

Figure E.4. *Magnitude of change in IL-6 determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in males of different physical maturity. Data redrawn from Chapter 5 and unpublished observations.*

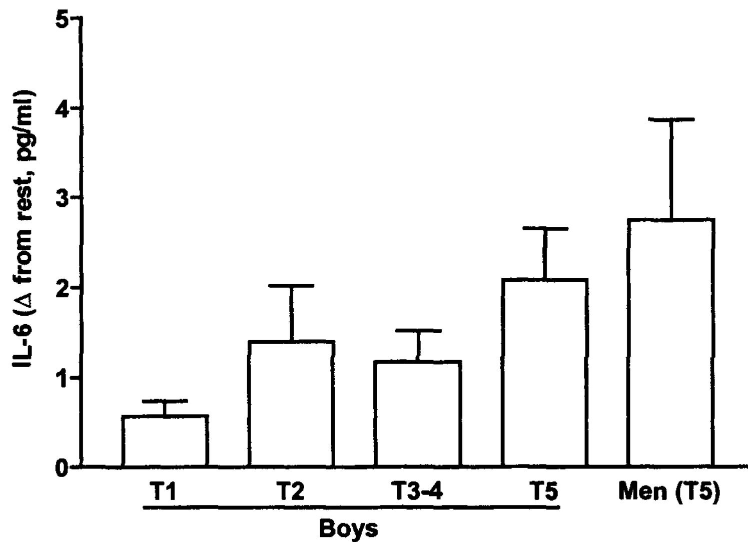

Figure E.5. *Magnitude of change in IL-6 determined 60 min following the end of exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in males of different physical maturity. Data redrawn from Chapter 5 and unpublished observations.*

APPENDIX F: FACTORS ASSOCIATED WITH SEX DIFFERENCES

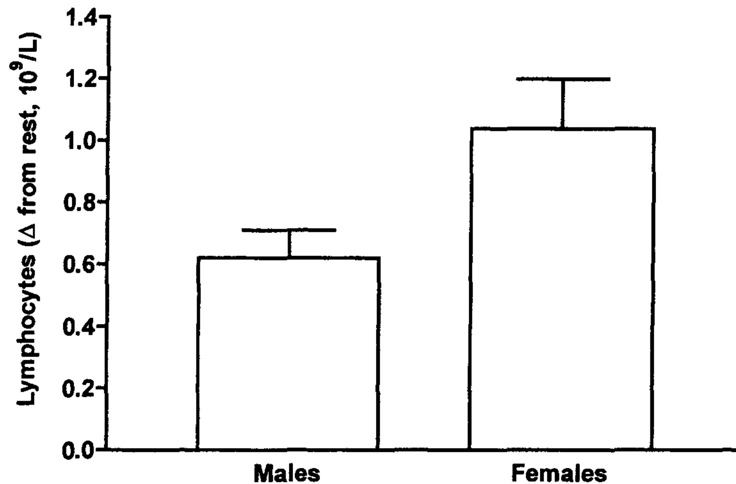

Figure F.1. *Magnitude of change in lymphocyte counts determined immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$ and 90 min cycling @ 65% $\dot{V}O_{2max}$) in males and females. Data redrawn from Chapters 5 and 6.*

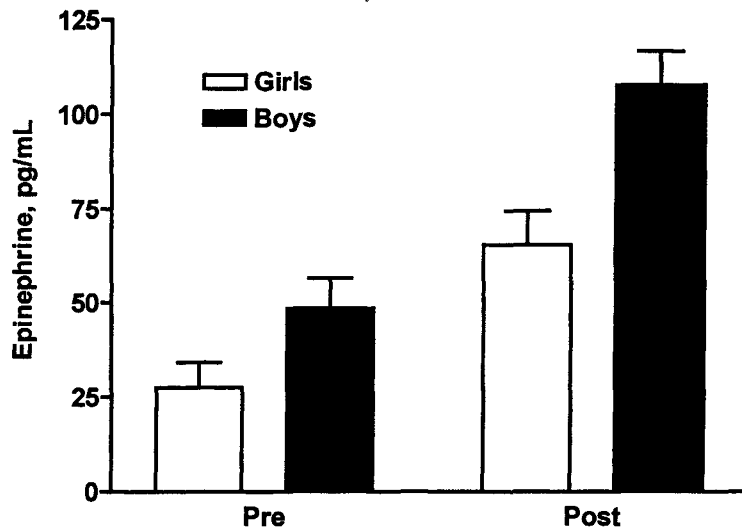

Figure F.2. *Epinephrine concentrations before and immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 14-yr-old boys and girls (unpublished observations). Pre, pre-exercise; Post, immediately after exercise.*

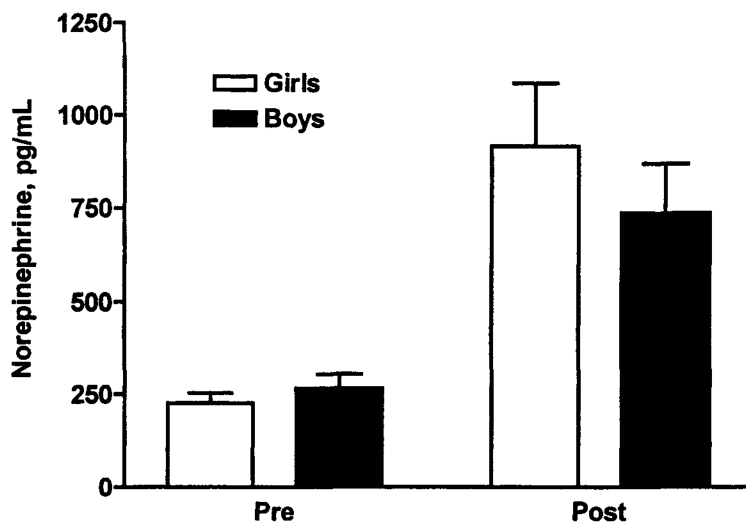

Figure F.3. *Norepinephrine concentrations before and immediately after exercise (60 min cycling @ 70% $\dot{V}O_{2max}$) in 14-yr-old boys and girls (unpublished observations). Pre, pre-exercise; Post, immediately after exercise.*