EFFECT OF EXERCISE ON NATURAL KILLER CELLS IN CHILDREN AND ADOLESCENTS

CHARACTERIZING THE EFFECTS OF ACUTE EXERCISE ON NATURAL KILLER CELL RECRUITMENT AND RECEPTOR EXPRESSION IN PRE-PUBERTAL AND POST-PUBERTAL YOUTH

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TITLE: Characterizing the effects of acute exercise on natural killer cell recruitment and receptor expression in pre-pubertal and post-pubertal youth

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LAY ABSTRACT

This study looked at how exercise can be used to change the behavior of Natural Killer cells. These are cells that are part of the immune system and help protect people from viruses and cancer cells by quickly detecting cells that don't belong in our bodies. They can also produce messenger chemicals to make other protective cells in the body aware of invaders. In this study children came into the lab and completed a different cycling exercise every time. In total, they completed four cycling exercises that included: 1) very hard cycling for 30 minutes; 2) very hard cycling in intervals, with 15 seconds of pedalling as hard and as fast as possible followed by a 1 minute rest, and repeating this for a total of 20 times; 3) comfortable cycling for 30 minutes; and 4) comfortable cycling in intervals. During each visit blood was collected from participants in order to count the number of natural killer cells there were before and after exercise as well as 30- and 60-minutes after the participants stopped cycling. Our study showed that in younger kids (8 to 10 years old), exercise doesn't change how many natural killer cells are in the blood. However, in teens (14 to 18 years old) we showed that pedaling in intervals increased the number of natural killer cells in the blood more than riding a bicycle for 30 minutes straight. Our research helps us understand how exercise can be used to make our immune system stronger.

Abstract

Natural Killer (NK) cells are recruited into circulation in response to physiological stress such as exercise. In adults, NK deployment and receptor expression are proportional to exercise intensity, and the cytotoxic $CD56^{dim}$ NK subset is preferentially deployed compared to immunoregulatory $CD56^{bright}$ subset. We know much less about the NK response to different exercise stimuli in children; however, pre-pubertal children are less responsive to acute exercise and recover faster than post-pubertal children and adults. The aims of this study were to (1) investigate the effects of exercise intensity and structure on NK recruitment and receptor expression, (2) compare the response among pre-pubertal and post-pubertal children, and (3) assess if factors such as fitness and physical activity were correlated with the magnitude of NK cell response.

Healthy, recreationally active, pre- and post-pubertal boys and girls were recruited from the Hamilton community (N=11; 5 pre-pubertal, 6 post-pubertal). At the initial study visit, participants completed an aerobic fitness test to determine $\dot{V}O_2$peak and ventilatory threshold. At the subsequent visits, participants performed one of four cycling structures in a randomized, counterbalanced order, including: high-intensity continuous (HI-CONT), high-intensity intermittent (HI-INT), moderate-intensity continuous (MI-CONT), or moderate-intensity intermittent (MI-INT) exercise. Blood was collected pre-, post-exercise, 30- and 60-minutes into recovery. NK cells, $CD56^{dim}$, and $CD56^{bright}$ NKs, activating (NKG2D and DNAM1) and inhibitory receptors (NKG2A and KIR2DL2/DL3) were quantified via flow cytometry. Participants were also outfitted with an accelerometer to measure physical activity. Three-way mixed ANOVA were used to examine effects of time, exercise and puberty on NK parameters, with Tukey's HSD post hoc where appropriate.

Pre-pubertal children showed no significant increase in the NK concentration in response to any of the exercise stimuli. Post-pubertal children, increased their NK cell concentration PRE to POST in all exercise stimuli except MI-CONT. Greater increases in NK concentration were seen POST HI-INT (124723 ± 91596 cells/mL) and MI-INT (109644 ± 84664 cells/mL), compared to HI-CONT (19931 ± 1492 cells/mL) and MI-CONT (17082 ± 9516 cells/mL), respectively ($p < 0.001$). Only increases in the expression of NKG2A were observed during REC1 (63.6 ± 13.7 %), and REC2 (64.2 ± 12.8 %) compared to PRE (57.9 ± 13.4%) ($p < 0.01$). However, the density of NKG2D, NKG2A and DNAM1 were all significantly increased at REC2 compared to PRE. Fitness (R^2= - 0.742) but not moderate-to-vigorous physical activity (R^2= 0.098) or sedentary time (R^2= 0.621) were significantly negatively associated with the magnitude of NK cell response.

We demonstrated that an acute bout of intermittent cycling (5 min of exercise) leads to greater NK recruitment and a more immunoregulatory NK environment than continuous cycling in post-pubertal children. Contrarily, we showed that pre-pubertal children are not responsive to acute exercise of 30-minutes or shorter. Across our cohort, exercise also upregulated the density of expression of both activating and inhibitory receptors. Future research should examine if the NK cell response to acute exercise is maintained with repeated exercise exposures or exercise training.

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LIST OF ABBREVIATIONS AND SYMBOLS

BMI	Body Mass index
DNAM1	DNAX Accessory Molecule 1
FMO	Flow Minus one
GH	Growth Hormone
HI-CONT	High-Intensity Continuous Cycling
HIIE	High intensity interval exercise
HI-INT	High-Intensity Intermittent Cycling
IFNγ	Interferon gamma
KIR	Killer-cell Immunoglobulin Like Receptor
KIR2DL2/DL3	Killer-cell immunoglobulin like receptor
LPA	Light Physical Activity
MHC Class I	Class 1 major histocompatibility complex
MFI	Median Fluorescence Intensity
MI-CONT	Moderate-Intensity Continuous Cycling
MI-INT	Moderate-Intensity Intermittent Cycling
MVPA	Moderate-to-Vigorous Physical Activity
NK	Natural Killer
NKG2A	Natural Killer Group 2A
NKG2D	Natural Killer Group 2D
PBMC	Peripheral Blood Mononuclear Cells
RCP	Respiratory Compensation Point

RPE	Ratings of Perceived Exertion
TNFα	Tumor Necrosis Factor Alpha
URTI	Upper Respiratory Infection
$\dot{V}O_2$	Volume of oxygen uptake
$\dot{V}O_2max$	Maximal volume of oxygen uptake (aerobic fitness)
VT	Ventilatory threshold

Chapter 1: Introduction

Natural Killer (NK) cells are a lymphocyte subset that recognizes tumour and virally infected cells without prior sensitization. They target and lyse aberrant cells that do not express the "self-marker" major histocompatibility complex-1 though a complex balance of activating and inhibitory receptor engagement[1,2]. Natural killer cells also appear to be the most responsive immune cells to an acute bout of exercise, demonstrating drastic but transient changes in number and activity[3]. In adults, the magnitude of NK cell response is known to depend on exercise intensity and to a lesser extent exercise duration. In fact, NK cell concentration in the blood increases proportionally with increases in exercise intensity in adults[4–6].

Although NK cell response to exercise has been well-characterized in adults, age-specific differences in physiological responses to exercise and NK cell phenotype make it difficult to translate existing findings to children. Compared with adults, children demonstrate a dampened immune reaction and faster recovery in response to physiological stress[7]. Moreover, children of the same chronological age but at different stages of pubertal development also demonstrate differences in the NK cell response to exercise, wherein late-/post-pubertal children experience more drastic overall NK cell deployment[8]. Puberty has also been correlated with a greater deployment of the immunoregulatory NK cell subtype post-exercise and a higher expression of the self-recognition killer immunoglobulin like receptor gene[9,10]. These findings suggest that exercise modulates the circulatory NK cell environment in children, however, the specific NK receptor response to exercise has yet to be examined in children and adolescents. This thesis represents the first efforts towards providing a comprehensive

understanding of the NK cell response to exercise of differing intensities and structures in healthy children and adolescents. Given the potential benefits of exercise for immune health, it is critical that we develop a more holistic understanding of the impact of exercise on NK cells in youth. Taken together, this study will provide critical insight into which different exercise stimuli shift the NK cell receptor balance to allow for greater activation, mobilization and activity.

1.1 Natural Killer Cell Overview

1.1.1 Natural Killer Cell Biology

Natural Killer cells are lymphocytes of the innate immune system primarily found in the spleen, liver, lymph nodes and bone marrow. Typically, NK cells account for roughly 5-20% of lymphocytes in circulation[2,11–13]. NK cells in the circulation cam be honed to a variety of tissues through engagement of cytokine and chemotactic receptors[14]. These cells perform key immunoregulatory roles and are able to recognize transformed aberrant cells such as tumor or virally infected cells without prior sensitization[11,15]. NK cells target any cells lacking a class I major histocompatibility complex (MHC), a marker found on nearly all healthy cells in the human body[1,16]. When MHC class I is present, it binds with inhibitory killer-cell immunoglobulin like (KIR) receptors or the NKG2 family of receptors, on the surface of NK cells[17]. These inhibitory receptors contain immuno-receptor-tyrosine-based-inhibitory motif domains in the cytoplasmic tail, which recruit phosphatases and impede signal transduction pathways needed for cell activation[18]. Contrarily, when MHC class I is downregulated, as is frequently observed in tumor and virally infected cells, the inhibitory stimuli are lost. In this case, the unsuppressed activation signals prompt NK cell activation[1,19]. Overall, NK cell activity is mediated by a

complex balance of activating, inhibitory and cytokine receptors engagement[20]. Once activated NK cells have two primary roles; to initiate a cytotoxic response and secrete cytokines and chemokines to engage the adaptive immune cells. However, different NK cell subtypes are responsible for each respective role.

1.1.2 NK Cell Subtypes

NK cells are derived from a CD34+ hematopoietic precursor in the bone marrow and can be subdivided into two functionally diverse subtypes based on the relative expression of cell surface marker CD56[12,21,22]. $CD56^{bright}$ cells account for roughly 10% of circulating NK cells, they are considered immature and predominantly specialize in cytokine secretion, with limited cytotoxic ability[13]. Once activated they secrete cytokines such as Interferon gamma (IFNγ), which is critical for shaping the T cell response, activating antigen presenting cells, inhibiting proliferation of aberrant cells, and activating the cytotoxic capacity of macrophages[23–25]. Consequently, this NK cell subtype is a critical cross-talker of the innate and adaptive immune system.

As $CD56^{bright}$ cells mature, CD56 is downregulated and CD57, a marker of maturation and differentiation, is upregulated thereby producing $CD56^{dim}$ cells[23,26,27]. The maturation from a $CD56^{bright}$ to $CD56^{dim}$ cell is a gradual, continuous process that includes changes in receptors, accompanied by changes in function. As cells undergo this transition, immunoregulatory capacity is diminished and cytotoxic capacity is acquired. The cytotoxic $CD56^{dim}$ NK cell fraction accounts for 90% of NK cells in circulation at rest. Upon target cell detection, an immunological synapse is formed, which stimulates movement of a secretory vesicle towards the synapse. The vesicle undergoes exocytosis and fuses with the cell membrane of the target cell, cytotoxic

material that includes perforin and granzyme is released, and caspase mediated apoptosis is initiated[28,29]. As CD56^{bright} cells mature they downregulate adhesion molecules such as CD62L, causing CD56^{dim} cells to have weakened tissue attachment properties[26]. This means that CD56^{dim} cells are more readily demarginated from the vascular endothelium in response to a chemical or physical stimulus. Although NK cell subpopulations are responsible for distinct roles, together they coordinate a rapid innate and adaptive immune responses.

1.1.3 NK Cell Activation and Regulation

Three theories have been proposed about how inhibitory and activating receptors interact to regulate NK activation. The 'missing self-theory' states that NK cell activation occurs due to the absence of inhibition (Figure 1). In other words, NK cells target and lyse any cell that doesn't contain an MHC class I molecule[30,31]. This theory was proposed by Karre and colleagues who demonstrated MHC Class I molecules are typically downregulated in tumor or virally infected cells[32]. A second, 'induced self-theory' has emerged, proposing that the interaction of stress ligands on target cells with activating receptors may override NK cell inhibitory signals (Figure 1)[31]. This alters the receptor balance in favor of NK cell activation and target lysis, even in the presence of MHC class I antigens. This theory is supported by the upregulation of stress inducible ligands such as MIC A/B and ULBP in tumor cells, which can interact with NKG2D activating NK cell receptors[33]. The third theory coined 'target interference' states that NK activating receptors fail to function in the presence of normal levels of MHC class I molecules on target cells due to the strong negative signals.

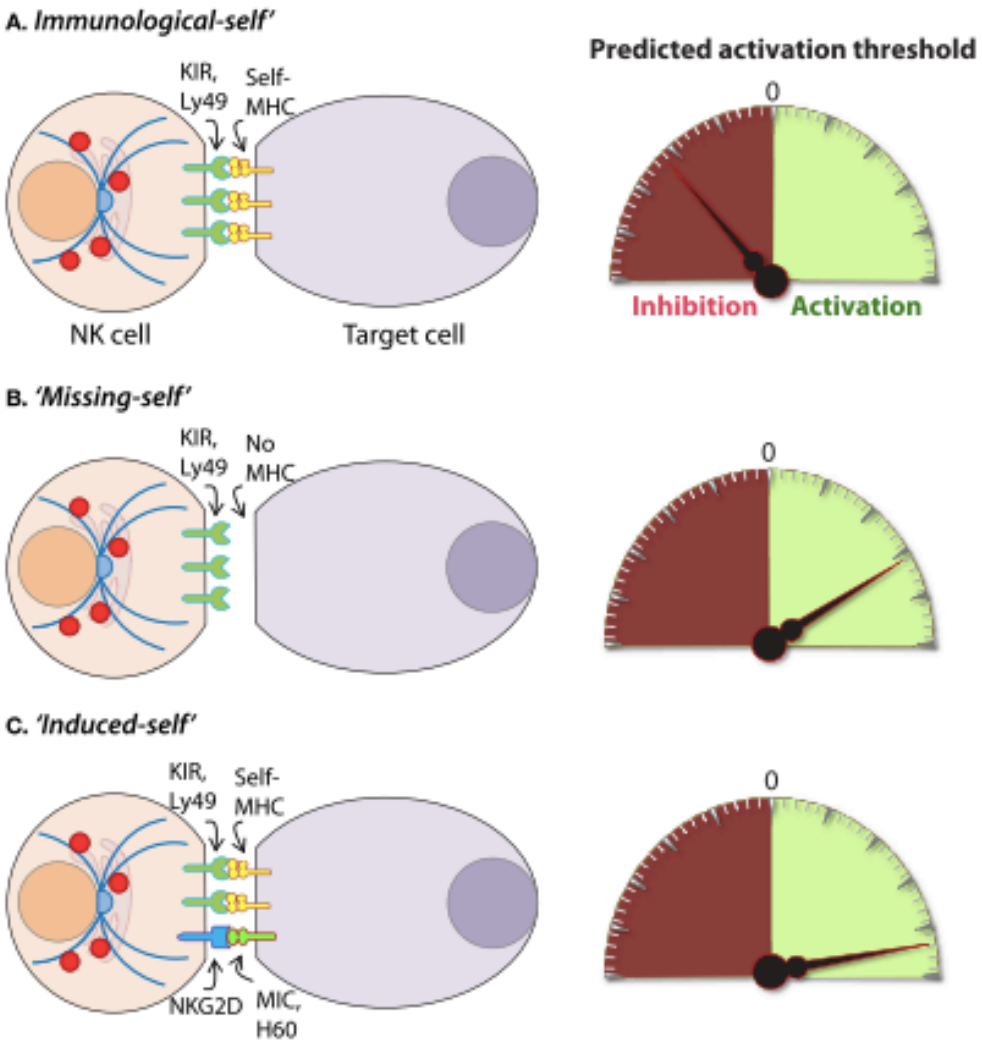


Figure 1: NK Cell Regulation and Activation Theories. Image adapted from Abel et al[30]. Activation of the lytic function of NK cells is dependent on activating and inhibitor receptor engagement. (A) When NK cells recognize autologous target cells, engagement of inhibitory receptors with self MHC's inhibits NK cell activation. (B) In the Missing-Self hypothesis, lack of self-recognition by the inhibitory receptors, diminishes the inhibitory stimuli and causes NK cell activation. (C) In the Induced Self-theory activation is regulated by a balance between activating and inhibitory receptor engagement. NK cells are only activated with the activating signal can override the inhibitory receptors.

As proposed by Malarkannan, NK cell regulation integrates elements of all three theories such that NK activation is mediated by a balance of the strength of the activating and inhibitory signals[34]. As a proof of this concept, Regunathan et al. used a

murine model to demonstrate that moderate levels of stress-inducible ligand H60 expression can interact with inhibitory Ly49 receptors (homologous to KIR's in humans) and block NK activation[35,36]. However, when H60 is expressed at pathological levels the activating signal overrides the inhibitory signal by binding to NKG2D and inducing target cell lysis. These experiments demonstrated the tight regulation of NK cell activation, and the critical role of receptor expression and engagement in managing NK function. Although there are more than a dozen activating and inhibitory receptors on NK cells, there are key receptors that play a role in pathogen detection and NK cell regulation.

1.1.4 Activating Receptors

Natural Killer Group 2D (NKG2D)

NKG2D receptor is constitutively expressed on NK cells and has a high degree of plasticity, allowing it to have a strong affinity to many different ligands[37,38]. This receptor typically recognizes stress inducible ligands that are structurally similar to MHC class I molecules, albeit lacking the β2-microglobulin subunit[37]. Examples of these include molecules such as MIC-A/B, which are highly expressed on epithelial tumors, and ULBP's expressed on other stressed or dying cells[39,40]. When NKG2D receptors bind directly to target ligands, they stimulate mobilization of the lytic granules to the NK cell surface as well as IFNγ cytokine production[37,41]. The significant role of this receptor has been demonstrated by Saito et al. who correlated the decreased expression of NKG2D receptors with impaired NK cell function in patients with gastric cancer[42].

DNAX Accessory Molecule 1 (DNAM1)

DNAM1 is a costimulatory adhesion molecule expressed on all human NK cells and is crucial in mediating immunosurveillance[43]. As DNAM1 interacts with target cell

ligands it stabilizes the interaction with the NK cell and activates other surface receptors. DNAM1 recognizes ligands such as CD155 and CD112, whose expression is upregulated on cancerous and virus-infected cells[44]. Upon detection of target ligands, DNAM1 associates with the *β*2-integrin LFA-1, which serves to stabilize the formation of a synapse between the effector NK cell and target cell[45]. Nevertheless, this is insufficient to activate NK cells alone, and co-regulation by other activating receptors is required to initiate a functional response. DNAM1 is also unique in that its directly involved in cross-talk with other immune cells such as dendritic cells that possess DNAM1 ligands[46]. Since the ligands recognized by DNAM1 are also commonly found in healthy cells, their expression is downregulated to prevent unnecessary activation.

1.1.5 Inhibitory Receptors

Natural Killer Group 2A (NKG2A)

NKG2A is a NK cell inhibitory receptor that functions as a heterodimer by forming a complex with CD94 subunit expressed on NK cells[38]. This receptor is free moving, cycles continuously between the plasma and cell surface and may be recruited to sites of target ligand[47]. NKG2A recognizes and binds a nonclassical MHC class I molecule encoded by the HLA-E gene, and though lowly expressed in healthy tissues, is overexpressed on aberrant cells, particularly in malignancies to evade detection[48]. Once the NKG2A/CD94 inhibitory complex engages with a ligand, there is downstream inhibition of the activation receptor cascade, rendering this receptor a key NK regulator[48,49]. Kamiya et al. demonstrated that NKG2A^{null} NK cells have greater anti-tumor killing capacity[50].

Killer Cell Immunoglobulin like Receptors (KIR2DL2/KIR2DL3)

The KIR family is a diverse group of transmembrane receptors. These are differentiated based on the number of extracellular immunoglobulin-like domains (2D vs. 3D) and the length of the cytoplasmic tail[51,52]. Those with long cytoplasmic tails are inhibitory, those with short cytoplasmic tails are activating. Two well-characterized inhibitory receptors, KIR2DL2 and KIR2DL3, are involved in binding HLA-C encoded MHC class I molecules which are expressed on most healthy cells[51,52]. The primary role of these receptors is to prevent NK-mediated attack against healthy cells[51]. Similar to the NKG2A/CD94 complex, inhibitory KIR engagement initiates an intracellular cascade which dephosphorylates the activating molecules and prevents activation. Blocking the KIR2DL2/2DL3 receptors has been shown to be clinically beneficial in potentiating NK cell activity against cancerous cells, particularly acute myeloid leukemias in mice[53].

1.2 Exercise as a Physiological Stressor

Stress can be defined as a stimulus which elicits a reaction in the brain that instructs the body to prepare for a fight-or-flight response[54]. Stressors may be psychological or physical in nature, but they elicit similar physiological changes which include both sympathetic nervous system and hypothalamic-pituitary-adrenal activation[55]. Exercise is a well-defined and reproducible model of physiological stress, that can be easily implemented to achieve short-term immune activation[56]. In the past decade, the capacity of exercise to provoke immune cell trafficking and redistribution has been of particular interest due to its application in enhancing immunosurveillance. Within only a couple minutes of exercise, catecholamines and glucocorticoids surge into circulation and cardiopulmonary changes take place[57,58]. This provides opportunity for

leukocytes, including monocytes, neutrophils and lymphocytes to enter the circulation and traffic to areas of potential immune challenges. This includes areas of ongoing tissue damage and inflammation such as highly susceptible mucosal surfaces like the lungs[14,59]. As such, exercise is an easily modifiable model for understanding how different physiological stress impacts the immune environment.

1.3 Natural Killer Cell Response to Exercise in Adults

1.3.1 Recruitment of Natural Killer Cells in Response to Acute Exercise

During a brief bout of exercise, lymphocytosis increases the concentration of lymphocytes in the blood by up to 70%[4,5]. Amongst the lymphocyte population, NK cells are the most responsive cells to an exercise stimulus. Increases in heart rate and cardiac output create a greater mechanical force on the vascular endothelium and cause lymphocytes to detach and be deployed into circulation[60]. Additionally, secretion of catecholamines like epinephrine and norepinephrine, which rapidly spike during physiological stress and remain elevated throughout, aid in deployment of lymphocytes[61,62]. In fact, a single two-minute bout of high intensity exercise increases circulating epinephrine levels by 4- to 6-fold[63]. Together, increased shear stress and catecholamines act synergistically to demarginate lymphocytes from the vascular endothelium into circulation. Epinephrine acts through high affinity of β-adrenergic receptors, which are found in highest density on NK cells[56,64]. Stimulation of these receptors lowers expression of adhesion markers such as CD44, triggering detachment from vascular endothelium and spleen and rapid mobilization into circulation[65,66].

Along with exercise-induced changes in lymphocyte counts, the composition of the lymphocyte population also changes with exercise. Due to the highly responsive

nature of NK cells, after a 60-minute low intensity muscle training exercise in young healthy adults there is a decrease in the proportion of T cells from 86% to 70% of circulatory lymphocytes[67]. This is further supported by Nielsen et al. who demonstrated, in a similar population, a seven-fold increase in the cytotoxic CD16+ proportion of NK cells immediately after a six-minute all-out rowing ergometer test[3]. Indeed, regardless of the exercise stimuli, NK cells demonstrate the most prominent lymphocyte response to exercise in the lymphocyte pool.

After exercise cessation, a period of lymphocytopenia will sometimes occur, wherein the number of circulating lymphocytes decrease up to 20% below baseline in adults[5,68]. This can last up to four hours. These changes parallel the decrease in the NK cells in circulation, which is hypothesized to be due to the cell redistribution to sites of potential injury or damage[69]. However, exact locations or patterns of distribution remain unknown. As the number of NK cells returns to baseline, circulating lymphocyte numbers concurrently approach pre-exercise values.

1.3.2 Effects of Exercise Intensity on NK Cell Number

The response to single bout of exercise is not homogenous, and the increase in NK cells in circulation is sensitive and directly related to the intensity of the exercise stimulus, at least in adults[6,70]. In young trained males, Nieman et al. demonstrated that the amount of NK cells in circulation roughly doubled in response to a high intensity running protocol compared to a moderate intensity protocol[4]. Similar results were also observed in a sample of women, where high intensity cycling resulted in 50% greater NK cell recruitment than moderate intensity[71]. In fact, Kendall et al. demonstrated a dose-dependent spike in NK cell response to exercise intensity by comparing 60

minutes of continuous cycling at 30%, 65% and 75% of individual maximum capacity, defined as maximal oxygen uptake ($\dot{V}O_2max$)[72]. As demonstrated by McMurray et al, these observations can be explained by corresponding changes in circulating catecholamines[73]. They examined the concentrations of circulatory catecholamines in response to three separate bouts of continuous cycling at 40%, 60% and 80% of $\dot{V}O_2max$. The authors reported that compared to cycling at 40% $\dot{V}O_2max$, cycling at 60% resulted in a 50% greater circulatory catecholamine concentration. Even more stark was the 150% - 200% greater catecholamine concentration noted when participants cycled at 80% compared to 60% $\dot{V}O_2max$. This study demonstrated not only the sensitivity of catecholamines to exercise intensity, but also the exponential relationship that exists at higher intensities[73,74]. Shear stress also increases proportionally to exercise intensity. As exercise intensity increases, heart rate and cardiac output increases as well, thereby elevating the shear stress[75]. Together, greater catecholamine stimulation of β-adrenergic receptors on NK cells and a stronger shear force cause more detachment of NK cells from the vascular endothelium.

Interestingly, an intensity dependent gradient is not observed during intermittent exercise. When the number of NK cells in circulation was compared immediately after fifteen 1-minute exercise bouts at either 30%, 60% or 90% of $\dot{V}O_2max$, no differences between the three stimuli were detected[76]. Furthermore, during recovery, no intensity dependent differences were observed in NK cell number[4,71,72]. As such, with the literature available, it's evident that NK cell recruitment increases proportional to exercise intensity only in response to continuous cycling. Furthermore, from what is observed in response to continuous cycling, exercise intensity does not alter the

number of NK cells in circulation during recovery nor the time required for NK cell number to return to baseline.

1.3.3 Effects of Exercise Duration on NK Cell Number

The effect of exercise duration on NK cell recruitment is sparsely addressed in literature. However, when Kendall et al. assessed whether the proportion of NK cells in circulation differed after 30 minutes and 2 hours of moderate intensity exercise, they showed no significant differences[72]. These findings were consistent with a review provided by Timmons et al. that showed no additional increases in circulatory NK cell number after 30 minutes of exercise[77]. Although catecholamines continue to increase beyond 30 minutes of exercise, onset of cortisol secretion during prolonged exercise counteracts these effects and drives NK cells out of circulation[74,78]. A comparison of the cumulative effects of exercise intensity and duration in adults has also been assessed. A 5-minute bout of high intensity cycling showed 50% greater NK cell recruitment than a 2-hour moderate intensity bout[79]. The process of recovery remained consistent in both protocols, with return to baseline occurring by 3-hours post exercise[79]. Although these results support the sensitivity of NK cell response to the exercise stimuli, how much exercise intensity and duration individually contribute to the changes remains unknown. In summary, evidence suggests that the magnitude of change may not be highly sensitive to the effects of exercise duration when it exceeds 30 minutes.

1.3.4 Effects of Exercise Structure on NK Cell Number

A recent body of evidence demonstrates that compared to continuous exercise, intermittent exercise possesses greater or equal cardiovascular and metabolic health benefits, despite having lower volume and shorter duration[80,81]. Beyond that, intermittent

exercise is also reported to be more enjoyable for participants and achieves superior or comparable increases in aerobic fitness[80,81]. A novel area of research has focused on understanding the effects of intermittent exercise on the immune system and specifically NK cell population. In adults, a direct comparison of 2 hours of intermittent vs. continuous cycling identified no differences in NK cell recruitment post-exercise or during recovery[82]. A more recent study by Turner et al. compared the effects of 20 minutes of continuous cycling at 80% $\dot{V}O_2$max to a high intensity interval exercise (HIIE) which included ten 1-minute intervals cycling at 90% $\dot{V}O_2$max interspersed with 1-minute cycling at 40% $\dot{V}O_2$max[83]. Their results contradicted Nieman's findings by showing that NK cells increased 200% more after continuous cycling compared to intermittent cycling[83].Whether these between-exercise differences are clinically meaningful is unclear, but intermittent training has already shown promising results in reducing NK cell-mediated tumor burden in patients with breast cancer[84]. Given the small body of literature about the effects of exercise structure on NK cell number, more research is required to understand how high intensity interval exercise compares to continuous exercise.

1.4 Changes in NK Subtypes in Response to Exercise

1.4.1 Recruitment of NK Cell Subtypes in Response to Acute Exercise

The effects of exercise on NK cells can be further stratified to understand specific effects on NK subsets. Several studies have found that exercise preferentially increases the proportion of $CD56^{dim}$ cells in healthy young adults[85,86]. This is postulated to be due to the decreased expression of adhesion markers on the $CD56^{dim}$ population as compared to the $CD56^{bright}$ population, and thus greater sensitivity to stress and rapid

mobilization into circulation[86]. This is true in response to both intermittent and continuous exercise where the number of $CD56^{dim}$ cells are higher immediately after exercise [83]. The concentration of both NK cell subsets then decreases 15- to 30-minutes after exercise, but a larger proportion of $CD56^{dim}$ cells moves out of the peripheral circulation. This propagates an elevated $CD56^{bright}$: $CD56^{dim}$ cell ratio during recovery[77]. The reasons NK cell subsets experience differential withdrawal from circulation are unclear. It may be related to a potential role for NK cells in post-exercise peripheral tissue and muscle repair, although the research on this remains entirely speculative[77,87]; During injury-induced liver inflammation, NK cells may be activated through engagement with damage-associated molecular patterns. Once activated, NK cells crosstalk with macrophages, which are recruited to the site of injury due to their critical role in liver tissue repair and recovery[88,89]. Since macrophages also play a critical role in muscle repair, it is plausible that a similar cascade of event occurs with exercise-induced muscle damage.

1.4.2 Changes in NK Receptor Profiles in Response to Exercise

Changes in NK cell number in response to exercise were characterized over 30 years ago in adults. More recent research has focused on how these changes in number correlate with function. Due to the well-established role of receptors in regulating NK cell activation, the effects of acute exercise on receptor gene expression has also become an area of growing interest[38]. Many investigations have focused specifically on changes in KIR genes, due to their diversity. Evidence from Maltseva et al. demonstrated an overall 1.8-fold increase in expression of genes of the KIR locus after a $\dot{V}O_2$max test, although specific receptor changes were not described[90]. In a

specific analysis of KIR2DL3 and KIR3DL2 inhibitory receptors, a 2 – 3-fold increase was observed after 20 minutes of intermittent exercise[91]. A comprehensive study by Shleptsova et al. further showed increases in both inhibitory KIR2DL3 and activating KIR2DS2 receptors post-exercise[92]. Engagement of KIR2D2 genes activates NK cells, and individuals who have greater expression of this gene and a downregulation of the inhibitory KIR2DL3 gene are seen to be more susceptible to developing an autoimmune condition[90]. Aligned with these changes, Shleptsova et al. showed a significant increase in the expression of PRF1, which encodes the perforins required to lyse target cells[92]. These findings suggest that increased expression of activating KIR genes may be related to a more cytotoxic NK phenotype; however, the authors did not directly examine the relationship between the KIR genes and NK cell killing capacity. Furthermore, the mechanism by which changes in NK cell phenotype directly link to function remains unclear, and a detailed understanding of how the balance of activating and inhibitory KIR genes changes in response to exercise has not been elucidated.

To date, no study has compared the effects of exercise intensity and duration on activating and inhibitory NK receptors. However, existing studies suggest a potential role for exercise to modulate expression of the activating NKG2D receptor, which plays a primary role in aberrant cell detection[37]. Although Bigley et al. showed no changes in NKG2D expression after 30 minutes of cycling at 15% above the lactate threshold, following a half-marathon NKG2D gene expression increased by about 50%[85,93]. Moreover, the transcription of the NKG2D receptor continued to increase more than 6 times 24 hours after the half-marathon exercise stimulus[93]. This was some of the first

evidence to demonstrate that acute exercise may modulate NK cell receptor balance up to 24 hours after activity, potentially improving immunosurveillance.

Exercise prompts both long and short-term epigenetic modifications, which can be directly related to changes to receptor expression. Histone acetylation increases DNA transcription and accordingly expression. Indeed, histone H4K5 acetylation is significantly positively correlated with NKG2D expression and both are elevated after intense exercise[89]. Conversely, histone and DNA methylation typically inhibits DNA expression and the demethylation of three unique sites in the DNA promoter of the KIR2DS4 gene has been positively correlated with greater gene expression post-exercise[90]. Although a detailed characterization of the epigenetic changes caused by exercise on all KIR genes is not available, emerging evidence demonstrates the ability of exercise to change methylation and acetylation patterns and thus expression. Beyond epigenetic modifications, another plausible mechanism by which receptor expression can be modulated is through cytokine or myokine secretion. *In vitro* experiments have shown that stimulation of NK cells by myokines such as IL-15, which is secreted from muscle after strenuous exercise, increases the expression of DNAM1, KIR2DL2/DL3 and NKG2D receptors[95]. However, these results should be interpreted with caution as *in vitro* concentrations do not accurately portray an exercise stimulus *in vivo*.

To date, no studies have directly assessed the effects of exercise on NKG2A. The knowledge that NKG2A must complex with CD94 to form a functional inhibitory receptor offers some preliminary information about possible changes in inhibitory function[47]. Horn et al. showed that after 2 minutes of all-out maximal cycling the mean fluorescence intensity of the CD94 marker decreased, and expression of CD94 on a per

NK cell basis was dampened[96]. Although these findings cannot be directly translated to NKG2A expression, it is plausible that this inhibitory receptor may be dampened after an exercise stimulus since CD94 complexes with NKG2 family receptors. Taken together, the existing literature suggests that a multitude of factors play a role in NK receptor regulation; however, the specific effect of exercise on NK receptor expression remains unclear and requires further investigation.

1.5. Comparison of NK Cells between Adults and Children

1.5.1 Resting NK Cell Values

There are no detectable differences in the relative percentage of NK cells in the circulatory lymphocyte pool between children, adolescents and adults[97,98]. Similarly, most studies that have examined differences in absolute NK cell counts in the peripheral circulation found that absolute NK cell counts at rest are comparable between school-age children and adults[7,99]. Conversely, Almeida-Oliviera et al. demonstrated that children have 60% more NK cells in circulation than do adults[100]. Although the disparity in these results is surprising, it might be due to differences in the definitions of NK cells used in these studies. While Almeida-Oliveria et al. defined NK cells as CD3-CD56+, the other two studies defined NK cells as CD3-CD56+CD16+ cells, thus only truly capturing changes in the most terminally differentiated mature cytotoxic portion of the NK cells, that have acquired expression of the CD16 molecule[12]. Therefore, these findings suggest that children and adults demonstrate comparable proportion of circulating cytotoxic NK cells and differences in the absolute counts of NK cell in circulation may be attributed to variance in the number of immunoregulatory or non-terminally differentiated NK cells.

1.5.2 NK Cell Receptors

The expression and density of receptors on NK cells, or the NK cell phenotype, may also change with growth, development and maturation. In work by Mahapatra et al. DNAM1 is expressed on a significantly greater number of $CD56^{bright}$ NK cells in children 5-10 years old (80.24 ± 11.19%) compared to adolescents 16-20 years old (68.38 ± 12.11%)[98]. The expression of NKG2D has been of particular interest, due to its pivotal role in NK cell activation and function. Changes are evident in early childhood compared to adulthood, where the density of NKG2D expression on NK cells of 5-year-old children was lower than that of adults[101]. Taken together, these findings provide an unclear image of the functional capacity changes throughout childhood and adolescence.

Evaluating changes NKG2A and KIR across growth and development is complex. As individual NK cells mature, they tend to undergo an isotype switch, downregulating expression of NKG2A receptors and upregulating KIR expression[27]. Manser et al. demonstrated that the proportion of NKG2A+ NK cells substantially decreased during childhood, without further downregulation after the second decade of life[102]. This was inversely related to the proportion of cells expressing KIR which increased during the first two decades of life with no further changes observed in adulthood[98,102]. As with other NK cell biology research, whether these differences are due to genetics, hormonal stimuli, or changes in the cytokine milieu throughout childhood remains to be elucidated.

1.5.3 NK Cell Response to Exercise

It has long been understood that children are not just smaller adults and have distinct physiological responses to exercise. From an exercise immunology perspective,

children experience a dampened NK cell response to exercise and faster recovery[7]. More specifically, Timmons et al. reported that in response to continuous, high-intensity exercise 10-year-old children experience a 78% increase in NK cell number, while adults experience a 236% increase[7]. Although there is a plethora of physiological differences between children and adults, these differences are commonly attributable to the cardiovascular and catecholamine responses to exercise. Upon exposure to comparable exercise stimuli, children are reported to have lower responsiveness of the β-adrenergic receptors to catecholamine stimulation and a significantly lower stroke volume[103–106]. These phenomena together suggest weaker chemical and physical stimulation on the NK cells in the endothelial vasculature, and thus decreased deployment into circulation. Differences between children and adults are also observed during recovery. Wherein children show full return to baseline NK cell numbers, the NK cell number remains below baseline one hour post-exercise in men[7]. One possible reason is that children possess a higher capillary density than adults, and this allows for faster NK cell redistribution to peripheral tissues after exercise and return to baseline values[107]. Taken together with the observations that children also experience faster cardiorespiratory recovery from exercise than adults, these findings suggest that children are able to re-establish baseline homeostatic balance faster than adults.

1.6 Effects of Puberty on NK Cell Recruitment and Subtypes

1.6.1 NK Cell Recruitment

It is important to note that the pediatric population is not homogenous, and the immune response to exercise changes throughout pubertal development. Children at later stages of pubertal development experience greater increases in NK cell number in

response to both aerobic and anaerobic exercise. In fact, NK cells demonstrate a progressively greater response to an a bout of anaerobic exercise across the pubertal spectrum (post-pubertal > peri-pubertal > pre-pubertal)[108]. NK cell recruitment was 465% of pretest values in post-pubertal boys as compared to 263% in pre-pubertal boys[108]. Similar results were observed in girls, whereby NK cell recruitment was 356% of pretest values in response to cycling in post-pubertal girls (Tanner 4 and 5) compared to 247% in peri-pubertal girls (Tanner 2)[109]. These differences may be explained by a variety of reasons, such as differences in both hormonal and cytokine milieu. For example, adolescent boys (Tanner 3-5) secrete significantly more Growth Hormone (GH) than pre-pubertal boys (Tanner 1) in response to an exercise stimulus[8,110]. Furthermore, the concentration of circulatory GH was significantly positively correlated with the number of $CD56^{dim}$ and $CD56^{bright}$ NK cells post-exercise[8]. Although the direct mechanism by which hormones such as GH modulate NK cell number and function remains poorly understood, Span et al. has shown that in adults who are GH-deficient there is decreased number and function of NK cells[111]. Other possible explanations for the differential recruitment across childhood is the changing level of baseline circulating tumor necrosis factor alpha (TNFα), which is higher in pre-pubertal boys[112]. In a cell culture model of NK cells co-incubated with endothelial cells, NK cell adherence was four- to seven-fold higher in cultures treated with TNFα[113]. A similar mechanism may serve to explain why physiologically, children at earlier pubertal stages experience lower NK cell deployment. Taken together, the available literature suggests a growth and maturation-related transition in the NK cell response to exercise.

1.6.2 Recruitment of CD56^{dim} and CD56^{bright} NK Cells

Pubertal status is also linked to differences in the recruitment of NK cell subpopulations, particularly in the context of CD56^{bright} cells. To examine this, studies have examined changes in both the concentration and proportion of NK cells. At rest, the concentrations of both CD56^{dim} and CD56^{bright} cells were comparable in pre-pubertal and late-pubertal boys[8]. Immediately after exercise boys at more advanced pubertal stages maintained significantly higher proportion of CD56^{bright} cells, and also demonstrated a significantly higher concentration of circulating CD56^{bright} cells relative to pre-pubertal boys[8]. Therefore, it appears that more advanced pubertal age may be related to a more immune surveillant and less cytotoxic NK cell environment. This may be indicative of a growth-related adaptation that improves the ability of the immune system to cross-communicate from the innate to adaptive immune system and recruit other immune cells such as monocytes during times of immunological stress.

1.7 Effect of Sex on NK cell Recruitment

Although this thesis will not be evaluating specific sex-related differences in our pediatric population, it is important to mention that hormonal variation in females can affect NK cell responses. In adults, no differences were detected in resting NK cell numbers in recreationally active healthy men and women, and similar results were seen in children and adolescents[114,115]. However, 14-year old girls experienced 29% greater lymphocyte recruitment, relative to age-matched boys after a 30-minute bout of continuous cycling[115]. In the same study, both CD56^{dim} and CD56^{bright} NK cell recruitment was higher in girls compared to boys[115]. This was hypothesized to be due to greater density of β-adrenergic receptors on NK cells in females, which in turn led to

greater sensitivity and responsiveness to exercise[116]. In pre-pubertal children, the NK response to exercise was similar in females and males, but greater NK recruitment was seen in late-/post-pubertal females compared to males[109]. This provides evidence that NK cell response may also be affected by puberty related onset of sex-differences between boys and girls.

1.8 Exercise Patterns in Children and Adults

As mentioned previously, intermittent exercise has garnered a lot of attention in the field of exercise science since it requires a lower time investment but yields comparable or greater health benefits. For children, intermittent exercise may also be appealing because it is in line with their natural "stop-and-go" movement behaviours. Nearly all bouts of vigorous activity in children last 15s or less and are interspersed amongst with breaks of low or no activity[117]. However, most studies used to understand the effects of NK cell environment to date, have used prolonged continuous exercise (e.g., 30 or 60 minutes of continuous cycling), which is not applicable to the typical movement behaviour in the pediatric population. Perez et al. compared the NK cell response to a laboratory protocol composed of ten 2-minute cycling bouts and an unregulated field activity during a soccer game in children. They found that field exercise induced only a 20% increase in NK cells, while a 150% increase was seen in response to the laboratory testing[118]. This validates the theory that while previous studies have provided important information about NK cell recruitment, their findings may not be as relevant to the pediatric population in free-living conditions. A study is needed to evaluate how NK cells respond to the more sporadic, short bursts of activity observed in children.

1.9 Fitness, Physical Activity and NK Cell Response

The idea that regular participation in physical activity is related to improved immune health has been well-described in the literature using the "J curve hypothesis" (Figure 2).

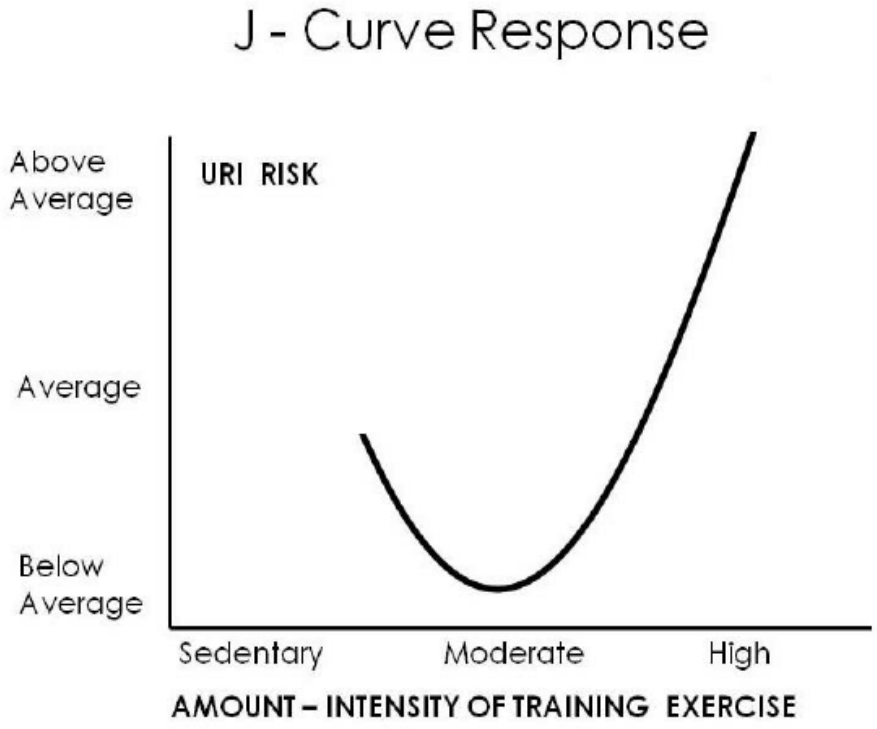


Figure 2: Risk of Upper Respiratory Infection in Response to Exercise Training. Image adapted from Hackney[119].

This theory, first proposed by Nieman et al., suggests that while the risk of an upper respiratory infection (URTI) decreases with moderate exercise training, excessive high-exertion training increases the risk even above that of sedentary young adults up to 6-fold[119–121]. More recent evidence also demonstrates that compared to individuals with low fitness, URTI persisted 46% and 43% fewer days in adults with high and

moderate physical fitness, respectively[122]. This is partly attributable to improved immunosurveillance due to transient recruitment of lymphocytes such as NK cells with regular exercise bouts lasting less than 60 minutes in duration[84].

Whether the immune response to acute exercise depends on an individual's fitness and habitual physical activity patterns remains contested in the literature. Furthermore, much of the current evidence has been focused on the elderly, who have distinct immune profiles and age-related concerns such as immunosenescence. Several studies in healthy adults have shown that trained adult cyclists and marathon runners with significantly higher aerobic fitness ($\dot{V}O_2max$) typically have a higher proportion of NK cells in circulation at rest[123,124]. Contrarily, other studies showed no significant differences in the concentration of NK cells at rest in elite athletes compared to recreationally active individuals[125,126]. Similarly, conflicting evidence has been observed in terms of the magnitude of lymphocytosis and NK cell recruitment with exercise between individuals. While one study showed no difference in NK cell recruitment between active and sedentary individuals, another demonstrated greater sensitivity of response in highly fit subjects compared to a moderately fit group[72,127]. Rhind et al. showed that a 12-week moderate training program significantly increased participant fitness and post-exercise circulatory NK cell concentration[128]. These findings suggest improved fitness is associated with a greater magnitude of NK cell response to exercise. Physiologically, this may be explained by greater baseline epinephrine and norepinephrine levels, which are also correlated with improved fitness[129]. It is plausible that greater baseline circulatory catecholamine values may be associated with a more pronounced hormone increase during exercise, leading to greater NK cell recruitment.

Only one known study to date has investigated the relationship between fitness and the immune response to acute exercise in children. This study showed that the resting concentration of NK cells were lower in swimmer children and adolescents compared to non-swimmers[130]. Interestingly, there were no between group differences in the NK cell concentrations post-exercise, but the fold change from pre-exercise values appeared greater in trained swimmers. It is important to note that in this study no objective measure of fitness was obtained from the children. Thus, although the swimmer group may have engaged in more formal exercise training it is unknown whether physiologically, they were in fact more fit or more active than the non-swimmer group. As such, more research with objective measures of fitness and physical activity in children is required in order to determine if those parameters play a critical role in modulating NK cell response to exercise.

1.10 The Importance of Studying the NK Cell Response to Exercise in Children

NK cells are the most responsive immune cell to exercise. While this has been repeatedly demonstrated in adults, and more recently in children, very little remains known about changes in NK cell phenotype and biology in response to exercise. *In-vitro,* acute exercise improved lysis of malignant cells. In mouse models, chronic exercise enhanced NK cell cytotoxicity and decreased tumor burden[85,131]. The aforementioned benefits of acute and chronic exercise, specifically in the context of cancer protection, are consistent with observations in adults[132]. Taken together, data from these model systems and from the adult literature demonstrate that exercise causes drastic improvements in NK cell immunosurveillance. However, before we can explore exercise and NK cell immunosurveillance in children, we need a better

understanding of the effects of different types of acute exercise on NK cell biology and phenotype.

1.11 Summary

Although there is an abundance of literature in adults examining the effect of exercise intensity, duration and structure on NK cells and, to a more limited extent, on NK receptors, this body of literature is entirely missing in the pediatric population. It is known that children have a dampened immune response to exercise compared to adults and that their circulatory immune profiles tend to recover to baseline more rapidly than adults. However, whether children are equally as sensitive to the exercise intensity and whether the exercise structure modulates their responsiveness remains uncharacterized. Importantly, the pediatric population is not homogenous and at different stages of puberty, there are distinct physiological and NK cell responses to exercise. As such, more detailed investigation is required to understand how NK cell numbers, receptor profiles, and eventually function, change in response to different exercise stimuli. More specifically, investigating how different pubertal groups respond to exercise of different intensities and structures will provide key information about which exercise protocol may be best suited to each pubertal group to establish desired NK cell effects. Lastly, while there is some evidence in adults suggesting the NK cell response is transformed based on by individual's fitness and level of physical activity, how tightly regulated this relationship is in children and adolescents remains unknown.

Chapter 2: Research Questions, Objectives, And Hypotheses

To fill these gaps in our knowledge, the purpose of this thesis is two-fold. The first goal of this study is to gain a better understanding of how exercise intensity and structure impact NK cell biology, which includes looking at changes in individual NK cell receptor expression and overall makeup of the NK cell pool in circulation in children at distinct pubertal stages. Together, our findings will aim to provide a more holistic understanding of NK cell responsiveness in the pediatric population. The second goal of our study is to examine how factors like physical activity and fitness are related to the NK cell response in children and adolescents.

2.1 Research Questions

To address the existing gaps in our knowledge of NK cell responses to exercise in the pediatric population, this thesis will address the following research questions:

1. What is the effect of exercise intensity and duration/structure on NK cell recruitment and activating/inhibitory receptor profiles in children?
2. Are there differences in the NK cell response to exercise in pre-pubertal children compared to late-/post-pubertal children?
3. Are differences in fitness and engagement in moderate-to-vigorous physical activity and sedentary time related to changes in the magnitude of NK cell response to exercise?

2.2 Objectives

1. To examine changes in NK cells, NK subsets, and receptor expression and density immediately after exercise and during recovery in response to high or moderate intensity, continuous or intermittent exercise.
2. To characterize differences in NK cells, NK subsets, and receptor expression and density between pre-pubertal children and late-/post-pubertal children.
3. To assess the relationship between fitness ($\dot{V}O_2max$), moderate-to-vigorous physical activity, or sedentary time and the magnitude of NK cell response to exercise in children.

2.3 Hypotheses

1. High-intensity exercise will elicit the greatest post-exercise increases in NK number. Furthermore, high-intensity exercise will also increase activating receptor expression while decreasing in the expression of inhibitory receptors immediately after exercise.
2. Late-/Post-pubertal children will experience the greatest increases in NK recruitment and changes in receptor expression to an acute bout of exercise.
3. Children with higher fitness who engage greater amounts of moderate and vigorous physical activity, and spend less time being sedentary will experience greater NK cell response to an acute bout of exercise.

Chapter 3: Methods

3.1 Recruitment of Healthy Recreationally Active Participants

Children who were healthy and recreationally active, defined as those who engaged in dedicated time to exercise 2 – 3 times a week[133], were recruited from the local Hamilton community. Children were further selected based on two criteria, chronological and pubertal status. Children ages 8 – 11 years and 15 – 18 years were invited to participate in the study. During the first visit, pubertal stage (described below) was verified to ensure that children in the younger age group were pre-pubertal (Tanner 1), while the older children were late-/post-pubertal (Tanner 4 – 5).

3.1.2 Recruitment Strategies

Recruitment was carried out using posters around the greater Hamilton community, by postings on our laboratory website, as well as through emails/phone calls to local sports clubs. Once interested parents or participants contacted the research staff, a phone call was scheduled where a screening questionnaire was administered. The parent/guardian (for those under 16 years) or the adolescent (for 16+ years), were asked to provide the participant's age, approximate height and weight, and participation in physical activity. We also administered a brief medical questionnaire to confirm eligibility based on health status. Although the ages selected generally reflect the appropriate pubertal stages, it was inevitable that some participants were either early maturers (i.e., a 10-year-old female may be peri-pubertal/Tanner 2), or late maturers (i.e., a 15-year-old male may be peri-pubertal/Tanner 3). As such, participants were informed there would still be a chance they may be excluded based on pubertal

development or weight status verification at Visit #1. All responses to the phone-administered screening questionnaire were confirmed at Visit #1.

3.1.1 Exclusion Criteria

Children and adolescents were excluded from this study based on:

Elite Athlete Status: Children who were classified as elite athletes were excluded from the study based on existing evidence suggesting that NK cell function is altered in elite trained adult athletes[69]. According to the International Olympic Committee an elite athlete is defined as a child completing at least 2 – 3 sessions a week of muscle strength training, along with 3 – 4 sessions of high intensity aerobic training lasting 40 – 60 minutes[134]. Thus, to ensure that the trainability effect did not act as a confounding factor, we excluded youth training more than 5 times a week or for more than 3 hours at a time.

Health Status: Children with any personal or sibling history of inflammatory conditions or asthma were excluded. Children who have siblings with inflammatory conditions may have a genetic predisposition that although does not cause overt symptoms may alter the immune inflammatory environment[135]. Additionally, children with asthma have been shown to have increased peripheral cytotoxicity[136]. Body mass index (BMI) percentile was calculated from height and weight measurements according to CDC guidelines and children who fell outside the healthy 5^{th} – 85^{th} percentile range were excluded. Children above the 85^{th} percentile were excluded based on existing evidence that chronic-low grade inflammation in children with obesity caused elevated baseline leucocyte values and dampened cellular responses to exercise[137] . Similarly,

undernutrition impaired immune function, therefore children below the 5th percentile were also excluded[138].

Medication Usage: Children taking any medication were excluded from the study to reduce any possible interference with the immune profiles of participants[139]. In order to capture the natural cyclical hormonal variation, girls taking any type or hormonal contraceptives in the last 6 months were excluded. NK cells are known to be sensitive to estrogen, one of the components of contraceptive pills. To avoid any confounding drug-related effects, girls with either an IUD or taking hormonal contraceptives were excluded[140].

Pubertal Development: Stage of pubertal development was confirmed at the first visit by self-report using Tanner's Scale of breast development for girls and pubic hair development for boys. The Tanner Scale is a commonly used, validated 5-stage scale with a diagram and a brief text description of male or female sex organs; the participant was asked to identify the diagram that most closely resembles them[141–143]. Girls in Tanner 1 indicated no breast development and were deemed pre-pubertal, while those in Tanner 4/5 indicated having a protruding nipple area above the breast and were deemed late-/post-pubertal. Similarly, boys in Tanner 1 reported no pubic hair and were deemed pre-pubertal, while those at Tanner 4/5 reported having pubic hair that spread towards or onto the thigh area and were deemed late/post-pubertal. Participants who self-identified as peri-pubertal or early pubertal (Tanner 2 or 3) stages were excluded so as to minimize the impact of the dynamic hormonal environments reported during puberty on our exercise effects[141,142].

Inability to Cooperate with Study Protocols: Children with exercise restrictions and/or those who could not follow study protocols were excluded.

3.2 Study Overview

Participants in this study were invited to complete five visits at the Child Health & Exercise Medicine lab at the McMaster Children's Hospital. During Visit #1 (preliminary visit), the study was explained to participants in detail. Participants and their parents were asked to fill out assent and/or consent forms, as appropriate (Appendix B). All information regarding eligibility was verified and physical activity and medical questionnaires (Appendix C) were completed with the participant and/or parent/guardian. Participants self-reported their Tanner stage. Anthropometric measurements were collected including weight and height, which were used to calculate BMI. Participants then completed an aerobic fitness test ($\dot{V}O_2\,max$) on a cycle ergometer, which provided data required to standardize the workloads for the subsequent visits. During visits #2-5 (experimental visits) participants were asked to complete one of four 30-minute protocols in a randomized, counterbalanced order. Blood samples were collected at rest (before exercise), immediately after exercise, and after 30, 60 minutes of seated recovery. Blood samples were analyzed for NK cell number and receptor profiles for each of the aforementioned timepoints. A detailed description of study visits and outcomes is provided below.

3.2.1 Visit #1 – Preliminary visit

The 1.5-hour preliminary session was used to verify eligibility, collect basic anthropometric data and standardize subsequent visits. Specific assessments included:

Questionnaires

In order to verify the phone screen, participants were asked the same medical questionnaire to ensure they do not have any medical conditions and were not taking any medication on a regular basis. To gather more information on the physical activity levels of the children and ensure they were recreationally active, a physical activity questionnaire was administered by the researcher to verify the child's frequency and type of organized and spontaneous activities.

Pubertal Stage Assessment

The participant were asked to self-report their pubertal development using the Tanner scale[141,142]. To do this, the participant was given a folder with a series of illustrated images of either breast development (for girls) or pubic hair (for boys) stages and instructed to circle the number they believe is most like them in a private setting. Participants returned the private folder and the researcher. While the participant was preparing for the next step of the assessment, the researcher verified that the participants were either Tanner 1 or Tanner 4/5 before completing the exercise test.

Anthropometric Assessments

Standing height and sitting height were measured at least in duplicate to the nearest 0.1 cm using a Harpenden stadiometer. Measurements within 0.4 cm of each other were averaged. Sitting height was used to calculate predicted years from age at peak height velocity, a secondary indicator of pubertal development[144]. Predicted years from age at peak height velocity represents the difference between the child's chronological age and the estimated age at which they experience maximal linear growth (i.e., a growth spurt). Linear growth occurs in tandem with pubertal development,

as the hypothalamic gonadal axis is activated[145]. Examining years from peak height velocity served as an additional confirmation that participants were in fact pre-pubertal and post-pubertal by ensuring that they were a number of years below and above their estimated age at peak height velocity, respectively. Weight was evaluated using a digital scale, with the participants in minimal clothing (i.e., shorts and a t-shirt) and no footwear. Weight was measured at least in duplicate to the nearest 0.1 kilograms, ensuring that values were within 0.1 kg of each other, and averaged. Body composition was evaluated using a non-invasive bioelectrical impedance analysis unit (InBody), outcomes included fat mass and lean mass, which were used to calculate percentage body fat. Height and weight were used to compute BMI as weight in kg ÷ height^2 in meters and converted into percentiles based on the CDC growth charts[146].

Maximal Aerobic Fitness Test

Next, aerobic fitness was measured using the gold standard maximal oxygen consumption ($\dot{V}O_2max$) assessment, which reflects the efficiency of oxygen transport (cardiorespiratory system) and oxygen utilization (muscle metabolism) during progressive exercise. This assessment was used to ensure we could standardize exercise intensities at subsequent visits. The *McMaster All-Out Progressive Continuous Cycling Test* was performed on a calibrated cycle ergometer (Lode, Groningen, The Netherlands) where workload increased by constant increments every 2 minutes[106]. This test is often described as the feeling of riding up a hill, and typically lasts between 8 to 12 minutes. Throughout the test, participants wore a mouthpiece connected to a calibrated metabolic cart (Vmax29, Sensor Medics) to allow for *direct* measurement of expired gases (O_2 and CO_2). Heart rate was recorded continuously using a heart rate

monitor (Bioharness) throughout the test and tracked by the researcher every minute of the test. Participants were asked to report their exertion level every 2 minutes using Borg's Rating of Perceived Exertion (RPE) scale. The test was terminated when participants reached maximal effort and were unable to maintain a cadence of 60-80 revolutions per minute, despite strong verbal encouragement. Other indicators used to verify maximal effort included either a heart rate of ≥180 bpm or a respiratory exchange quotient ≥1.0[147]. The maximal heart rate threshold was achieved during a graded exercise test by over 95% of children in a large cohort study[147]. The highest 20-sec $\dot{V}O_2$ was considered the $\dot{V}O_{2max}$ and peak mechanical power was defined as the prorated highest power attained during the test.

Physical Activity (between visit #1 and #2)

At the end of the preliminary study visit, participants were given an ActiGraph wGT3X-BT accelerometer to wear for 7 days[148]. An accelerometer is a small, unobtrusive device that measures acceleration, thereby providing an objective method of measuring movement (physical activity and sedentary time) in free-living settings. Participants were outfitted with the accelerometer and instructed to wear it over their right hip during all waking hours of the day, with the exception of water activities (ie. showering and swimming). Children were provided with a log and asked to indicate times the accelerometer was worn and removed (Appendix D). Movement of the y-plane (vertical) was captured in 3-sec intervals and analyzed to quantify levels of physical activity. Upon return, accelerometer data were downloaded and cleaned to remove any non-wear time. Only participants who wore the accelerometer for at least 10 hours a day, and a minimum of 3 days were included in the final analyses. Acceleration data

was converted into activity counts, and time spent in activities of different intensities were calculated with the validated Evenson cut points[149]. The average amount of time per day spent in moderate, vigorous, moderate-to-vigorous, and sedentary time are reported both as a % of wear time (average activity per day / time spent wearing the accelerometer per day) and minutes per day.

3.2.2 Visit #2-5 – Experimental Visits

Following their preliminary visit, participants were scheduled for four experimental visits that were at least 4 days, but no more than 2 weeks, apart (with exceptions of when participants were sick). The 4 days were selected to ensure that any delayed muscle onset soreness induced by the previous visit had subsided, and thus would not affect the children's ability to cycle[150].

Experimental Controls

The day before their study visit, a researcher contacted the participants and their families to remind them of their visit, and to confirm that they have not been sick in the 2 weeks leading up to the visit. If a participant was sick, visits were rescheduled to 2-weeks post-illness to minimize the effect on NK cells. For a given participant, all visits took place within 2-3 hours of each other. For all participants, experimental visits took place in the afternoon in order to avoid diurnal variations in cortisol and growth hormone that may affect exercise responses[151]. The post-menarcheal female participants began their first experimental visit at the end of their menstrual cycle to ensure that they progressed through the study during similar phases of their cycle. Prior to each visit participants were asked to refrain from vigorous physical activity for 24 hours, in order to avoid confounding exercise effects. Participants were also asked to avoid eating junk

food and having caffeine 24 hour before their visit in order to avoid the potential for both a high-fat diet and caffeine to supress the immune system[152]. To further standardize nutritional status across all visits, the participants were asked to log their food and beverage intake 24 hours before the first exercise visit. They were then provided with a copy of this log and asked to replicate their diet as closely as possible for subsequent visits. Finally, participants were asked to refrain from eating or drinking (with the exception of water) for 4 hours before coming into the lab[7].

Ventilatory Threshold and Experimental Visit Workload Calculation

The ventilatory threshold (VT) was calculated using the V-slope method and used to calculate exercise intensity for all experimental visits[153]. To do this, oxygen uptake ($\dot{V}O_2$) was plotted against expired carbon dioxide ($\dot{V}CO_2$) from the $\dot{V}O_2$max test. The respiratory compensation point (RCP) was identified visually as a marked increase in both $\dot{V}O_2$ and $\dot{V}CO_2$ that appears as a gap in the plotted data. The data set was truncated by excluding all values above RCP to more clearly identify VT. The line of identity was then plotted (y = x line), and VT was defined as the $\dot{V}O_2$ point that deviated above, and never returned to or below, the line of identity (Figure 3). VT was calculated independently by 2 investigators (IU/JO).

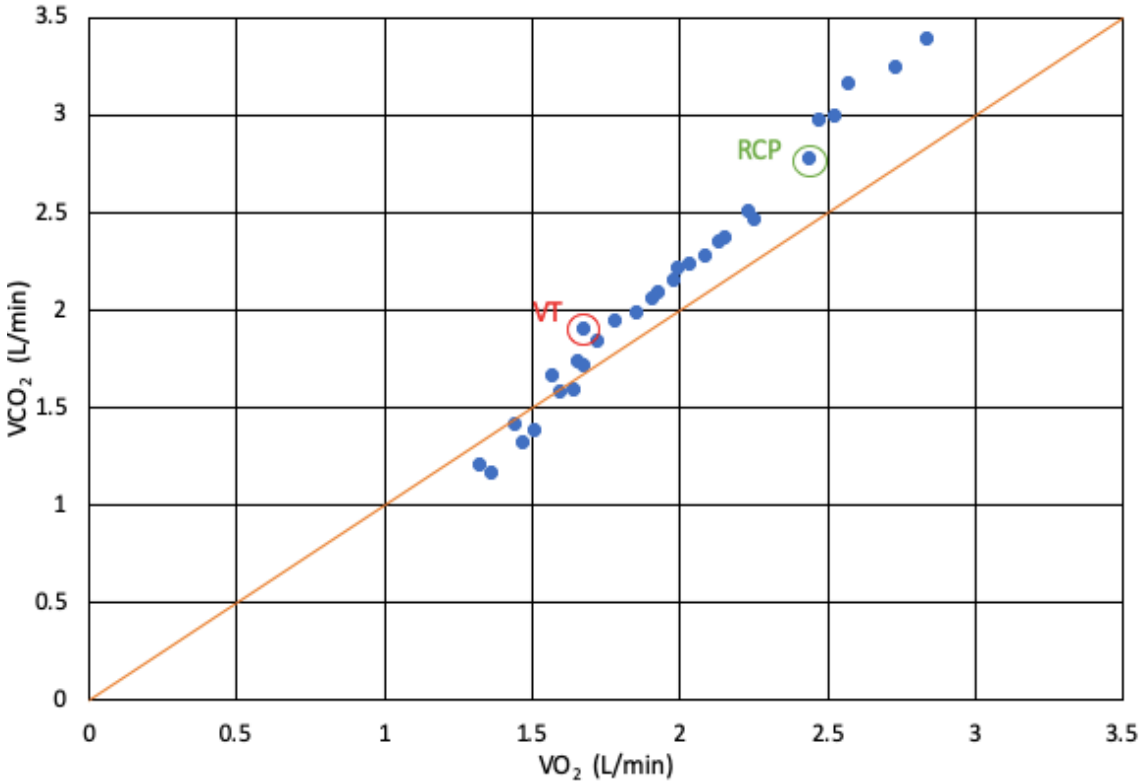


Figure 3: An example of VT and RCP identified using the V-slope method in a participant.

Next, workload was plotted against $\dot{V}O_2$ from the $\dot{V}O_2$max test, and used to interpolate workloads at fixed intensities for each of the experimental sessions. The moderate intensity protocols were set to the workload equivalent to resting $\dot{V}O_2$ plus 75% of the difference between VT and resting $\dot{V}O_2$ values. High intensity protocols were set at a workload corresponding to VT plus 25% of the difference between VT and $\dot{V}O_2$max. This methodology standardized intensity to the participant's maximal capacity, and also considered their anaerobic threshold and ability to efficiently utilize oxygen[154].

Experimental Visit Exercise Protocols

Participants were randomly assigned in a counterbalanced fashion to complete one of four exercise cycling protocols (Figure 4) on a cycle ergometer:

1) Moderate intensity continuous cycling (MI-CONT), a standard exercise in adults.

2) Moderate intensity, intermittent all-out cycling (MI-INT): that will allow for direct comparison of exercise intensity;

3) High intensity, continuous cycling (HI-CONT) - the exercise model which has been used in previous pediatric NK cell studies[7];

4) High intensity, all-out intermittent cycling (HI-INT) - representative of the typical activity patterns reported in children[155];

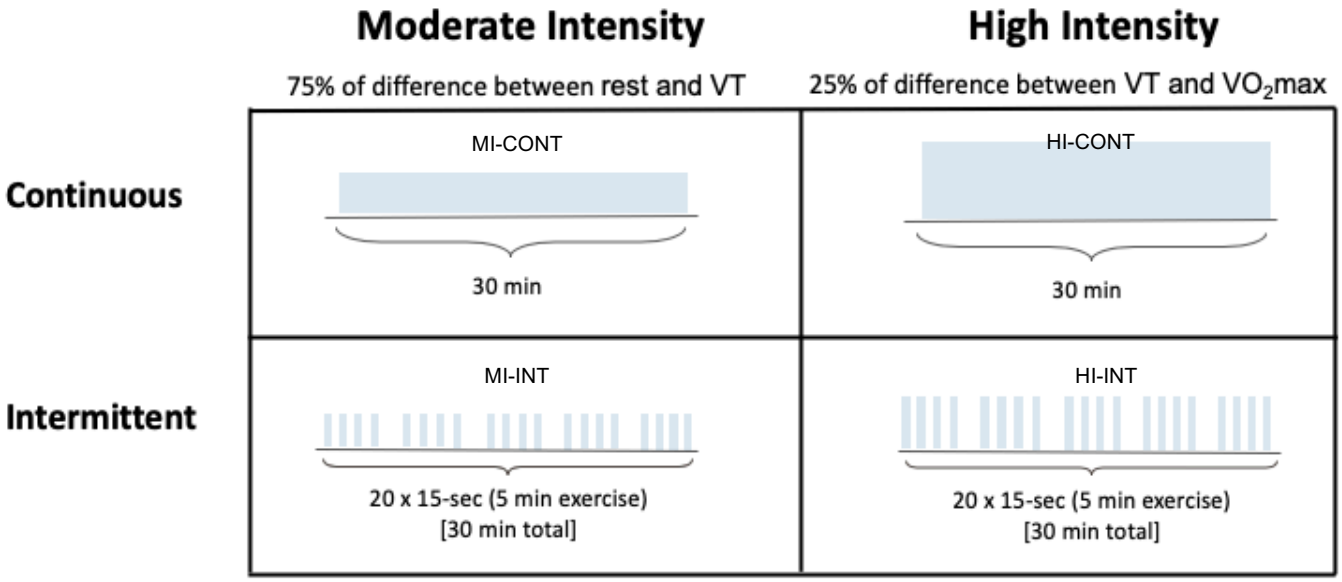


Figure 4: Summary of Exercise Protocols

At each visit, throughout the course of the exercise, heart rate and ratings of perceived exertion were monitored. Gas collection (O_2 and CO_2) was also used to verify that the participants were exercising at the prescribed intensities during the continuous exercise protocols. No gas collection took place during the intermittent protocols due to the short timing of the rest periods. Workloads were adjusted to ensure that exercise intensity was maintained, as defined in Table 2, throughout the exercise protocol.

Heart Rate and Self-Reported Rating of Perceived Exertion

During each cycling protocol, participants were asked to report their level of exertion using Borg's 6 – 20 Rating of Perceived Exertion Scale, which has been validated for use in children[156]. Participants were asked to point at the chart to identify how hard they felt like they were working. During intermittent exercise, they were asked to rate their exertion every 5 minutes. During continuous exercise, they were asked to rate their exertion every 2 minutes. Ratings of 6 – 11 represented an area requiring little effort. Ratings of 12 – 16 were represented working hard, but manageable and expected during exercise. Ratings of 17 – 19 represented the participant felt they were working very hard, and 20 suggested they had attained maximal exertion[156]. Participants were also outfitted with Bioharness, which tracked and recorded heart rates continuously before and during exercise. For each continuous protocol, heart rate was recorded every 2 minutes. During each intermittent protocol, heart rate was recorded roughly 10-15 seconds after each cycling bout (when heart rate peaked). This was done in order to verify that the participants were at roughly 64% - 76% of their max during moderate intensity exercise and 76% - 93% of their max during high intensity exercise[157].

3.3 Blood Collection, Processing and Preparation for Analysis

3.3.1 Blood Collection and Processing

For ease of blood collection, an indwelling catheter was placed in the antecubital region of the arm at the start of each experimental session by an experienced researcher (Dr. Obeid or Dr. Brian Timmons). The catheter was cleaned and flushed using a sterile saline solution before and after each blood draw. Blood samples were

collected at four timepoints during each experimental visit: at rest (PRE), post-exercise (POST), 30 minutes into recovery (REC1) and at 1 hour of recovery (REC2). Serum and plasma from each sample were collected and stored at -80°C in 0.5 ml aliquots. Peripheral Mononuclear Cells (PBMC's) were isolated and cryopreserved in 1.5 mL aliquots for future studies[158,159]. For the purposes of this thesis, whole blood staining was used to quantify the number of NK cells and characterize receptor profiles.

3.3.2 Natural Killer Cell and Receptor Whole Blood Staining

For each time point, 4 individual aliquots of whole blood (100 μL each) were prepared, including: unstained sample, NK cell panel only, activating panel, and inhibitory panel (Table 2). All antibodies were purchased from Miltenyi Biotec Inc. First, 2 μL of each stain was added to the respective panels as outlined in Table 2. Next, the samples were vortexed and refrigerated for 20 minutes, after which 2 mL of room temperature 1x lysing solution (9-parts ddH_2O:1-part 10x BD Bioscience lysing buffer) was added and the samples, vortexed and incubated at room temperature for 10 minutes. Each sample was then centrifuged for 5 minutes at 400 × g at room temperature. The supernatant was discarded, and the sample was washed using 2 mL of MACS buffer (phosphate-buffered saline, 0.5% bovine serum albumin, and 2mM EDTA prepared by mixing 1-part MACS BSA Stock Solution: 20-parts autoMACS Rinsing Solution, Miltenyi Biotech Inc.). The samples were vortexed and centrifuged for 5 minutes at 400 × g at room temperature. The supernatant was discarded and 500 μL of BD Cytofix (BD Bioscience) was added to preserve the samples. The samples were refrigerated for a maximum of 1 week prior to analysis.

Table 1: Summary of monoclonal antibodies – conjugated fluorochromes for each tube.

Unstained	NK Only Panel	Activating Panel	Inhibitory Panel
n/a	CD45-APC700	CD45-APC700	CD45-APC700
	CD3-VioBlue	CD3-VioBlue	CD3-VioBlue
	CD56-PE	CD56-PE	CD56-PE
		NKG2D-PE770	NKG2A-FITC
		DNAM1-FITC	KIRDL2/DL3-PE770

3.4 Flow Cytometric Analysis

3.4.1 Fluorescence Minus One Preparation

Fluorescence Minus One tubes (FMOs) were prepared for each colour in the receptor panel in order to account for any interference of stain colors. A total of six pre-exercise sample FMOs were prepared, with samples collected from participants at two visits. To do this, four extra 100 μL whole blood aliquots were collected. For the activating panel, two FMOs were prepared: 1) all cell stains except NKG2D-PE770, and 2) all stains except DNAM-FITC. Similarly, for the inhibitory panel, two FMOs were prepared: 1) all stains except KIR2DL2/DL3-PE770, and 2) all stains except NKG2A-FITC. FMO samples were prepared and analyzed together with the full stain panels.

3.4.2 Flow cytometer Preparation

All flow cytometer analyses were conducted on a 7-channel Miltenyi MACSQuant Analyzer. On each analysis day, the flow cytometer was calibrated using MACSQuant calibration beads (Miltenyi Biotec Inc.). Prior to sample analysis, compensations were completed for the fluorochromes included in the two receptor panels using MACS Compensation Bead Kits (Miltenyi Biotec Inc.). The compensations were applied to all

the samples processed in a given day in order to control for spectral overlap between channels and fluorochromes.

3.4.3 Gating for Natural Killer Cells

All gating protocols were performed in FloJo software. For each sample, leukocytes were gated using the common leukocyte marker CD45 (Figure 5A). $CD45^{+}$ events were further analyzed in order to identify the lymphocyte population using known information about their size (forward scatter) and granularity (side scatter) (Figure 5B). NK cells were then identified from the lymphocyte pool as cells that were CD3-CD56+ (Figure 5C). Natural Killer cell subtypes were delineated based on the intensity of the CD56 marker. Cells with a high density of CD56 expression were classified as $CD56^{bight}$ (Figure 5D). Cells with lower density of CD56 expression were classified as $CD56^{dim}$ (Figure 5D). This gating protocol was repeated for the NK cell only sample, activating and inhibitory panels.

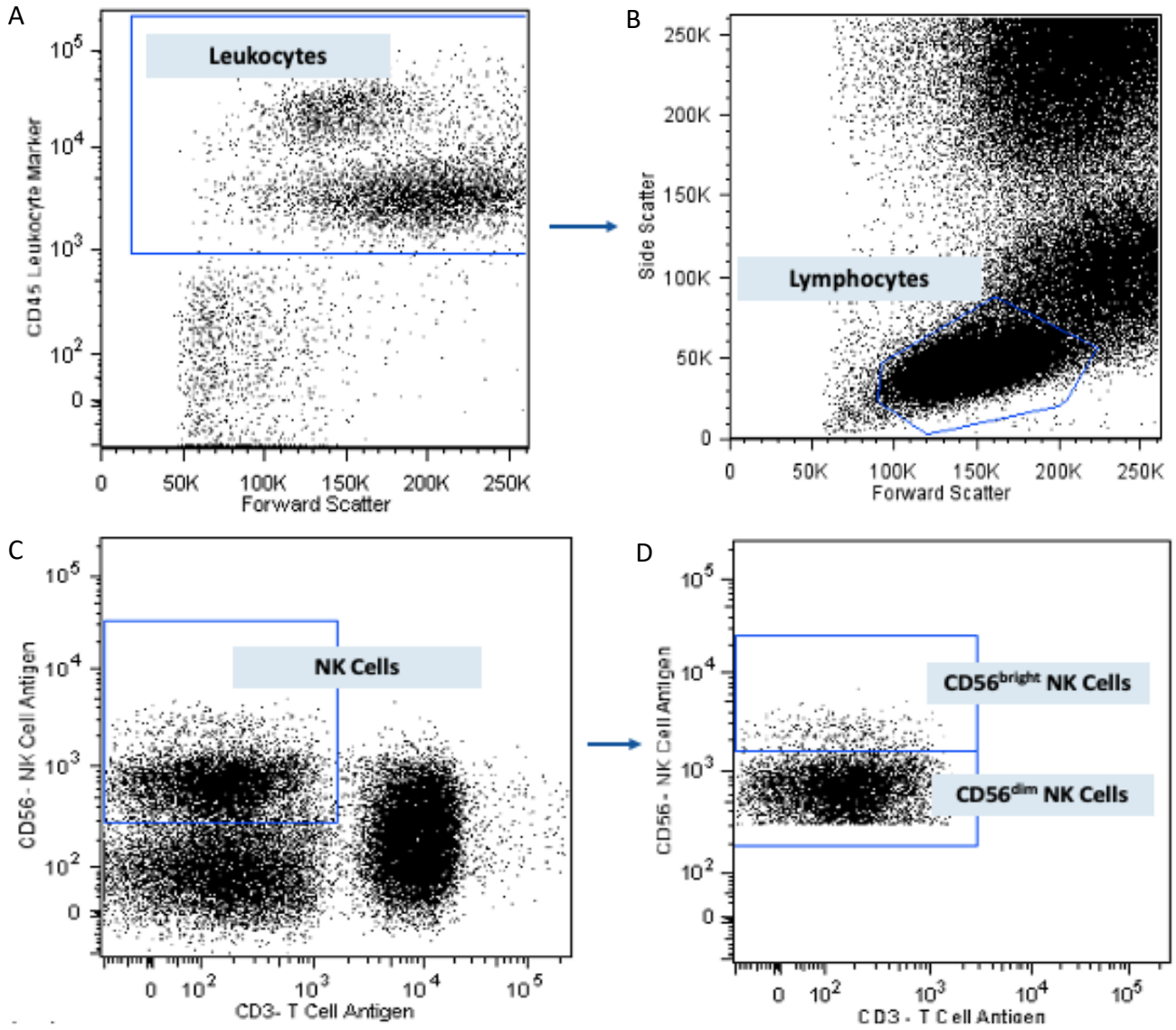


Figure 5: Natural Killer Cell Gating Strategy

3.4.4 Activating and Inhibitory Receptor Panels

After gating for NK cells, as outlined above, individual histograms were generated used to identify the number of cells that expressed each receptor ($CD45^+CD3^-CD56^+$ and $NKG2D^+$ or $DNAM1^+$ or $NKG2D^+$ or $KIRDL2/DL3^+$). The threshold for positive events on the histograms were confirmed using the mean thresholds identified from each receptor's respective FMO analyses. The concentration

cells and the percent of NK cells that expressed each receptor was reported. The median intensity fluorescence (MFI) for each receptor was also calculated using positive events from respective histograms.

3.5 Statistical Analyses

All data are presented as means ± standard deviation. The **primary objective** of the project was aimed at characterizing the effect of exercise intensity and structure on NK cell recruitment and receptor profile. The **secondary** objective of the study was to assess whether there were differences in the NK cell response between pre-pubertal and post-pubertal youth. Prior to conducting a three-way ANOVA to evaluate the interactions between exercise x time x puberty a one-way repeated measures ANOVA was used to compare the PRE values across all four visits. If the ANOVA revealed no differences at PRE (i.e., not significant, $p > 0.05$), a three-way ANOVA was completed using the raw NK total, subsets, and receptors outcomes (expressed per ml or percent or MFI). However, if the one-way ANOVA identified differences at PRE between exercises ($p < 0.05$ for any two or more exercises), the data were converted fold changes relative to PRE to account for these differences (i.e., all PRE values were adjusted to 1, and remaining timepoints were some factor of PRE). Fold changes were then submitted to the three-way ANOVA. The three-way mixed method ANOVA was used in order to compare NK outcomes across two within factors (exercise [4 levels: HI-INT, HI-CONT, MI-INT, MI-CONT], and time [4 levels: PRE, POST, REC1, REC2]) and one between factor (pubertal status [2 levels: pre-pubertal, late/post-pubertal). Where appropriate a Tukey's honestly significant differences post-hoc test was used to determine pair-wise differences. STATISTICA for Windows 7 software was used for all

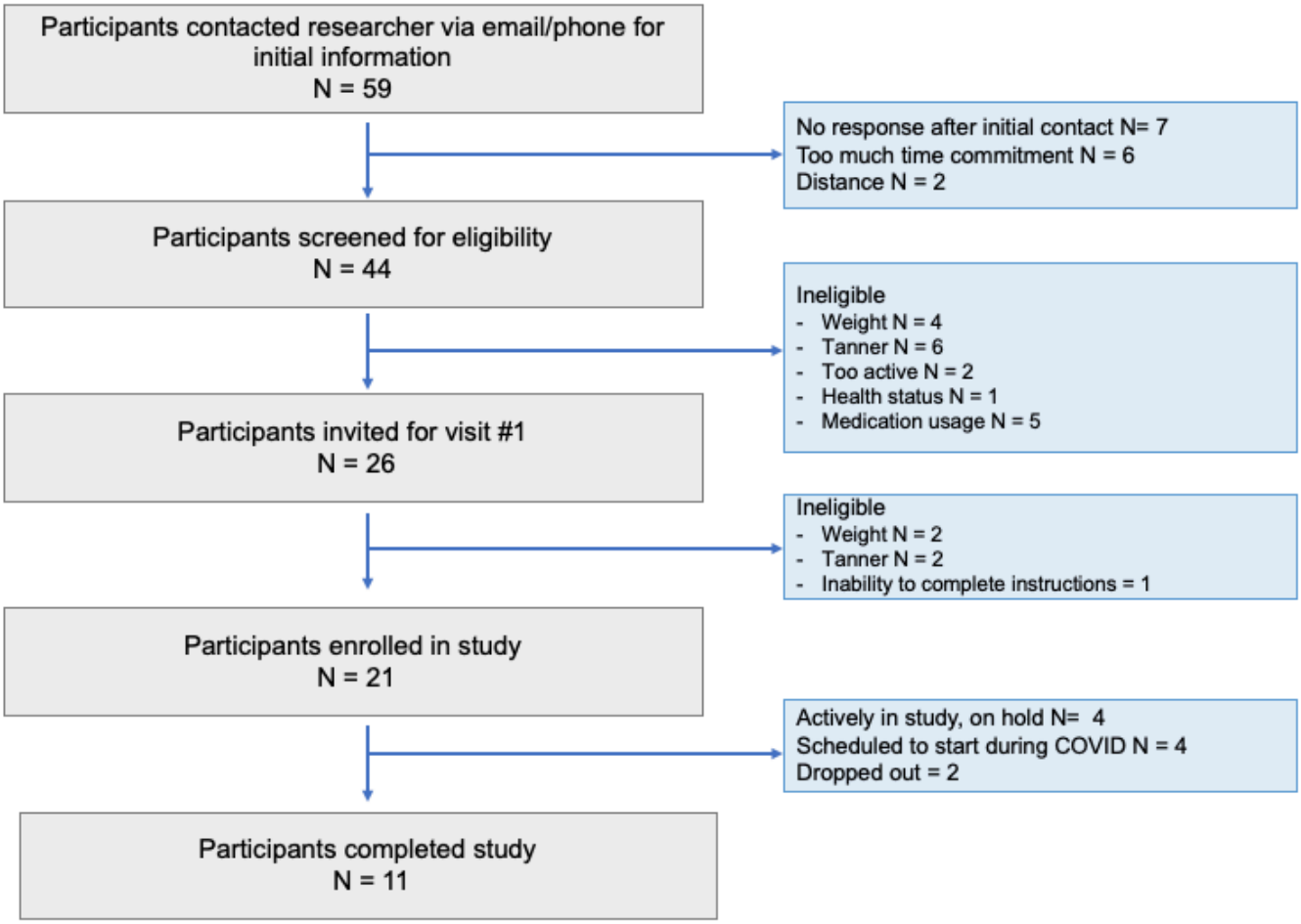
analyses. To address the **third objective,** Pearson Correlations were calculated to examine the relationships between fitness, MVPA, and sedentary time (as a percent of time) and change in NK cell response (as an average of all four visits). Exploratory analysis using a hierarchal linear regression was also conducted to evaluate the relationships between fitness, puberty and magnitude of NK cell change. All analyses for the third objective were conducted using SPSS Version 9.0 software for Mac.

Chapter 4: Results

4.1 Participant Characteristics

During the recruitment process 59 participants contracted the researcher for additional information on the study after either seeing a study poster or hearing a recruitment presentation. Following this, 44 individuals were screened for eligibility and 26 participants were invited for an initial visit after being deemed eligible during the phone screen. 21 participants had enrolled in the study, of those 11 have completed the study and 8 participants have consented to participation but are on hold due to COVID.

Figure 6: Eligibility Flow Chat



4.1.1 Descriptive Characteristics

Descriptive participant characteristics are presented in Table 3. Data were collected from a total of 11 participants (45% females). On average both the pre-pubertal and post-pubertal children were within average height and weight percentiles for their sex and age. All children were within healthy BMI percentiles and body fat percentage[160]. As expected, children in the pre-pubertal group were roughly 2.9 years away from reaching their peak height velocity (PHV), while the post-pubertal kids were roughly 3.0 years post their YPHV. All pre-pubertal kids self-reported as Tanner 1, two post-pubertal children reported to be Tanner 4 and four reported as Tanner 5.

Table 2: Descriptive Participant Characteristics

Participant Characteristic N = 11 (6M, 5F)	Pre-pubertal	Post-pubertal	All	Minimum	Maximum
Sex	2F, 3M	3F, 3M	-	-	-
Age (years)	10.0 ± 0.9	17.2 ± 1.0	13.9 ± 3.9	8.6	18.3
Tanner	Tanner 1	2 (Tanner 4) 4 (Tanner 5)	-	-	-
Height Percentile	53.5 ± 13.8	38.7 ± 27.4	45.4 ± 22.6	1.9	77.0
Weight Percentile	47.4 ± 22.5	60.6 ± 17.8	54. ± 20.2	23.0	89.4
BMI Percentile	42.0 ± 25.0	70.2 ± 11.3	57 ± 23	17	85
Percent Body Fat	15.6 ± 3.4	21.7 ± 8.5	18.9 ± 7.1	9.2	32.6
Years to Peak Height Velocity	-2.9 ± 0.2	3.0 ± 0.9	0.3 ± 3.2	-3.2	3.9

Values are reported as mean ± standard deviation.

4.1.2 Fitness Outcomes

Descriptive fitness outcomes are presented in Table 4. Independent samples t-tests revealed that the pre-pubertal children had significantly lower absolute $\dot{V}O_2max$ values (t (9) =3.602, p = 0.006, absolute peak power t (9) =2.750, p = 0.020), and

ventilatory threshold (t (9) = 3.363, p = 0.008). However, when normalized to weight, pre-pubertal children displayed greater relative $\dot{V}O_2$max values (t (9) = 3.357, p = 0.008) and no significant difference in relative peak power (t (9) = 0.457, p = 0.658) compared with post-pubertal children. No significant differences were observed in the VT as a percent of $\dot{V}O_2$max between the two groups (t (9) = 1.281, p = 0.232). Given the differences in size between participants, a wide range of intensity workloads were used for experimental sessions.

Table 3: Participant Fitness Parameters.

N = 11 (6M, 5F)	Pre-pubertal	Post-pubertal	All	Minimum	Maximum
Absolute $\dot{V}O_2$max (L/min)	1.5 ± 0.2**	2.5 ± 0.6	2.1 ± 0.7	1.3	3.6
Relative $\dot{V}O_2$max (ml/kg*min)	49.8 ± 6.2*	39.4 ± 4.1	44.1 ± 7.3	35.3	55.0
Absolute peak power (watts)	94 ± 16 *	189 ± 75	146 ± 73	75	285
Relative peak power (W/kg)	3.0 ± 0.3	2.9 ± 0.7	2.9 ± 0.6	2.1	3.6
$\dot{V}O_2$ at VT (L/min)	1.0 ± 0.2 *	1.5 ± 0.2	1.2 ± 0.3	0.8	1.8
VT as a percent of $\dot{V}O_2$max	63.9 ± 7.0	58.6 ± 6.7	61.0 ± 7.1	50.7	71.8
Power output MI protocols (watts)	39 ± 8	64 ± 25	53 ± 23	28	111
Power output HI protocols (watts)	65 ± 13	114 ± 33	92 ± 35	51	155

*Values are reported as mean ± standard deviation; * denotes p < 0.05; ** denotes p < 0.01.*

4.1.3 Physical Activity

Valid accelerometer data were available for 10 participants. One participant was not included in analyses because they did not meet the criteria of wearing the accelerometer for at least 3 days for ≥10 hours. On average, participants engaged in 52 ± 8 minutes of daily MVPA and spent 443 ± 45 minutes per day being sedentary.

Independent t-tests revealed there were no significant differences in active or sedentary time between the pre-pubertal and post-pubertal children. As a percent of wear time, children spent 8.6 ± 1.4% of their day in MVPA, with no differences observed between the pre-pubertal and post-pubertal children. Post-pubertal children spent a significantly higher proportion of time being sedentary compared with pre-pubertal children (79.5 ± 4.6 % vs. 73.9 ± 7.4 %, t (8) = 3.686, p <0.01). Light physical activity (LPA) was significantly greater in pre-pubertal compared to post-pubertal children both in minutes/day (139.1 ± 24.0 min/d vs. 73.1 ± 22.7 min/d, t (8) = 4.591, p <0.01) and as a percent of wear time (19.8 ± 3.8 % vs. 7.3 ± 4.2 %, t (8) = 4.930, p <0.01).

Table 4: Physical Activity Characteristics

Participant N = 10 (6M, 5F)	Prepubertal	Post-pubertal	All	Min.	Max.
Sedentary time (min/day)	439 ± 51	447 ± 38	443 ± 45	380	518
Sedentary time (% of day)	68.3 ± 5.0	79.5 ± 4.6 **	73.9 ± 7.4	63.3	86.3
LPA (min/day)	139.1 ± 24.0	71.3 ± 22.7 **	105.2 ± 42.0	39.6	169.8
LPA (% of day)	19.8 ± 3.8	7.3 ± 4.2 **	13.5 ± 7.6	1.8	25.1
MVPA (min/day)	55 ± 9	49 ± 6	52 ± 8	40	67
MVPA (% of day)	8.6 ± 1.8	8.7 ± 1.1	8.6 ± 1.4	6.7	11.1

*Values are reported as mean ± standard deviation; * denotes p < 0.05; ** denotes p < 0.01.*

4.2 Natural Killer Cell Concentration and Proportion

4.2.1 NK Cell Concentration

A significant time effect was observed for NK cell concentrations ($F(3,27) = 18.226$, $p < 0.001$). The concentration of NK cells circulating at POST (58,741 ± 9,301 cells/mL) was significantly greater than those observed at PRE (15,423 ± 59,992 cells/mL), REC1 (11,668 ± 7,737 cells/mL), and REC2 (9,629 ± 5,247 cells/mL), $p < 0.001$. No significant main effects were observed for exercise or puberty.

A significant time × exercise × puberty interaction was observed (Figure 7, $F(9,81) = 3.295$, $p < 0.01$). Tukey's post-hoc analysis revealed no significant differences across all time points and exercises in pre-pubertal children (Figure 7A). In post-pubertal children, no significant difference was observed in NK cell concentrations at PRE across all four protocols (Figure 7B). A significant increase in concentration of NK cells PRE to POST occurred in each exercise visit except MI-CONT (*, $p < 0.001$). We observed between-exercise differences in the proportion of NK cells at POST, wherein the total NK cell concentration was higher for HI-INT (124,723 ± 91,596 cells/mL) compared to both MI-CONT (17,082 ± 9,516 cells/mL) and HI-CONT (19,931 ± 1,492 cells/mL; a, $p < 0.001$). POST NK cell concentrations for MI-INT (109,644 ± 84,664 cells/mL) were significantly greater than POST for MI-CONT (b, $p < 0.001$). No significant difference was detected at POST between MI-INT and HI-INT. No significant differences in the NK cell concentration were observed at REC1 or REC2 across all four exercise protocols (Figure 7B). The ANOVA table is included in Appendix A (Table A1).

4.2.2 NK Cell Proportion

A significant time effect was also observed in the proportion of NK cells ($F(3,27) = 76.558$, $p < 0.0001$). The proportion of NK cells at POST (18.2 ± 8.4%) was significantly greater than those observed at PRE (9.5 ± 4.2%), REC1 (7.6 ± 3.3%), and REC2 (6.6 ± 2.3%), $p < 0.01$. Furthermore, PRE values were significantly higher than those observed at REC2, $p < 0.01$. A significant puberty effect was also observed ($F(1,9) = 15.180$, $p < 0.01$), wherein post-pubertal children have significantly higher overall proportion of NK cells (12.7 ± 8.2%) than pre-pubertal children (7.8 ± 2.9%). There was no significant main effect of exercise ($F(3,27) = 1.581$, $p = 0.217$).

A significant time × exercise × puberty interaction was observed (Figure 5, $F(9,81) = 2.421$, $p < 0.05$). Tukey's post-hoc analysis revealed no significant differences across all time points and exercises in pre-pubertal children (Figure 7C). In post-pubertal children, there were no differences in the proportion of NK cells at PRE across the four exercise protocols (Figure 7D). There was a significant increase in the proportion of NK cells PRE to POST in each exercise visit (*, $p < 0.001$). We observed between-exercise differences in the proportion of NK cells at POST, where HI-INT (29.2 ± 8.9%) was higher than both MI-CONT (18.7 ± 5.6%) and HI -CONT (21.9 ± 4.8%) exercise stimuli (a, $p < 0.001$). POST NK cell values for MI-INT (25.7 ± 5.8%) were significantly greater than MI-CONT (b, $p < 0.001$). No significant differences were observed at POST between MI-INT and HI-INT. No significant differences in NK cell proportion were observed at REC1 or REC2 across all four exercise protocols (Figure 5D). The ANOVA table is included in Appendix A (Table A2).

4.2.3 Pubertal Group Comparison

There were no significant differences at PRE between pubertal groups. In post-pubertal children, the increase in NK cell concentrations from PRE to POST were 12-fold higher for MI-INT and 7-fold higher for HI-INT compared to pre-pubertal children (#, $p < 0.001$). The increase in the proportion of NK cells from PRE to POST were 8-fold higher for MI-INT and 3-fold higher for HI-INT in post-pubertal children compared to pre-pubertal children (#, $p < 0.001$). No significant differences were observed at REC1 or REC2.

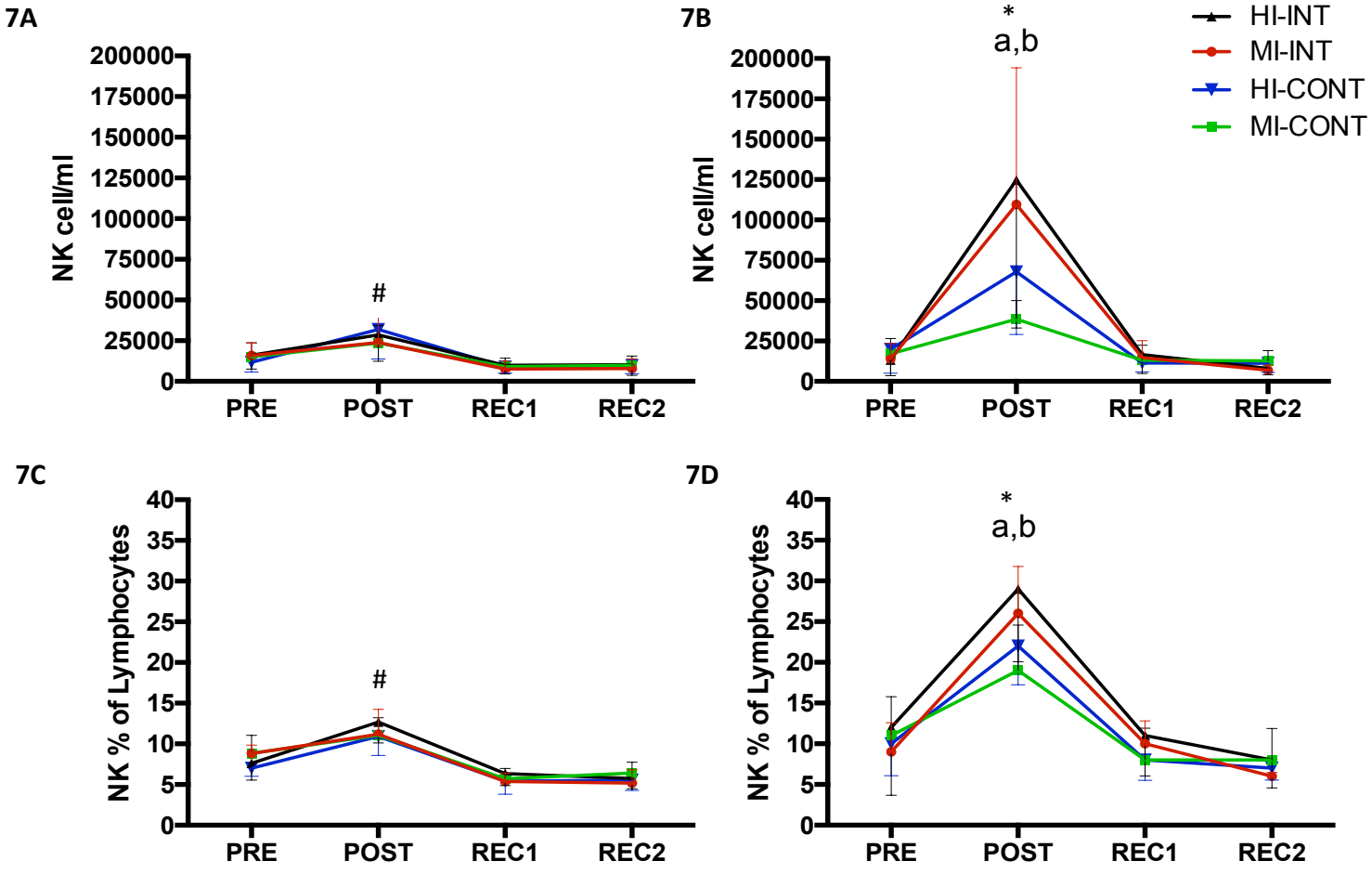


Figure 7: Natural Killer Cell Proportion and Concentration. 7A – pre-pubertal NK concentration, 7B – post-pubertal NK concentration, 7C – pre-pubertal NK as a proportion of lymphocytes, 7D – post-pubertal NK as a proportion of lymphocytes. a = HI-INT > HI-CONT and MI-CONT, b = MI-INT > MI-CONT, * = PRE < POST, # = POST post-pubertal > POST pre-pubertal

4.3 NK Cell Subtype ($CD56^{dim}$ and $CD56^{bright}$) Concentration

4.3.1 $CD56^{dim}$ Concentration

A significant time effect was observed for concentration of $CD56^{dim}$ cells ($F(3,27) = 17.930$, $p < 0.0001$). The concentration of $CD56^{dim}$ NK cells at POST (53,332 ± 56,151 cells/ml) was significantly greater than PRE (13,169 ± 8,248 cells/ml), REC1 (9,053 ± 6,472 cells/ml), and REC2 (7,483 ± 4,464 cells/ml), $p < 0.001$. No significant exercise or puberty effect was observed for the concentration of $CD56^{dim}$ cells.

A significant time × exercise × puberty interaction was observed for $CD56^{dim}$ cells (Figure 8A/B, $F(9,81) = 3.160$, $p < 0.01$). Tukey's post-hoc analysis revealed no significant differences in $CD56^{dim}$ cells across all time points and exercise protocols in pre-pubertal children (Figure 8A). In post-pubertal children, the $CD56^{dim}$ concentration was not different at PRE across the four exercise protocols. There was a significant increase in the $CD56^{dim}$ cell subset from PRE to POST in response to HI-INT, MI-INT and HI-CONT exercise (*, $p < 0.001$). The concentration of $CD56^{dim}$ NK cells at POST were significantly higher for HI-INT (115,139 ± 88,563 cells/ml) compared to MI-CONT (33,549 ± 9,283 cells/ml) and HI-CONT (62,709 ± 36,892 cells/ml) (a, $p < 0.001$, Figure 8B). The POST MI-INT $CD56^{dim}$ NK cell concentration (99,656 ± 77,017 cells/ml) was significantly greater than POST MI-CONT and HI-CONT (b, $p < 0.001$). No significant differences in the concentration of $CD56^{dim}$ were observed at POST between MI-INT and HI-INT. No differences in the concentration of $CD56^{dim}$ cells were observed at REC1 and REC2. The ANOVA tables are included in Appendix A (Table A3).

4.3.2 CD56^{bright} Concentration

A significant time effect was observed for concentration of CD56^{bright} cells ($F(3,27) = 20.330$, $p < 0.0001$). The concentration of CD56^{bright} NK cells at POST (5,409 ± 5,604 cells/ml) was significantly greater than those observed at PRE (2,255 ± 1,250 cells/ml), REC1 (2,615 ± 1,549 cells/ml), and REC2 (2,146 ± 1,098 cells/ml), $p < 0.001$. No significant exercise or puberty effect was observed for the concentration of CD56^{bright} cells.

A significant time × exercise × puberty interaction was observed for CD56^{bright} cells (Figure 8C/D, $F(9,81) = 2.59$, $p < 0.05$). Tukey's post-hoc analysis revealed no significant differences in CD56^{bright} cells across all time points and exercise protocols in pre-pubertal children (Figure 8C). In post-pubertal children, the CD56^{bright} concentration was not different at PRE across the four exercise protocols. Significant PRE to POST increases in the CD56^{bright} subset were only observed in response to HI-INT and MI-INT (*$p < 0.001$). The concentration of CD56^{bright} (Figure 8D) cells at POST was significantly higher for HI-INT (9,584 ± 3,944 cells/ml) compared to both MI-CONT (5,029 ± 3,178 cells/ml) and HI-CONT (5,128 ± 3,073 cells/ml) exercise protocols (a, $p < 0.001$). The concentration of CD56^{bright} NK cells at POST for MI-INT (9,988 ± 8,456 cells/ml) was significantly greater than POST for MI-CONT and HI-CONT (b, $p < 0.001$). No significant differences in the concentration of CD56^{bright} were observed at POST between MI-INT and HI-INT. No differences in the concentration of CD56^{bright} cells were observed at REC1 and REC2. ANOVA tables are included in Appendix A (Table A4).

4.3.3 Pubertal Group Comparison

There were no significant differences in the PRE concentrations of $CD56^{dim}$ or $CD56^{bright}$ across pubertal groups. The increase in the concentration of $CD56^{dim}$ cells from PRE to POST was 12-fold higher for MI-INT and 9-fold higher for HI-INT in post-pubertal children compared to pre-pubertal children (#, $p < 0.001$). The increase in concentration of $CD56^{bright}$ cells from PRE to POST was 12-fold higher for MI-INT and 18-fold higher for HI-INT in post-pubertal children compared to pre-pubertal children (^, $p < 0.05$).

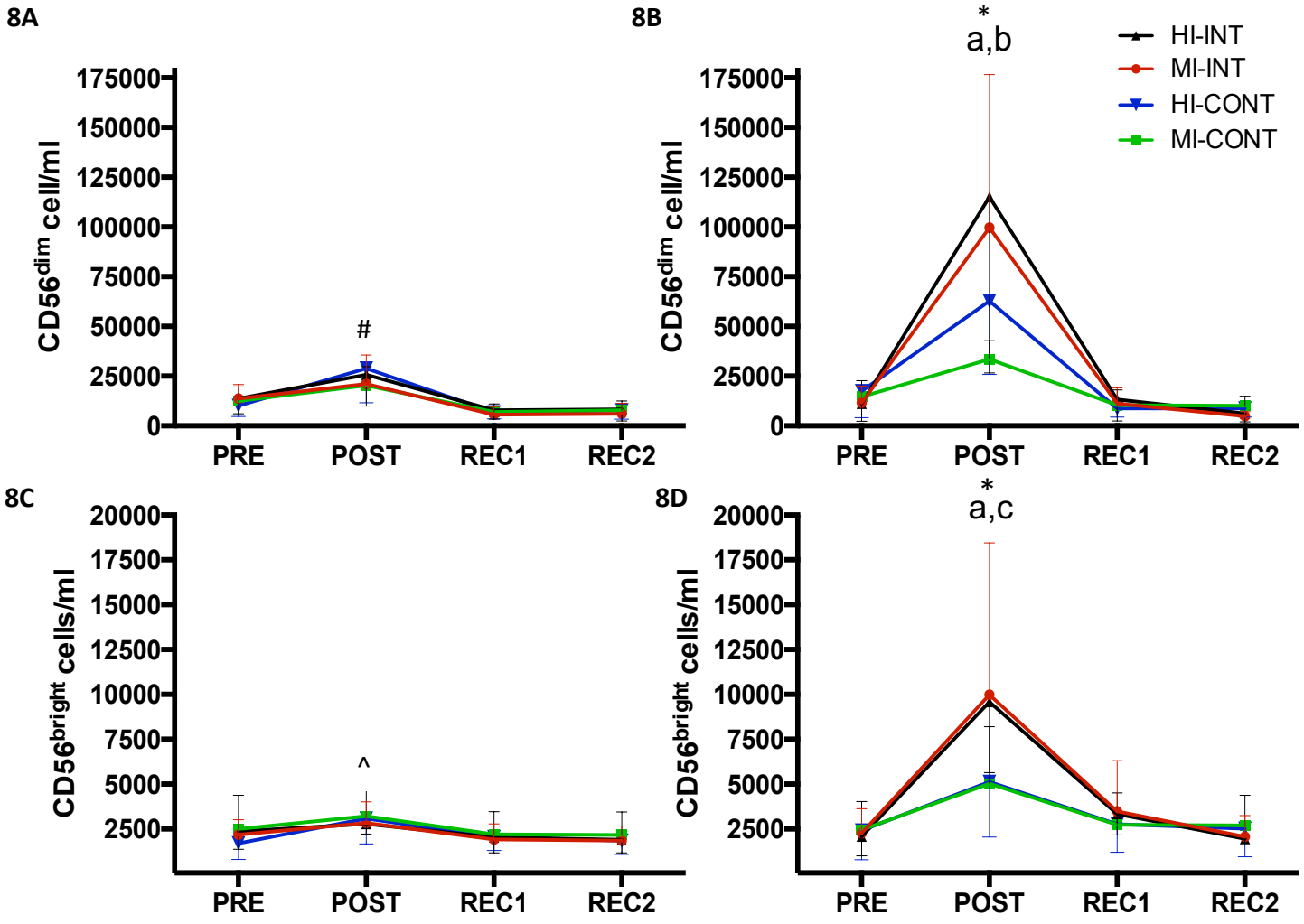


Figure 8: Natural Killer Cell Subtype Concentration. 8A – pre-pubertal $CD56^{dim}$ concentration, 8B – post-pubertal $CD56^{dim}$ concentration, 8C – pre-pubertal $CD56^{bright}$ concentration, 8D – post-pubertal $CD56^{bright}$ concentration. a = HI-INT > HI-CONT and MI-CONT, b = MI-INT > MI-CONT, c = MI-INT > MI-CONT and HI-CONT, * = PRE < POST, # = pre-pubertal < post-pubertal ($p < 0.001$), ^ = pre-pubertal < post-pubertal ($p < 0.05$)

4.4 NK Cell Subtype Proportions

All effects detected in $CD56^{dim}$ cells as a proportion of total NK cells are presented below. Although not presented, the $CD56^{bright}$ subset demonstrated the exact inverse relationships as $CD56^{dim}$ proportion by virtue of the fact that they are complementary (% $CD56^{dim}$ + % $CD56^{bright}$ = 100% NK). A significant time effect was observed for $CD56^{dim}$ cells ($F(3,27) = 52.280$, $p < 0.0001$). The proportion of $CD56^{dim}$ cells at PRE (84.4 ± 5.5 %) was significantly lower than POST (88.9 ± 4.6 %), and significantly greater than REC1 (76.0 ± 7.0 %), and REC2 (75.5 ± 8.7 %), $p < 0.001$. No significant main effects of exercise or puberty were observed in the proportion of $CD56^{dim}$ cells. A significant time × exercise × puberty interaction was observed (Figure 9, $F(9,81) = 2.050$, $p < 0.05$) (Figure 9A). In all exercise protocols except HI-INT, there was a significant decrease in the proportion of $CD56^{dim}$ cells at REC1 relative to PRE (*, $p < 0.05$). By REC2, the proportion of $CD56^{dim}$ cells returned to PRE values in every exercise except MI-INT (#, $p < 0.001$).

The proportion of $CD56^{dim}$ cells was not significantly different at PRE between pubertal groups or across all four visits. In post-pubertal children, PRE $CD56^{dim}$ values were not significantly different across visits. There was a significant increase in the proportion of $CD56^{dim}$ cells during MI-INT and HI-INT exercise stimuli at POST in post-pubertal children (^, $p < 0.05$). The proportion of $CD56^{dim}$ cells was not different at REC1 across the four exercise protocols. We observed between exercise differences in the proportion of $CD56^{dim}$ cells at REC2 (Graph 9B), with lower values for HI-INT (68.8 ± 15.6 %) compared to both MI-CONT (79.0 ± 6.6 %) and HI-CONT (78.1 ± 5.5 %) exercise protocols (a, $p < 0.001$). At REC2 values for MI-INT (71.0 ± 8.5 %) were lower

than both MI-CONT and HI-CONT (b, $p < 0.05$) (Figure 9B). No significant differences at REC2 were detected between MI-INT and HI-INT. Importantly, in all exercise protocols except MI-CONT, the proportions of $CD56^{dim}$ cells at REC2 are significantly lower than PRE ($p < 0.001$). The ANOVA tables are included in Appendix A (Table A5 – A6).

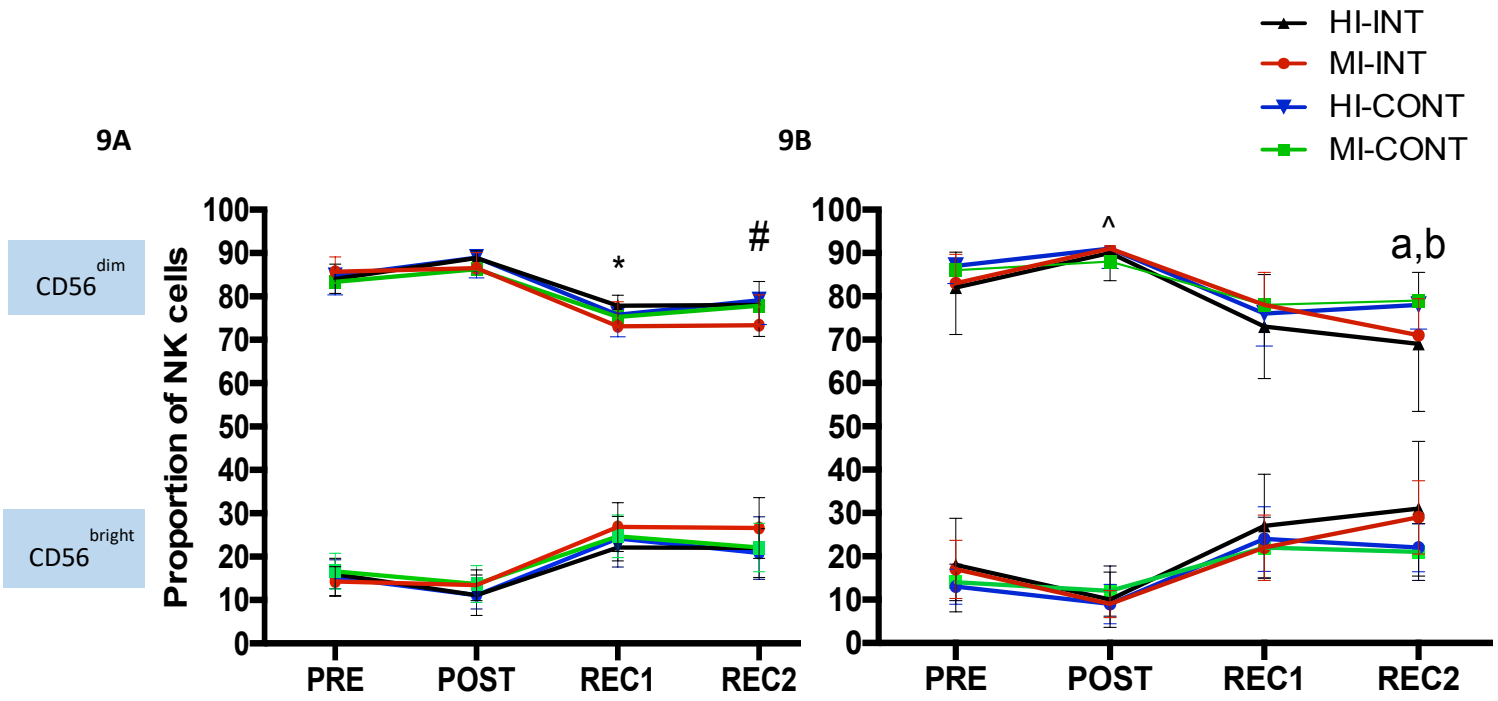


Figure 9: NK cell subsets as a proportion of total NK cells. 9A – pre-pubertal $CD56^{dim}$ and $CD56^{bright}$ as a proportion NK total, 9B – post-pubertal $CD56^{dim}$ and $CD56^{bright}$ as a proportion of NK total. * = REC1 < PRE $CD56^{dim}$, # = REC2 < PRE $CD56^{dim}$, ^ = POST > PRE $CD56^{dim}$, a= HI-INT < MI-CONT and HI-CONT and b = MI-INT < MI-CONT and HI-CONT

4.5 Activating and Inhibitory Receptor Characterization

4.5.1 Activating and Inhibitory Receptor Expression

Since no exercise or pubertal group main effects or interactions were observed for activating or inhibitory receptors, all receptor data presented below are collapsed together to illustrate time effects. A significant time effect was observed in the proportion of NK cells expressing NKG2D ($F(3,27) = 3.902$, $p < 0.05$, Figure 10A). The proportion of NK cells expressing NKG2D at POST (64.5 ± 20.5 %) was significantly lower than REC1 (69.1 ± 16.8 %), (*, $p < 0.05$). No significant differences in the expression of NKG2D were detected between PRE (65.5 ± 18.4 %), REC1 or REC2 (67.1 ± 17.2 %). Exercise did not affect the proportion of NK cells expressing DNAM1 (Figure 10A).

A significant time effect was observed in the proportion of NK cells expressing NKG2A ($F(3,27) = 45.02$, $p < 0.001$). The proportion of NK cells expressing NKG2A at POST (53.8 ± 14.2 %) was significantly lower than PRE (57.9 ± 13.4%), REC1 (63.6 ± 13.7 %), and REC2 (64.2 ± 12.8 %), (#, $p < 0.01$). There were no significant differences between REC1 and REC2, and both were significantly greater than PRE (^, $p < 0.001$). Exercise did not have any effect on the proportion of NK cells expressing KIR2DL2/DL3 (Graph 10B). The ANOVA tables are included in Appendix A (Tables A7 – A10).

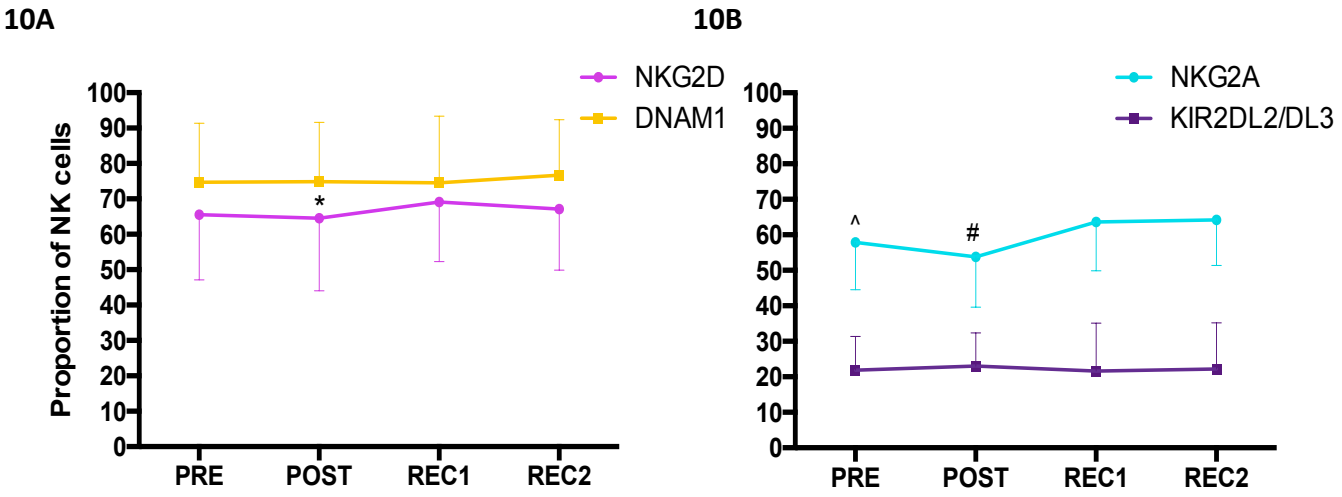


Figure 10: Activating and Inhibitory Receptor Expression. 10A – Activating receptor proportion, 10B – Inhibitory receptor proportion. * = POST < REC1, ^ = PRE < REC1 and REC2, # = POST < PRE, REC1 and REC2

4.5.2 Activating and Inhibitory Receptors Median Fluoresce Intensity (MFI)

Since neither exercise protocol type nor puberty had an effect on the MFI of NKG2D, DNAM1, and NKG2A, all the receptor data presented below are collapsed by exercise and pubertal group to illustrate time effects. A significant time effect was observed for the MFI of NKG2D ($F(3,27) = 18.98$, $p < 0.001$, Figure 9A). The MFI of NKG2D at PRE (2,395 ± 300) and POST (2,361 ± 297) were not different, and both were significantly lower than REC1 (2,549 ± 369) and REC2 (2,523 ± 361) (* and #, $p < 0.001$). No significant differences were detected between REC1 and REC2. A significant time effect was also observed in the MFI of DNAM1 ($F(3,27) = 8.603$, $p < 0.001$). The MFI of DNAM1 at PRE (1,002 ± 199) and POST (985 ± 165) exercise were significantly lower than REC2 (1,052 ± 203) (^, $p < 0.01$), but not REC1 (1,018 ± 188). A significant time effect was observed for the MFI of NKG2A ($F(3,27) = 21.5$ $p < 0.001$, Figure 9B). The MFI of NKG2A at PRE (4,864 ± 1,573) was not different from POST (4,534 ± 1,874), and both were significantly lower than at REC1 (5,774 ± 2,124) and REC2 (5,891 ± 2,074) (* and #, $p < 0.001$). No significant differences were detected between REC1 and REC2.

No significant main effect of time, exercise or puberty were observed in the context KIR2DL2/DL3 MFI. However, a significant time × exercise × puberty effect was observed ($F(9,81) = 4.852$, $p < 0.001$, Figure 9C). Tukey's post-hoc analysis revealed no significant differences across all time points and exercise protocols in pre-pubertal children (Figure 9C). In post-pubertal children (Figure 9D), the MFI of KIR2DL2/DL3 receptor at PRE was comparable across all exercise visits. The MFI at REC1, was significantly lower for MI-INT (3,085 ± 602) compared to both HI-CONT (3,688 ± 964)

and HI-INT (3,829 ± 1,189) exercise stimuli (a, $p < 0.001$). However, no significant differences were observed between HI-CONT and HI-INT or MI-CONT (3,412 ± 842) and MI-INT. At REC1 both HI-INT and HI-CONT display greater MFI compared to PRE values; MFI was only still elevated at REC2 compared to PRE for HI-INT ($p < 0.001$). At REC2, the MFI of KIR2DL2/DL3 receptor was significantly higher in response to HI-INT (4,007 ± 1,226) compared to HI-CONT (3,382 ± 764), MI-CONT (3,328 ± 739) and MI-INT (3,150 ± 535) (b, $p < 0.001$). No significant differences were detected between pre-pubertal and post-pubertal children. The ANOVA tables are included in Appendix A (Tables A11 – A14).

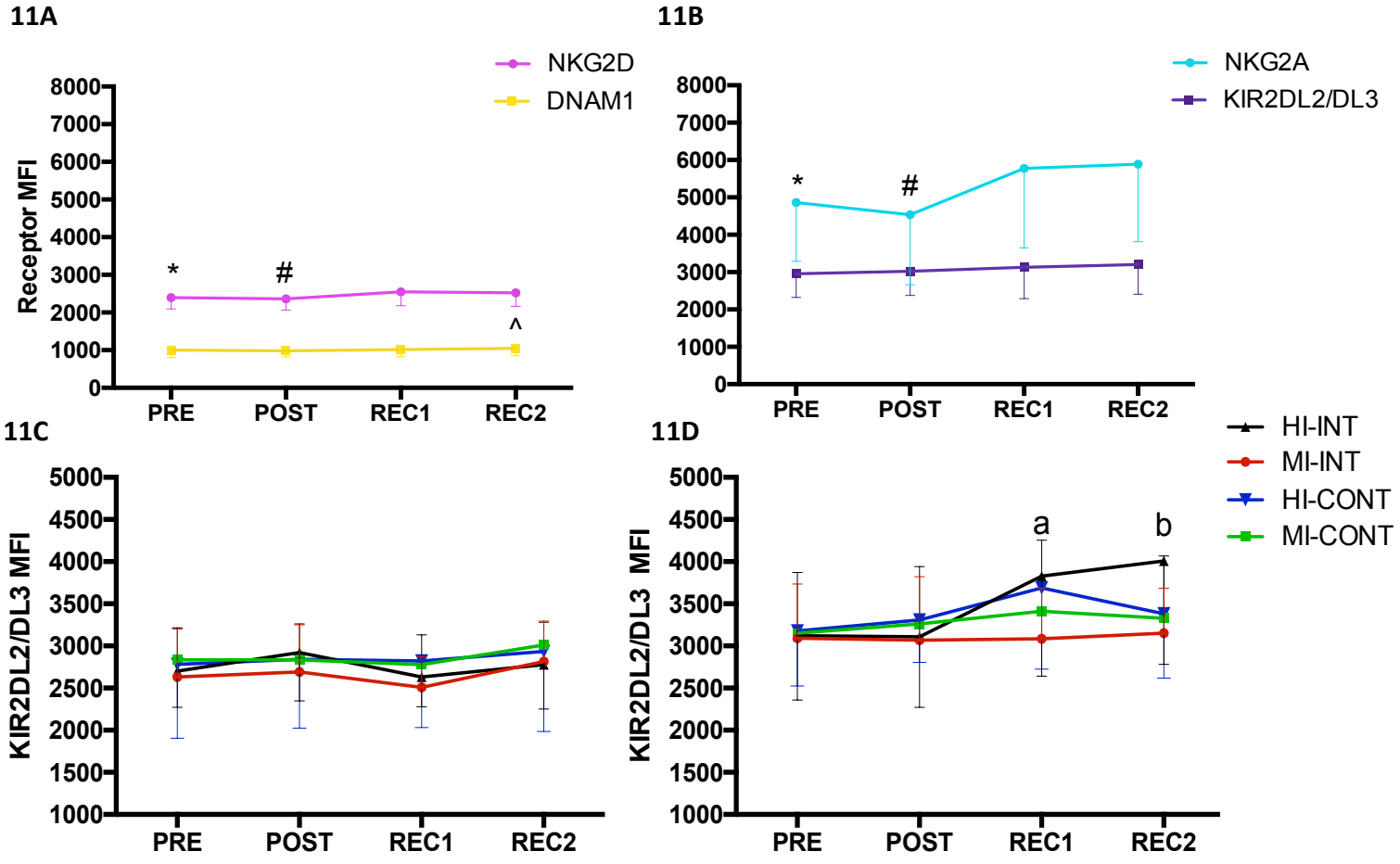


Figure 11: Activating and Inhibitory Receptor MFI. 11A – Activating receptor MFI, 11B – Inhibitory Receptor MFI, 11C – pre-pubertal KIR2DL2/DL3 MFI, 11D – post-pubertal KIR2DL2/DL3 MFI. * = PRE < REC1 and REC2, # = POST < REC1 and REC2, ^ = REC2 > PRE and POST, a = MI-INT < HI-CONT and HI-INT, b = HI-INT > HI-CONT, MI-CONT and MI-INT.

4.5.3 Pubertal Group Comparison

To assess if the expression and density of the activating receptors were different across pubertal groups at PRE, a series of t-tests were carried out. There were no significant differences in the expression ($t(9) = 0.900$, $p = 0.39$) or MFI ($t(9) = 0.118$, $p = 0.91$) of the NKG2D receptor between pre-pubertal and post-pubertal children (Figure 12A/C). There were no significant differences in the expression ($t(9) = 1.47$, $p = 0.18$) or MFI ($t(9) = 0.325$, $p = 0.76$) of the DNAM1 receptor between pre-pubertal and post-pubertal children (Figure 12B/D).

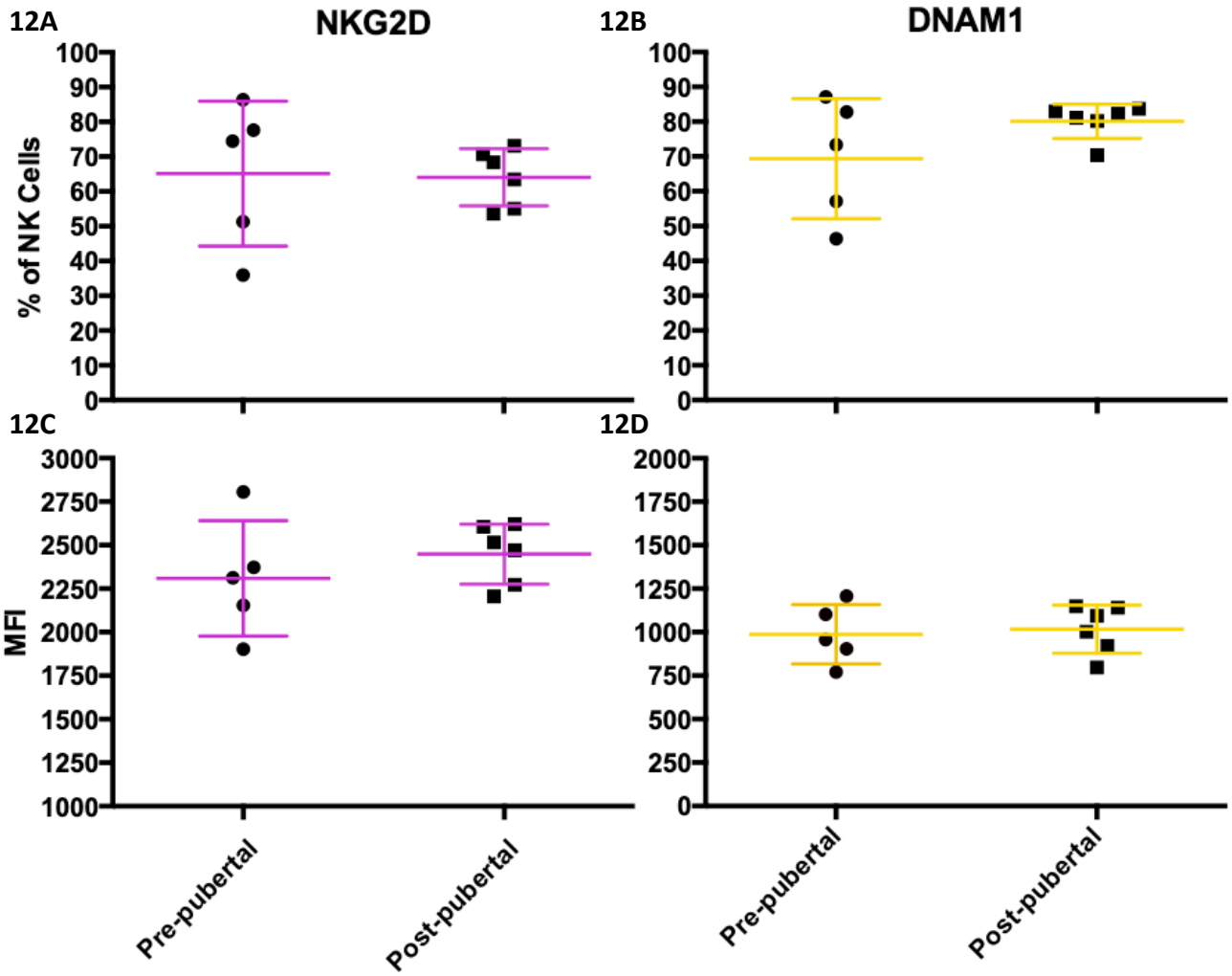


Figure 12: Activating NKG2D and DNAM1 Receptor Expression and MFI Pubertal Comparison. (12A) NKG2D receptor expression (12B) DNAM1 Receptor Expression (12C) NKG2D MFI (12D) DNAM1 MFI. All graphs are presented as mean ± standard deviation.

To assess if the expression and density of the inhibitory receptors were different across pubertal groups at PRE, a series of t-tests were carried out. There were no significant differences in the expression (t (9) =0.141, p = 0.89) or MFI (t (9) = 0.255, p = 0.80) of the NKG2A receptor between pre-pubertal and post-pubertal children (Figure 13A/C). There were no significant differences in the expression (t (9) =0.049, p = 0.96) or MFI (t (9) =0.977, p = 0.35) of the KIR2DL2/DL3 receptor between pre-pubertal and post-pubertal children (Figure 13B/D).

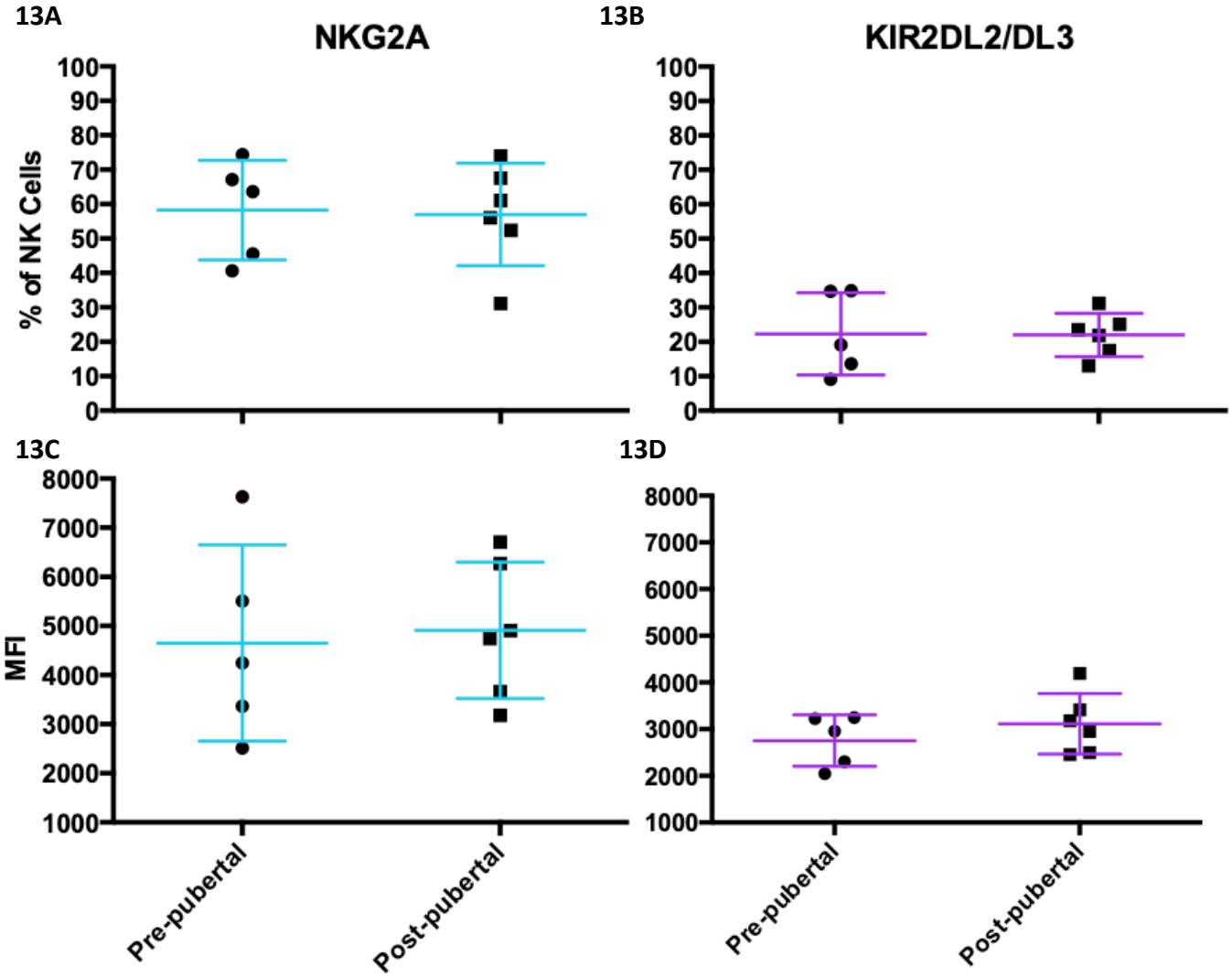


Figure 13: Inhibitory NKG2A and KIR2DL2/DL3 Receptor Expression and MFI Pubertal Comparison. (13A) NKG2A receptor expression (13B) KIR2DL2/DL3 Receptor Expression (13C) NKG2A MFI (13D) KIR2DL2/DL3 MFI. All graphs are presented as mean ± standard deviation.

4.6 Measurements of Exercise Intensity

4.6.1 Heart Rate (HR)

HR was collected throughout each visit to objectively track how hard participants were working and to verify exercise intensity at each visit. During the continuous exercise, HR was recorded every two minutes; during intermittent exercise, HR was recorded after every bout. For both types of exercise, the average beats per minute across all 30-minutes are displayed in Figure 14. Two one-way ANOVAs were used to evaluate differences in HR for each pubertal group separately. In the pre-pubertal children, there was a significant exercise effect ($F(3,16) = 6.166$, $p < 0.01$). Tukey's post-hoc analysis revealed that average HR was significantly lower during the MI-CONT (139 ± 8 bpm) compared to HI-INT (170 ± 13 bpm) and MI-INT (166 ± 18 bpm). No significant differences were observed between HI-CONT (161 ± 9 bpm) and MI-CONT. In the post-pubertal children, a significant exercise effect was also observed ($F(3,20) = 7.817$, $p < 0.01$). Significantly lower average HR was noted during MI-CONT (130 ± 21 bpm) compared to HI-CONT (164 ± 21 bpm), MI-INT (175 ± 15 bpm) and HI-INT (175 ± 16). There were no differences in HR by exercise protocol between pubertal groups.

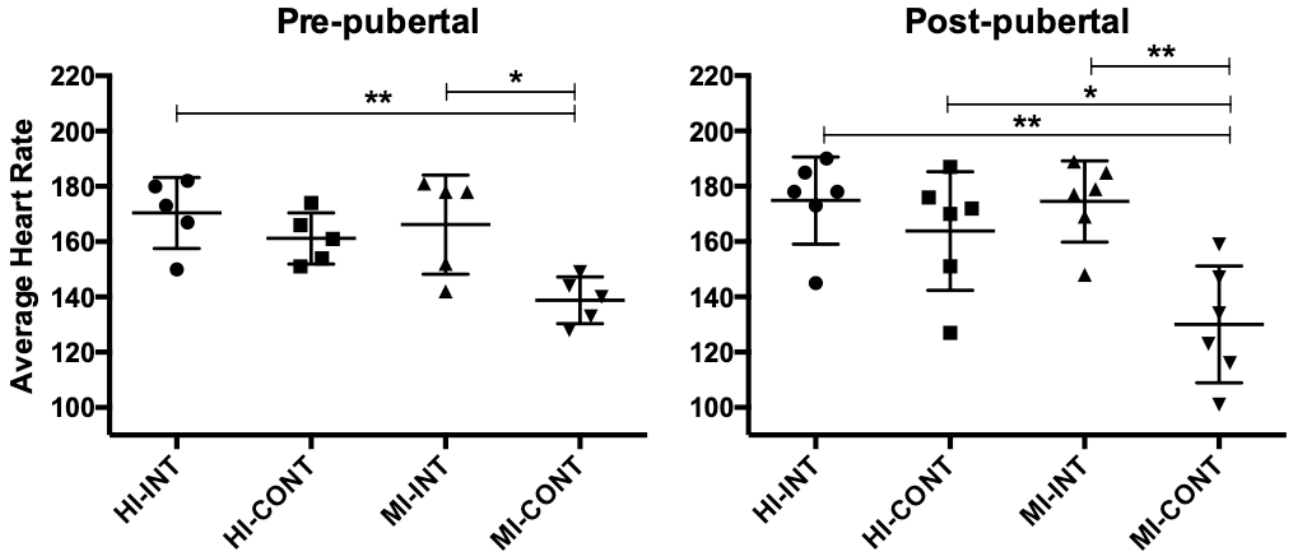


Figure 14: Average heart rate for each exercise protocol by pubertal group (*, $p < 0.05$) (**, $p < 0.01$). All graphs are presented as mean ± standard deviation.

4.6.2 Ratings of Perceived Exertion (RPE)

Self-reported RPE was collected in an effort to subjectively track exercise intensity and values averaged for each visit. Two one-way ANOVAs were used to evaluate differences in the average RPE across all four exercise visits separately for each pubertal group. Pre-pubertal children did not demonstrate significant differences in their RPE across HI-INT (12 ± 2), MI-INT (14 ± 3), MI-CONT (11 ± 2) or HI-CONT (14 ± 2) ($F(3,16) = 1.910$, $p = 0.169$). In post-pubertal children there was a significant exercise effect ($F(3,16) = 7.273$, $p < 0.01$). Average RPE at MI-CONT (10 ± 2) was significantly lower than HI-INT (16 ± 1) and MI-INT (13 ± 2) but not HI-CONT (14 ± 3).

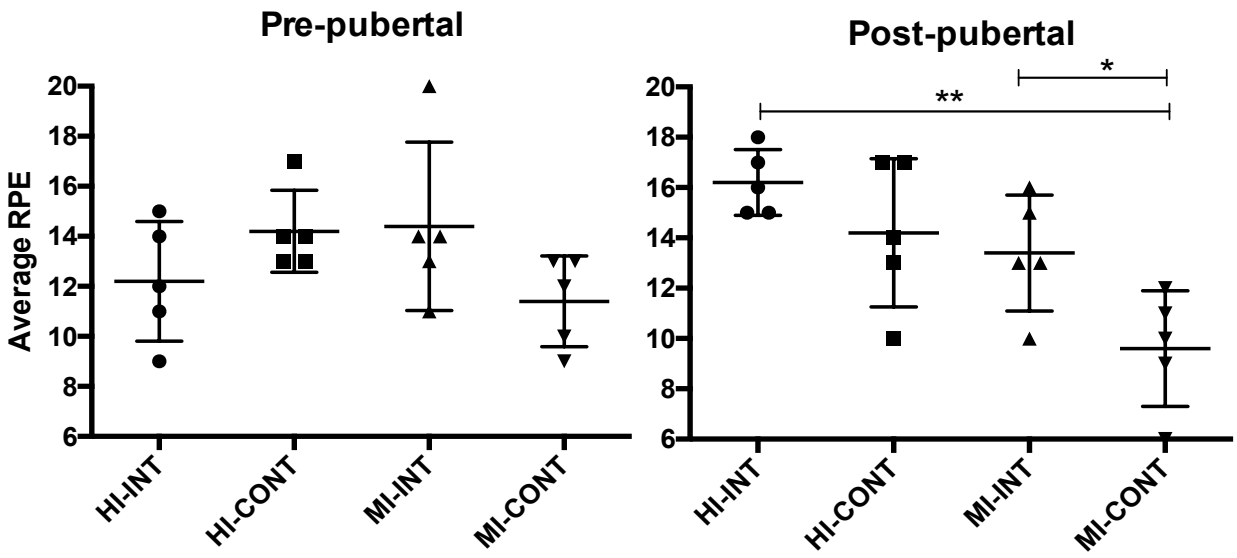


Figure 15: Average RPE for each exercise protocol by pubertal group (*, $p < 0.05$) (**, $p < 0.01$). All graphs are presented as mean ± standard deviation.

4.7 Relationship between fitness, MPVA, Sedentary time and Δ NK concentration

A Pearson correlation was used to investigate the relationships between change in NK cell (Δ NK = POST – PRE concentration), fitness, MVPA and sedentary time. Only fitness, expressed as relative $\dot{V}O_2$max, was negatively related to Δ NK (R^2= - 0.742).

Table 5: Correlation of fitness, MVPA and sedentary time with Δ NK (*, p < 0.01)

Factors	Pearson Correlation with Δ NK	P value
Fitness ($\dot{V}O_2$ Relative)	-.742	.009*
% MVPA	.098	.788
% Sedentary Time	.621	.056

4.8 Exploratory Analysis

Given that both NK cell response and fitness varied by puberty in our sample, and existing literature suggesting differences in fitness by puberty, we conducted an exploratory analysis to estimate the independent contributions of fitness and puberty to Δ NK with exercise. To do this, we utilized a hierarchal regression which first assessed the relationship of fitness alone with Δ NK concentration and then the relationship of both fitness and puberty. The overall regression model was significant (F (1,8) = 5.982, p = 0.026 (Table 6). When the relationship of fitness alone was examined, it accounted for approximately 55% of the variability in the NK cell response. In model 2, puberty explained an additional 4.9% of the variability in NK cells response. Although the model remained significant, each factor alone did not contribute significantly to predicting Δ NK concentration.

Table 6: Hierarchal Linear Regression Results (*, p < 0.05, ** < 0.01)

Model	R2	F	Df1	Df2	Sig.	SS	MS
Model 1: Fitness ($\dot{V}O_2$ Rel.)	0.550	11.004	1	9	0.009**	9.886×10^9	9.886×10^9
Model 2: Fitness ($\dot{V}O_2$ Rel.) Puberty	0.599	5.982	1	8	0.026*	1.077×10^{10}	5.385×10^9

	Unstandardized Coeff.				95% Confidence Interval	
Model	B	Std. Error	t	Sig.	Lower Bound	Upper Bound
Model 1:						
(Constant)	233088.37	57918.01	4.024	.003	102068.72	364108.02
Fitness ($\dot{V}O_2$ Rel.)	-4299.92	1296.26	-3.317	.009	-7232.27	-1367.58
Model 2:						
(Constant)	208926.98	62894.67	3.322	.011	63891.62	353962.33
Fitness ($\dot{V}O_2$ Rel.)	-2862.49	1946.25	-1.471	.180	-7350.58	1625.57
Puberty	-27003.46	27250.48	-.991	.351	-89843.19	35836.27

Chapter 5: Discussion

To our knowledge, this is the first study that has directly compared the immune response to exercises of differing intensities, durations, and/or structures in children and adolescents. We demonstrated that in healthy pre-pubertal children, 30-minute of cycling even at high intensity may not be a sufficient physiological stressor to induce a NK cell response. Our findings also confirm that in post-pubertal children, an acute bout of exercise can transiently alter the concentration and proportion of NK cells in circulation. In these adolescents, we found that the magnitude of NK cell response is primarily determined by the exercise structure and that intermittent exercise causes a significantly greater increase in the circulatory NK cell concentration compared to continuous exercise. Our study is also the first of its kind to characterize changes in activating and inhibitory receptor profiles in response to an acute bout of exercise in children. We observed that the concentration of NK cells in circulation expressing the NKG2A inhibitory receptor increased in the one-hour recovery after exercise. However, we also noted that the density of activating NKG2D and inhibitory NKG2A receptors were elevated after exercise. Lastly, we aimed to better understand whether fitness and physical activity levels in children and adolescents were correlated with the magnitude of the NK cell response to an acute bout of exercise. We demonstrated, that in our cohort, fitness but not MVPA or sedentary time, was significantly negatively correlated with changes in NK cell concentration from PRE to POST exercise. This suggests that children who were more fit exhibited a smaller NK cell response.

5.1 Characteristics of Pre-Pubertal Compared to Post-Pubertal Children

Both the pre-pubertal and post-pubertal children were screened based on the same BMI percentile thresholds, health status and physical activity eligibility criteria. As such, by design, there were no significant differences in BMI percentile or percent body fat between groups. However, the pre-pubertal children had higher aerobic fitness (49.8 $\pm$ 6.2 ml/kg/min) than post-pubertal children (39.4 $\pm$ 4.1 ml/kg/min), when normalized to their body weight (*, $p < 0.05$, Table 3). These differences in fitness can be attributed to a number of possible factors. First, lower fitness may be the result of decreases in MVPA with age. The decline in engagement in MVPA is a well-characterized phenomenon in adolescents[161–163]. A longitudinal study by Nader et al. tracked children from ages 9 to 15 years[161] and demonstrated roughly a 40-minute decline per year in the time spent per day in MVPA in both boys and girls. Remarkably, in our sample the amount of time (minutes/day) spent in moderate-to-vigorous physical activity was not significantly different between pubertal groups. However, in our cohort adolescents spent a greater percentage of their time being sedentary (79.5 $\pm$ 4.6%) relative to pre-pubertal children (67.3 $\pm$ 5.0%) (Table 4). This is consistent with findings by Carson et al. that have shown that sedentary behavior is significantly negatively correlated with aerobic fitness[164]. Upon further investigation, this negative correlation was evident in our participants as well, wherein those who spent the most time being sedentary were also the least fit (R^2= -.758, $p< 0.05$). Second, the sex composition of our post-pubertal sample may also contribute to differences in fitness. Adolescent girls experience a smaller gain in lean muscle mass (that drives oxygen uptake) and larger increases in fat mass compared to adolescent boys[165]. Since girls typically have lower muscle mass,

their oxygen demands are lower and normalized to their body mass, which has increased due to increases in fat mass, their $\dot{V}O_2$max decreases, by approximately 2% per year in girls[163]. As such, with half of our post-pubertal sample being composed of adolescent females, lower aerobic fitness is to be expected. We had three girls and three boys in the post-pubertal group successfully complete the study. Although our sample is too small to reliability assess significance, we see that on average adolescent girls had a lower percent of fat-free mass (72 $\pm$ 5% fat-free mass) than adolescent boys (85 $\pm$ 6 %). This is in line with our observation that adolescent girls (37.5 $\pm$ 0.7 ml/kg/min) tended to have slightly lower fitness than adolescent boys (41.3 $\pm$ 3.1 ml/kg/min). The lower fitness values in our female participants may be contributing to overall lower fitness in our post-pubertal sample. Additionally, qualitative observations in other studies suggest that adolescent females tend to not reach a point of total exertion in the same way adolescent boys do. This does not appear to be a likely explanation for the differences in fitness in our sample since adolescent boys (191 $\pm$ 9 bpm) and girls (197 $\pm$ 14 bpm) reached comparable maximal heart rates during their fitness tests. In summary, with half of our post-pubertal sample being composed of adolescent females, lower aerobic fitness compared to the pre-pubertal group values can be expected due to puberty-related differences in body composition.

Interestingly, in our sample we did not observe a difference in VT as a percent of $\dot{V}O_2$max between our pre-pubertal and post-pubertal participants. This suggested that even though we saw an overall decline in aerobic fitness in the post-pubertal group, oxygen utilization efficiency was similar in our pre-pubertal and post-pubertal children. As explained by Reybrouch et al. this is likely due to the involvement of different

physiological mechanisms in VT and $\dot{V}O_2$max determination[166]. While $\dot{V}O_2$max is driven by maximal cardiac output and the extraction of O_2 in peripheral tissues, VT depends on the body's ability to clear lactic acid. Therefore, our findings suggest that the efficiency of the metabolic machinery is similar in the post-pubertal and pre-pubertal children in our sample.

5.2 NK Cell Changes in Pre-Pubertal Children

We hypothesized that a 30-minute high-intensity protocol would be sufficient to elicit a profound immune response in both pre- and post-pubertal children, as described in adults[77]. However, our findings indicate otherwise. In the pre-pubertal cohort in this study, none of the exercise protocols elicited a significant increase in NK cell concentration post-exercise (Figure 7A). Although the pattern of response appeared similar to that observed in the post-pubertal cohort, these modest increases did not reach statistical significance. This finding is consistent with Timmons et al. who showed that 30-minutes of high intensity cycling did not significantly alter the concentration of NK cells (Figure 7A/7C)[7]. Although pre-pubertal children experience a significantly greater spike in norepinephrine compared to post-pubertal children, they also exhibit decreased β-adrenergic receptor density and sensitivity on NK cells, which may render the stimulus insufficient to mobilize NK cells into circulation[8,167,168]. Additionally, pre-pubertal children secrete significantly less GH in response to an acute exercise bout compared to pubertal or post-pubertal children, thereby decreasing the initial stimulus on NK cells[110,169].

Another possible reason the post-exercise spike in NK cells is not evident in pre-pubertal children is due to a greater and more rapid cortisol increase compared to post-

pubertal children. Cortisol has a counterregulatory effect that causes rapid redistribution of NK cells to peripheral tissue sites[56]. A serial time course analysis of cortisol secretion in response to exercise in children is not available. However, post-exercise salivary cortisol has been shown to be negatively correlated with pubertal stage[170]. Typically, cortisol secretion begins within about 10-15 minutes of high intensity cycling, but does not peak until about 20 – 30 minutes of exercise cessation in adults[171]. However, given that children experience more rapid recovery to physical stressors, it is plausible that the compensatory cortisol secretion peaks earlier than is observed in adults or adolescents and causes faster and earlier NK cell redistribution[109,172].

The few studies in children that have compared hemodynamic and hormonal responses to continuous and intermittent exercise at various intensities have shown that structure-specific differences are not observed as long as exercise duration remains equal[173,174]. In our study, while cycling duration was not the same (5-min vs. 30-min), the exercise stimulus was spread out over the same time (30 min) for the intermittent and continuous sessions, and pre-pubertal children still did not experience significantly different NK cell responses across any of our exercise protocols. Our intermittent exercise, which included 20 × 15-sec bouts of all-out cycling distributed over 30 minutes evidently caused a similarly pronounced counterregulatory response, causing similar NK cell redistribution as observed in the continuous protocols[108]. Moreover, even despite a lower heart rate during MOD-CONT, NK cell recruitment was comparable across all exercise protocols, further suggesting that endocrine regulation may be a key driver of NK cell recruitment. Alternatively, it's also plausible that the lack of NK cell changes post-exercise in pre-pubertal children (Figure 7A/7C) was indicative of this

population being more resistant to immune perturbance and oxidative stress build up in response to exercise[175]. As such, the dampened stress response required less extensive recovery and faster return to baseline.

The degree to which the NK cell pool is differentiated is also linked differences in recruitment. Bigley et al. has previously shown that the more differentiated $CD56^{dim}$ NK cells, which are characterized by downregulated NKG2A expression and upregulated KIR expression, are preferentially deployed by acute exercise compared to $CD56^{bright}$ cells[85]. To assess whether a less differentiated NK cell pool could be contributing to lower recruitment in the pre-pubertal cohort, resting proportion of $CD56^{dim}$ and $CD56^{bright}$ NK cells were assessed (Figure 7A/7B). In our cohort, no differences in the resting proportion of the NK cell subtypes were detected, although this is not consistent with findings by Timmons et al. who showed that children in Tanner 3-5 had a greater proportion of $CD56^{bright}$ cells than children in Tanner 1[8]. Therefore, in our cohort of children, differences in the degree of differentiation in our NK cell pool did not explain why pre-pubertal children experienced dampened recruitment.

5.3 NK Cell Changes in Post-Pubertal Children

In our post-pubertal cohort, we observed a larger increase in the concentration and proportion of NK cells at POST for HI-INT compared to both HI-CONT and MI-CONT exercise (Figure 7B/7D). This is an unexpected finding as previous studies have shown that acutely, cardiovascular and metabolic parameters as well as muscle adaptations are similar across both high intensity intermittent and continuous exercise in young physically active adults[176,177]. For example, Foster et al. looked at parameters of left ventricular function in response to 15 minutes of steady state and interval cycling

and found no significant differences in heart rate, cardiac output, blood pressure or systemic vascular resistance in adults[178]. This is consistent with our findings which demonstrated no differences in the average heart rate or RPE between the HI-INT or HI-CONT exercise stimuli (Figure 14/15). As such, it appears that in our sample cardiovascular changes may not explain differences in NK cell recruitment in response to high intensity cycling. It may also be indicative of a ceiling effect, in which the exhaustive effort of both exercise protocols caused similar stress stimuli on NK cells. Presently, two studies directly compared continuous cycling to intermittent cycling of the same duration in adults, one found no significant differences in the NK cell response and the other identified greater recruitment in response to continuous [82,83]. Consistent with the idea of a ceiling effect, Nieman et al. showed that both the continuous and intermittent exercise caused a comparable concentration of epinephrine post-exercise[82]. However, this study used a prolonged, 2-hour cycling protocol. Exercise of this duration is known to cause different hormonal, muscular and immune responses compared to short duration acute exercise[56,69,179], which limits our ability to definitively apply these findings to our results.

Hormonal differences between continuous and intermittent exercise may explain some of our observations. A study carried out by Bally et al. characterized the metabolic and hormonal responses to high intensity intermittent exercise compared to a moderate bout of continuous cycling in young adults with diabetes. They demonstrated significantly greater increases in catecholamines and GH after 30-minutes of intermittent exercise compared with continuous exercise[180]. Both of these hormones stimulate NK cell detachment from the vascular endothelium and cause rapid

mobilization into circulation. However, since different exercise intensities were used for the continuous and intermittent protocols in the study above, it is difficult to discern how much of the difference is due to exercise intensity and what part is driven by exercise structure. Unfortunately, there is also a lack of consensus in literature about the role of endocrine hormones in altering the NK cell response between continuous and intermittent exercise. For example, Karagiogos et al. showed greater increases in GH after intermittent exercise in healthy adult males, supporting findings in diabetic adult cohorts and children with cystic fibrosis[180–183]. Contrarily, Sills et al. showed GH did not change significantly between exercise protocols in diabetic children[182]. Inconsistent findings have been reported with respect to the response of cortisol as well. In one study, cortisol increased more after intermittent exercise compared to time-matched continuous exercise, but in another study, cortisol equally decreased after both continuous and intermittent exercise[184,185]. The lack of standardization between studies is a major limitation of comparing their respective findings. Indeed, the between-study variability in the length of intermittent bouts (10 seconds vs. 5 minutes), length of rest periods (30 seconds vs. 4 minutes) and intensities at which the continuous and intermittent protocols were carried out (45% of $\dot{V}O_2$max vs. 80% of HR) represent major obstacles in interpreting the existing literature.

Notwithstanding these limitations, it is likely that the greater responsiveness of NK cells we observed in our cohort after intermittent exercise is primarily due to catecholamine activity. While there are no studies directly examining the effects of two types of exercise on concentrations of epinephrine and norepinephrine in circulation, a between-study comparison shows promising results. Bracken et al. reported that in

response to 6 all-out cycling sprint exercises, interspersed with 30 second rests, epinephrine increased by 6-fold, while norepinephrine increased by 15-fold[186]. Conversely, a 20-minute bout of cycling at 60% $\dot{V}O_2$max showed a roughly a 7-fold increase in epinephrine and a 3-fold increase in norepinephrine[73]. Given the similarities of these exercise protocols to our own study, we hypothesize that our intermittent protocol may have also resulted in a greater catecholamine response, and in turn, a larger NK cell response in post-pubertal children. Additionally, in our sample no differences were observed between the HI-INT and MI-INT, which suggests that during intermittent exercise the structure of the exercise rather than the intensity determines the NK cell response. This is likely due to the similar cardiac stress that the intermittent exercise induced irrespective of intensity.

5.4 Role of Cytokines in mediating NK Cell Response in Post-Pubertal Children

The role of cytokines in mediating the NK cell response to exercise is also noteworthy. One study compared the secretion of TNFα after a continuous protocol and an intermittent protocol that included longer, 5 × 4-minute cycling bouts interspersed with 5-minute breaks. The authors reported a significantly lower concentration of TNFα in circulation post intermittent exercise compared to continuous exercise in healthy adults[187]. This is consistent with cell model observations that NK cells are more adherent to vascular endothelium in the presence of TNFα [113,187]. Therefore, it is plausible that lower TNFα concentrations during intermittent exercise make it easier for NK cells to detach from the vascular endothelium and enter circulation. Nevertheless, it is important to mention that increases in TNFα were not observed in previous studies in response to either continuous or intermittent exercise in healthy children [7,181].

5.5 NK Cell Response to Moderate and High Intensity Continuous Exercise

Post-exercise, no significant differences were observed for NK cells between the HI-CONT and MI-CONT exercise stimuli (Figure 7B). This is surprising since both visually and statistically, HI-CONT resulted in significantly greater POST NK values compared to PRE while MI-CONT did not. Although the lack of between-visit statistical difference is likely a result of the small sample size, heart rate differences might explain the difference in NK cell response (Figure 14). MI-CONT resulted in a significantly lower average heart rate compared to HI-CONT. Furthermore, exploratory analysis also showed that heart rate was significantly positively correlated with the magnitude of NK cell response ($R^2 = 0.202$, $p < 0.05$). This is consistent with a strong body of existing literature that suggests greater exercise intensity is correlated with higher shear stress and catecholamine secretion[56,74,180].

5.6 $CD56^{dim}$ and $CD56^{bright}$ NK cell subset response to exercise

In both pre-pubertal and post-pubertal children, the effects of exercise on concentrations of both NK cell subsets were similar to those observed in the overall concentration of NK cells (Figure 8). However, when we analyzed changes in the proportions of the two NK cell subtypes in post-pubertal children, we observed an increase in the proportion of $CD56^{dim}$ cells at POST relative to PRE for both the HI-INT and MI-INT stimuli. This is consistent with the literature which shows that $CD56^{dim}$ cells have a lower density of adhesion markers, higher density of β-adrenergic receptors, and thus tend to mobilize more drastically from the spleen and vascular bed in response to a physical stimulus[188–190].

In our pre-pubertal children, we observed a decrease in the proportion of $CD56^{dim}$ cells and an increase in the proportion of $CD56^{bright}$ cells at REC1 relative to PRE (figure 8A). However, at REC2, the proportions of the NK cell subtypes returned to PRE values in all exercise protocols except MI-INT. In our post-pubertal cohort at REC2, we noted a persistent decrease in the proportion of $CD56^{dim}$ cells and increase in the proportion of $CD56^{bright}$ cells in all but MI-CONT exercise stimuli (Figure 8B). We believe that this may be related to the fact that pre-pubertal children recover from physiological stressors faster than adolescents and adults. A similar theory was postulated by Timmons et al. who rationalized that in their study, children didn't demonstrate the same 30-minute post-exercise immunosuppression as adults because they simply were able to return to a baseline homeostasis quicker[7,8]. We also propose that the higher proportion of $CD56^{bright}$ cells at REC1 in pre-pubertal but not post-pubertal children is a result of more rapid redistribution of $CD56^{dim}$ cells to sites of peripheral tissue damage such as muscle[77].

To date, we are the first study to investigate how different exercise structures affect NK subtype distribution in the pediatric population. We showed in our post-pubertal adolescents that intermittent exercise caused a more pronounced increase in the proportion of $CD56^{bright}$ cells at REC2 compared to continuous exercise (Figure 9B). We hypothesize that this is because intermittent exercise, with its all-out nature, may be causing more peripheral cellular damage. However, contrary to our hypothesis, Combes et al. showed that intensity-matched equal volume continuous exercise causes airway epithelium damage while intermittent exercise does not[191]. Nevertheless, this is only one peripheral tissue, and the literature comparing tissue damage in response to all-out

exercise to continuous cycling remains extremely limited. As such, we hypothesize that peripheral cellular damage may lead to greater deployment of $CD56^{dim}$ cells to for repair, leaving behind a greater proportion of $CD56^{bright}$ cells in circulation after both MI-CONT and HI-CONT. While peripheral muscle or tissue damage may be more substantial after intermittent exercise, the greater proportion of immunoregulatory $CD56^{bright}$ cells left behind in circulation may be indicative of greater immunosurveillance rather than an immunocompromised environment[23]. However, further investigation is required to test this hypothesis in our pediatric cohort.

5.7 Activating and Inhibitory Receptor Expression

To address whether exercise is able to modulate NK cell phenotype we evaluated the expression of four key activating and inhibitory receptors. We assessed the effect of exercise on the expression of these receptors in the overall NK cell population as well as on a per cell basis or density by looking at the MFI's.

5.7.1 Expression of Activating NKG2D Receptor

We found no significant differences in the number of NK cells expressing NKG2D receptors after exercise or during recovery (Figure 10A). Although this is a novel finding in the pediatric population, it is consistent with findings in adults, wherein Bigley et al. showed no change in the expression of NKG2D after 30-minutes of cycling 15% above VT[192]. Given the similarities between the exercise stimuli in Bigley's study and ours, it seems likely that the NKG2D receptor responds similarly in children and adolescents as it does in adults. However, young adults that ran a half marathon demonstrated an increase in the NKG2D gene expression 15 minutes after exercise[193]. This suggests that a prolonged exercise stimulus might be required to modulate the expression of

NKG2D expression. The expression of NKG2D is also upregulated in response to IL-15, a myokine that is secreted from muscle after strenuous exhaustive exercise[95]. It is plausible, that our 30-minute acute bout exercise was not a sufficient stressor to substantially warrant an upregulation of the NKG2D receptor. However, given the novelty of this work, far more research is required in children to understand if exercise can mediate changes in NKG2D expression.

5.7.2 Expression of Activating DNAM1 Receptor

In terms of DNAM1, the accessory molecule involved in detection of virulent and malignant cells, we found no change in the proportion of cells expressing the receptor across all time points (Figure 10A). While myokines released after strenuous exercise can upregulate the expression of DNAM1 *in vitro*, there have been no other studies that have looked at the effects of exercise on DNAM1 expression *in vivo*[95].

5.7.3 Expression of Inhibitory NKG2A Receptor

Across our entire sample, we saw significantly lower proportion of NK cells expressing the NKG2A receptor at POST compared to PRE (Figure 10B)[48]. This was followed by an increase in the proportion of NK cells expressing NKG2A at both REC1 and REC2 compared to PRE. Exercise acts as a physiological stressor. This means that once exercise begins, similar “fight-or-flight” triggers are experienced by the sympathetic nervous system and hormones, such as catecholamines are secreted. As NK cells are recruited into circulation during exercise, they become readily available to detect pathogens[56–58]. To do this, NKG2A receptor expression may be downregulated immediately post-exercise to allow the NK cells to be more readily activated. However, as time since exposure to the stressor (i.e., exercise) passes and no target cells are

detected, a compensatory upregulation of the inhibitory receptors may occur in order to prevent overactivation of the NK cell pool[34]. Given the fact we did not see significant increases in NKG2D after exercise, it is plausible that a decrease in the overall inhibitory signal POST transiently shifts the balance towards activation[34–36].

The changes in the proportion of cells expressing NKG2A may also be linked to the changing proportion of $CD56^{dim}$ and $CD56^{bright}$ cells in circulation with exercise. At POST, we saw a greater proportion of $CD56^{dim}$ cells. These cells do not readily express a high density of the NKG2A receptor[23]. However, throughout recovery we see a greater proportion of $CD56^{bright}$ cells in circulation. These immature NK cells inherently express a high density of NKG2A receptors[23]. In summary, the shift towards a higher proportion of $CD56^{bright}$ cells at post-exercise in our sample of children may also contribute to increases the number of cells expressing the NKG2A receptor.

5.7.4 Expression of Inhibitory KIR2DL2/DL3 Receptor

Across our entire sample, there was no significant difference compared to PRE in the proportion of NK cells that expressing KIR2DL2/DL3 receptor during any of the timepoints (Figure 10B). Although there are no existing studies that investigated the effect of exercise intensity or structure on the expression of KIR2DL2/DL3 receptors, strenuous exercise such as a $\dot{V}O_2$max test or intermittent cycling has been shown to increase the overall expression of the KIR family of genes[10,90]. The KIR gene family contains over a dozen genes, and further research is required to establish how different exercise stimuli impact the KIR2DL2/DL3 gene specifically.

5.8 Activating and Inhibitory Receptor Density

5.8.1 Density of NKG2D and NKG2A Receptors

Across our entire sample, compared to PRE and POST the density of the NKG2D receptor was significantly greater at REC1 and REC2 (Figure 11A). The exact same pattern of response was seen with the NKG2A receptor (Figure 11B). This created an interesting dichotomy whereby the activating and inhibitory NKG2 family of receptors are upregulated at the same time. Since we observed a similar pattern of response with both receptor densities, we believe exercise has overall stimulatory effect on the NKG2 receptor family. We speculate this to be because of IL-2 which is released during periods of acute stress, and increases the density of both NKG2D and NKG2A receptors *in vitro* [95,194]. To expand on this, we hypothesize that during recovery, once the NK cells are in circulation and the stress stimulus has subsided, all receptors of the NKG2 family are upregulated due to IL-2 stimulation. Unfortunately, without *in vitro* analysis, we cannot determine in which way the receptor balance is shifted. This is important because the shift in NK cell activation or inhibition dictates how equipped they are to detect and kill pathogenic cells.

5.8.2 Density of DNAM1 Receptor

In our sample of children, the receptor density of DNAM1 was greater at REC2 compared to PRE (Figure 11A). While the number of cells expressing DNAM1 does not change, the intensity with which the receptor is expressed increased an hour post exercise. This is indicative of the beneficial effects of exercise, suggesting improvements in immunosurveillance in the NK cell population. In our study, we did not collect blood 24 hours after exercise, a time point that could provide further detail about

whether the effects of exercise on NK receptors such as DNAM1, which aids in viral detection, are sustained[43,44]. Given that NKG2D expression was elevated even 24 hours after exercise in the study by Zimmer et al., it is plausible that changes in DNAM1 expression with exercise extend beyond the 1-hour mark we investigated[93].

5.8.3 Density of Inhibitory KIR2DL2/DL3 Receptor

Although no overall time effect was observed for KIR2DL2/DL3 receptor density, there was a significant time by exercise by puberty interaction (Figure 9C/9D). In pre-pubertal children, no significant changes in the density of the KIR2DL2/DL3 were observed, which is consistent with the lack of detectable NK response to exercise in the pre-pubertal cohort. In the post-pubertal children, there appears to be an exercise intensity-driven effect on KIR2DL2/DL3 expression. Our findings demonstrate that during recovery from high intensity exercise the density of KIR2DL2/DL3 expression is increased. We hypothesize that this observation could be as a result of cellular and epigenetic modifications in NK cell gene expression, which may be exercise intensity dependent. The role of KIR genes is to detect self-cells and protect them from autologous attack[52,195]. Given that high intensity exercise elicits significant physiological stress, the KIR response observed in this study may be a mechanism employed to prevent NK cells from unregulated self-killing during post-exercise recovery[51,52]. Contrarily, it may demonstrate that high intensity exercise leads to a period of immunosuppression, where the NK cells are temporarily unable to detect and clear pathogens, a concern previously discussed throughout literature[76,77].

5.8.3 Pubertal Differences Receptor Expression and Density (MFI)

For all of the receptors at PRE, there were no differences in the expression (concentration) or density (MFI) of the receptors between the pre-pubertal and post-pubertal children (Figure 12/13). These observations were inconsistent with previous findings that showed that NK cells of younger children had a greater concentration of $CD56^{bright}$ cells that expressed DNAM1, and a lower density of NKG2D expression[98,101]. The lack of differences we observed in the context of DNAM1 may be due to the fact that we normalized to the total NK cell population rather than only $CD56^{bright}$ cells. This was consistent with observations by Mahapatra et al. also noted no differences in the expression of DNAM1 across the entire NK cell population suggesting that pubertal differences are driven by $CD56^{bright}$ cells [98]. Sundstrom et al. observed significant differences in the MFI of NKG2D between 5-year-old children and adults[101]. The narrower age difference between our pre- and post-pubertal groups may explain the discrepancy between our findings. It is plausible that either changes in NKG2D expression already began in school aged children, or adult-onset changes occur later in life.

The lack of differences in the receptor expression of KIR2DL2/DL3 and NKG2A in our sample is surprising since previous literature has shown age-related upregulation of KIR genes and downregulation of NKG2A on NK cells during the first two decades of life[27,102]. However, we recognize that large between subject variability, compounded with a small sample sizes of only 5-6 children per pubertal group make it uniquely difficult to discern any pubertal effects (Figure 13).

5.9 Relationship between Fitness, MVPA, Sedentary Time and NK Response

Physical activity is essential to maintaining a healthy lifestyle throughout growth and development. The current movement behaviour guidelines focus specifically on the importance of engaging in at least 60-min of daily MVPA to support cardiovascular, musculoskeletal and immune health[122,196]. The guidelines have also recommended reducing sedentary time which based on existing literature has shown to independently predict health risks. Tremblay et al. reported in a systematic review that greater sedentary time is associated with higher BMI, unfavorable body composition, lower fitness, and lower self-esteem scores in youth ages 5-17[197]. We observed that in our sample fitness and sedentary time, but not MVPA, were negatively corelated (R^2 = -0.758). We also noted that fitness but not MVPA or sedentary time was linked to change in NK cell response (R^2 = -0.742) (Table 6). This could imply that the relationship between fitness and NK cell change is influenced (or moderated) by an individual's sedentary time. Due to limitations in our sample size, we were not powered enough to conduct the moderation analysis; however, this relationship should be further examined with more participants.

The negative relationship between fitness and NK cell response in our participants was rather unexpected. Ferry et al. showed trends towards greater NK cell response in trained compared to untrained individuals[197]. Indeed, Boas et al. had previously suggested that trained children and adolescents have a greater NK fold change from pre- to post-exercise[108]. We found the opposite relationship, but we are also the only study that used an objective measure of fitness rather than self-reported training level as a proxy for fitness in children. This finding in our cohort also did not align with studies

in adults that have shown that more fit individual experience a greater NK cell response Since many of these studies were conducted in adults, their results may not be transferable to our pediatric cohort[72,127]. The pediatric years are unique in that they are characterized by continuous growth and development, mediated by growth factors such as IGF-1. Proinflammatory cytokines released during exercise, such as IL-6 and TNFα, may inhibit the function of growth factors such as IGF-1[175]. Therefore, it is plausible that immune responsiveness in childhood is restricted so as to maintain proper growth and development. Conversely, the larger immune response to physiological stress observed in adults may serve to maintain a healthy systemic environment.

Our exploratory findings demonstrate that together, fitness and puberty significantly predict (Table 7) the magnitude of NK cell response; however, independently neither puberty nor fitness significantly contribute to the relationship. This is likely due to the substantial overlapping effect of the two parameters, which negates any independent effects. We previously showed that our pre-pubertal children were more fit than the post-pubertal participants, but what remains unclear is whether fitness level or pubertal development is driving the difference in NK cell response between the groups. Our study was designed in a way that purposely excluded fitness extremes. Children who were either very physically active/identified as elite athletes (and likely high fit), and those who were not engaging in any dedicated time to structured physical activity (and likely low fit) were not eligible for study participation. Without these two extremes, it is even more difficult to accurately assess the strength of the relationship and independent contributions of puberty and fitness to the exercise-induced NK cell response.

6.0 Novelty of Findings

The primary goal of this thesis was to provide the first direct investigation of the effect of exercise intensity and structure on NK cell response in healthy, typically developing children and adolescents. An additional goal was to assess if fitness and/or physical activity could predict the magnitude of NK cell response to exercise. This thesis established several novel findings:

1. Pre-pubertal children do not show a significant change in NK concentration or proportion in response to moderate or high intensity, continuous or intermittent cycling. From an applied perspective, if the goal of the exercise is to boost the immune system, we demonstrated that a 30-minute exercise protocol in pre-pubertal children is insufficient. Conversely, this preliminary evidence suggests that any of the aforementioned protocols may be used in pre-pubertal children when immune activation is unfavourable such as in children with chronic inflammatory conditions.
2. We showed for the first time in post-pubertal children that as little as 5-minutes of intermittent cycling can elicit a greater NK cell response than 30-minutes of strenuous continuous cycling. This is an entirely new area of investigation in the pediatric population. Importantly, it supports the notion that low volume, more enjoyable intermittent exercise is also immunologically beneficial in post-pubertal children. This is exciting because it falls directly in line with the typical movement patterns of children, making it more feasible for implementation in a real-world setting.

3. The overall finding here suggests that with intermittent exercise, there is a change in the composition of the NK cell pool that favours $CD56^{bright}$ cells. This immunoregulatory subtype is important in communicating with other immune cells in order to mount responses to foreign invades and pathogens. Upregulation of this subtype of NK cells for at least an hour post-exercise could be important for maintaining a vigilant immune system that protect the body.
4. This is the first study to investigate the change in receptor expression and intensity in response to exercise in children. We provided the first evidence that exercise can alter activating and inhibitory receptor expression. However, without characterizing the complete NK cell repertoire, it is impossible to piece together what this means for NK cell activation and function.
5. Exploratory analysis revealed that the magnitude of the NK cell response to exercise is related to both fitness and puberty. This finding may provide insight into individual factors that should be considered when designing exercise interventions for children. For example, knowing an individual's fitness level may help identify the exercise intensity required to elicit a desired immune response.

7.0 Limitations

The most significant limitation of our study is sample size. Given the number of parameters being examined and within subject variability (e.g. receptor expression) across four visits, we were underpowered to definitively detect exercise-induced changes in NK cells by exercise protocol and pubertal status. The study began in May of 2019, and unfortunately due to COVID-19, was halted in March of 2020. Although we completed all testing sessions for 11 participants and have partial results for 4

participants (on hold), we were not able to reach our target of 24 participants. Although we are confident in our outcomes that demonstrated a large magnitude of change, like NK cell numbers PRE to POST in post-pubertal children, the small sample size significantly limited our ability to interpret results where the effect size was much smaller and variability was greater, like receptor expression and density. Nevertheless, this thesis is a part of a larger project, which will re-evaluate these research questions with after all 24 participants are recruited.

Another limitation of our study is the lack of a familiarization visit. Although we counterbalanced the order of visits, many participants were anxious and nervous during their first visit since bloodwork was not a routine practise for them. Psychological and physiological stress are known to cause a similar sympathetic nerve system stimulation that cannot be disentangled[55]. Thus, the stress and anticipation of a blood draw could potentially have affected the NK cell measurements throughout the visit. Heart rate was not measured during the resting blood sample collection; however, all children began cycling with a normal resting heart rate. Nevertheless, catecholamine circulation and sympathetic nervous system stimulation from the previous stressor may have already triggered NK cell mobilization, thereby skewing PRE outcomes.

A third limitation of the study is related to the measurement of PA. Although accelerometry is heavily utilized methodology validated for use in children, it is not compatible with water wear[149]. Out of the 10 participants who provided completed accelerometers logs, 3 reported taking off the accelerometer for at least a 3 – 4 hours, on at least 2 separate days throughout the week for swim-related activities. Although these participants still met the daily accelerometer requirements for analysis, this

accounted for approximately a third of our participants, who were likely engaging in MVPA that was not captured by the accelerometer. This missing data may have altered the relationship between physical activity and the NK cell response to exercise.

Lastly, we measured 2 activating receptors and 2 inhibitory receptors as a first step towards understanding NK cell phenotype with exercise. NK cells express a host of chemokine, honing, inhibitory and activating receptors that all interact to regulate NK cell activation. Although these were beyond the scope of this project, characterizing a greater number of receptors would provide a more complete understanding of changes in NK cell phenotype in response to exercise.

8.0 Implications and Future Directions

Throughout the study there we collected a plethora of biological information that will be critical in addressing the next steps of our study. First, we have collected plasma samples that can be useful in answering whether differences in catecholamines, GH and cortisol are truly responsible for differences in NK cell response during and after exercise. A better understanding of hormonal profiles before and after exercise gives us insight into how sensitive endocrine hormones are to different exercise stimuli. Using this information, we can aim to better link the endocrine response to changes in NK cells. Second, we also collected and cryopreserved purified peripheral blood mononuclear cells. The abundance of cells that were collected from our population can be used to answer a multitude of interesting questions. The cells can be used to characterize other receptors of interest that we were unable to examine at this step of the project. Although we focused on four receptors, there are more than a dozen others that are of interest in regulating NK cell biology. Some of these include cytokine

receptors, which respond to changes in circulating levels of cytokines post-exercise such as IL-12 and IL-15, as well as chemotactic receptors which could be used to glean more information about NK cell honing.

Using stored plasma and frozen PBMC's we can also carry out cross culture experiments to answer questions about what modulate the NK cell response throughout puberty. In particular, we could investigate whether it is inherently the NK cells themselves that respond differently or whether it's the endocrine and cytokines environment in the blood. One set of experiments could focus on incubating resting NK cells of the pre-pubertal group with separate exercised serum from both our pre-pubertal and post-pubertal children. This will allow us to characterize the role the circulatory environment has in modulating the NK cell response. A second layer of analysis could focus on incubating NK cells from pre-pubertal and post-pubertal children with one group's exercise serum to evaluate whether the NK cells themselves elicit a different response. Ultimately, the key question of interest is how do all of these findings translate to NK function? We can answer these questions too through cytoxicity assays using our preserved cells and a cancer cell line. These next steps are critical in helping us further develop our understanding of the effects of exercise on the NK cells specifically in the pediatric population.

Beyond the aforementioned next steps for the project, the overall goal is to provide insight into which exercise protocol, based on its acute effects, should be used to design an exercise intervention for promoting immune health in children. Armed with the information obtained from this study, we will be able to take the next steps of designing an effective, evidence-based exercise intervention to examine the effects of

repeated exposures (i.e., exercise training) on immune health. Based on our preliminary findings, it would be of particular interest to examine the NK cell response after a training program where participants performed either a high intensity continuous cycling or moderate intensity intermittent training program. With this, changes in NK cell receptors could be tracked to better understand how exercise training may change the NK cell phenotype.

The field of pediatric exercise immunology is still relatively young. Very few studies have assessed the immune-related benefits of exercise training in immunocompromised populations, such as children with cancer[198]. These studies lack a detailed characterization of NK cells. However, down the line this understanding is critical to help researchers and clinicians understand how to effectively integrate exercise training into the standardized care of patients whose immune system could benefit from a boost. Our study provides some of the first evidence of both differences in NK number, subset and receptor responses to exercise of different intensities and structures, which will hopefully, with more information help inform the designs of these interventions.

9.0 Conclusion

Acute exercise causes transient changes in the immune system[5]. When repeated, these transient changes have the potential to cause cumulative changes in immune health[199]. For example, in adults, regular moderate intensity exercise decreases the risk of viral infections, improves tumor detection and enhances anti-tumor cytotoxic activity[119,132]. Our findings provide us with a foundation for understanding exercise-based regulation of NK cell biology and mobilization in children. Specifically, in the

context of understanding whether NK cell phenotype and response in this population is altered by exercise intensity or structure. In this project we identified that 5-minutes of moderate intensity intermittent cycling can elicit more robust acute NK cell responses than rigorous 30-minutes of high intensity continuous cycling. This is promising because it suggests that exercise in children can be used to induce a robust immune response. Furthermore, it demonstrates that lower volume exercise done using an intermittent protocol, which is typical of patterns of physical behavior of children, is able to more efficiently cause increases in NK cell circulation. Further investigation is warranted to better understand if beyond the transient acute changes, a training program could be clinically beneficial. For example, if the goal is to boost immune activity, information is required to provide insight into which type of exercise is best equipped to do so. On the contrary, in conditions where the immune system may be overactive, evidence that sheds light into which exercise structures may be safest to avoid NK cell activation, while retaining the other physical and mental benefits of exercise, is crucia

10.0 References

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Appendix A: ANOVA Tables

Table A1: ANOVA table for NK cell concentration

Variables	SS	Df	MS	F	P value
Time	6.40E+10	3	2.13E+10	18.23	.000*
Exercise	2.91E+09	3	9.70E+08	2.3	0.1
Puberty	1.14E+10	1	1.14E+10	4.05	0.075
Time x Puberty	2.58E+10	3	8.59E+09	7.34	.001*
Exercise x Time	1.03E+10	9	1.15E+09	3.33	.002*
Puberty x Exercise	2.51E+09	3	8.35E+08	1.98	0.141
Time x Exercise x Puberty	1.02E+10	9	1.14E+09	3.29	.002*

Table A2: ANOVA table for NK cell proportion

Variables	SS	Df	MS	F	P value
Time	3398	3	1133	76.6	.000*
Exercise	106	3	35	1.58	0.22
Puberty	1091	1	1091	15.18	0.004*
Time x Puberty	820	3	273	18.5	.000*
Exercise x Time	181	9	20	4.00	.000*
Puberty x Exercise	61	3	20	0.9	.45
Time x Exercise x Puberty	110	9	12	2.42	.017*

Table A3: ANOVA table for $CD56^{dim}$ concentration

Variables	SS	Df	MS	F	P value
Time	5.60E+10	3	1.87E+10	17.93	.000*
Exercise	2.65E+09	3	8.83E+08	2.35	0.095
Puberty	9.47E+09	1	9.47E+09	4.04	0.075
Time x Puberty	2.22E+10	3	7.41E+09	7.13	.001*
Exercise x Time	9.17E+09	9	1.02E+09	3.28	.002*
Puberty x Exercise	2.12E+09	3	7.05E+08	1.88	0.157
Time x Exercise x Puberty	8.83E+09	9	9.81E+08	3.16	.003*

Table A4: ANOVA table for $CD56^{dim}$ proportion of NK cells

Variables	SS	Df	MS	F	P value
Time	5484	3	1828	52.3	.000*
Exercise	171	3	57	0.79	0.51
Puberty	-	1	-	0	1
Time x Puberty	153	3	51	1.46	0.25
Exercise x Time	306	9	34	3.67	.001*
Puberty x Exercise	214	3	71	0.98	0.42
Time x Exercise x Puberty	171	9	19	2.05	0.044*

Table A5: ANOVA table for $CD56^{bright}$ concentration

Variables	SS	Df	MS	F	P value
Time	2.78E+08	3	9.27E+07	20.33	.000*
Exercise	1.21E+07	3	4.02E+06	0.8	0.506
Puberty	9.70E+07	1	9.70E+07	2.82	0.127
Time x Puberty	1.32E+08	3	4.41E+07	9.67	.000*
Exercise x Time	4.38E+07	9	4.86E+06	2.03	0.046*
Puberty x Exercise	1.79E+07	3	5.97E+06	1.19	0.334
Time x Exercise x Puberty	5.58E+07	9	6.20E+06	2.59	.011*

Table A6: ANOVA table for $CD56^{bright}$ proportion of NK cells

Variables	SS	Df	MS	F	P value
Time	5483	3	1828	52.3	.000*
Exercise	171	3	57	0.78	0.514
Puberty	-	1		0.00	0.998
Time x Puberty	153	3	51	1.46	0.248
Exercise x Time	305	9	34	3.66	.001*
Puberty x Exercise	214	3	71	0.98	0.42
Time x Exercise x Puberty	171	9	19	2.05	0.044*

Table A7: ANOVA table for NKG2D as a proportion of NK cells

Variables	SS	Df	MS	F	P value
Time	488	3	163	3.902	.019*
Exercise	2651	3	884	1.185	0.334
Puberty	3	1	3	0.001	0.976
Time x Puberty	242	3	81	1.933	0.148
Exercise x Time	522	9	58	1.51	0.158
Puberty x Exercise	3008	3	1003	1.345	0.281
Time x Exercise x Puberty	412	9	46	1.191	0.312

Table A8: ANOVA table for DNAM1 as a proportion of NK cells

Variables	SS	Df	MS	F	P value
Time	139	3	46	2.035	0.133
Exercise	1777	3	592	0.757	0.528
Puberty	3334	1	3334	1.476	0.255
Time x Puberty	19	3	6	0.275	0.843
Exercise x Time	217	9	24	1.076	0.389
Puberty x Exercise	685	3	228	0.292	0.831
Time x Exercise x Puberty	165	9	18	0.82	0.6

Table A9: ANOVA table for NKG2A as a proportion of NK cells

Variables	SS	Df	MS	F	P value
Time	3170	3	1057	45.02	.000*
Exercise	560	3	187	3.32	.035*
Puberty	379	1	379	0.13	0.728
Time x Puberty	63	3	21	0.9	0.456
Exercise x Time	214	9	24	1.72	0.098
Puberty x Exercise	103	3	34	0.61	0.614
Time x Exercise x Puberty	161	9	18	1.29	0.254

Table A10: ANOVA table for KIR2DL2/DL3 as a proportion of NK cells

Variables	SS	Df	MS	F	P value
Time	51	3	17	0.276	0.842
Exercise	1112	3	370	2.617	0.071
Puberty	182	1	182	0.118	0.739
Time x Puberty	60	3	20	0.323	0.809
Exercise x Time	190	90	21	1.171	0.325
Puberty x Exercise	130	3	43	0.305	0.821
Time x Exercise x Puberty	228	9	25	1.404	0.2

Table A11: ANOVA table for NKG2D MFI

Variables	SS	Df	MS	F	P value
Time	1.09E+06	3	3.62E+05	18.98	.000*
Exercise	1.35E+06	3	4.49E+05	2.27	0.103
Puberty	5.61E+05	1	5.61E+05	0.55	0.477
Time x Puberty	7.00E+04	3	2.33E+04	1.22	0.321
Exercise x Time	2.20E+05	9	2.45E+04	1.83	0.076
Puberty x Exercise	6.67E+05	3	2.22E+05	1.12	0.357
Time x Exercise x Puberty	1.36E+05	9	1.51E04	1.13	0.352

Table A12: ANOVA table for DNAM1 MFI

Variables	SS	Df	MS	F	P value
Time	1.07E+05	3	3.56E+04	8.603	.000*
Exercise	1.25E+05	3	4.16E+04	0.512	0.677
Puberty	3.24E+04	1	3.24E+04	0.099	0.76
Time x Puberty	779	3	260	0.063	0.979
Exercise x Time	5.47E+04	9	6081	1.217	0.296
Puberty x Exercise	2.73E+05	3	9.12E+04	1.123	0.357
Time x Exercise x Puberty	4.49E+04	9	4993	0.999	0.448

Table A13: ANOVA table for NKG2A MFI

Variables	SS	Df	MS	F	P value
Time	5.67E+07	3	1.89E+07	21.5	.000*
Exercise	1.72E+07	3	5.74E+06	1.97	0.142
Puberty	1.66E+05	1	1.66E+05	0	0.956
Time x Puberty	5.23E+06	3	1.74E+06	1.98	0.14
Exercise x Time	5.25E+06	9	5.83E+05	1.49	0.166
Puberty x Exercise	1.09E+07	3	3.62E+06	1.24	0.314
Time x Exercise x Puberty	5.52E+06	9	6.13E+05	1.57	0.139

Table A14: ANOVA table for KIR2DL2/DL3 MFI

Variables	SS	Df	MS	F	P value
Time	1.38E+06	3	4.61E+05	2.334	0.096
Exercise	1.84E+06	3	6.14E+05	1.396	0.265
Puberty	1.31E+07	1	1.31E+07	2.262	0.167
Time x Puberty	1.33E+06	3	4.42E+05	2.238	0.107
Exercise x Time	9.62E+05	9	1.07E+05	2.784	.000*
Puberty x Exercise	7.26E+05	3	2.42E+05	0.55	0.652
Time x Exercise x Puberty	1.68E+06	9	1.86E+05	4.852	.000*

Appendix B: Parental Consent and Assent

McMaster University Health Sciences

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PARENT CONSENT FORM

Title of Study:	Characterizing the effects of **EX**ercise **i**ntensity and **D**uration on natural killer cell response at distinct stages of growth and development **(EXiD Study)**
Local Principal Investigator:	Dr. Joyce Obeid, PhD (Department of Pediatrics)
Principal Investigator:	Inna Ushcatz, MSc Candidate (Faculty of Health Sciences)
Funding Source:	Natural Sciences and Engineering Research Council of Canada

INTRODUCTION

Your child is being invited to participate in a research study conducted in Dr. Joyce Obeid's lab, and led by Inna Ushcatz. We are inviting your child because they are healthy. In order to decide whether or not you and your child want to be a part of this research study, you should understand what is involved and the potential risks and benefits. This form will give you detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you and your child wish to participate. Take your time to make your decision. Feel free to discuss it with your family.

WHY IS THIS RESEARCH BEING DONE?

The benefits of exercise have been studied for a long time in adults. In fact, many studies show that regularly exercise can improve a person's ability to fight off infection. This is because exercise can help improve Natural Killer cells, which are a type of immune cell in your body that are fast at recognizing invaders and killing them. However, studies in adult have also shown that natural killer cells can behave differently depending on how intensely or how long people exercise. What is surprising is that we don't know much about how these cells act in children and adolescents and so we are unable to develop a way to use exercise to improve the ability of the natural killer cells to do their job. As children grow and mature, their bodies change and with that, their immune system matures as well. The goal of the study is to understand the relationship between exercise and the body's ability to protect itself at different stages of development.

WHAT IS THE PURPOSE OF THIS STUDY?

We have a couple of questions to answer in this study. Our main goal is to see how different types of exercise affect the number and activity of the Natural Killer cells. We are also interested in understanding how growth and development play a role in this process by looking at differences in pre-pubertal and pubertal boys and girls.

WHAT WILL MY RESPONSIBILITIES BE IF THEY TAKE PART IN THE STUDY?

If your child volunteers to participate in this study, we will ask them to come to our lab at McMaster University on **5 occasions over a 5-week period**. These visits will be to conduct exercise testing in the laboratory. We will schedule these visits at your convenience.

Visit #1 will take about 2 hours to complete, and will include the following:

1) We will measure your child's height and weight. We will also ask your child to stand on a machine that will help us measure how much muscle and fat they have in their body. This machine does not hurt, it just requires your child to stand still for 2 minutes.
2) We will ask your child to assess their puberty status using a questionnaire.
3) We will assess your child's fitness level on a special bicycle. This exercise test (called a "VO_{2max} test") lasts 8 to 12 minutes and feels like your child is riding up a hill. During the test, we will ask your child to wear a face mask over their mouth and nose, this will help us measure how much oxygen they are breathing during the test. We will also ask your child to wear a heart rate monitor on their chest so we can monitor their heart rate throughout the test.
4) Before going home, we will give your child a small belt to take home for the week. This device is called an accelerometer, and it will help us measure your child's movement – your child's physical activity and sedentary time. It's like a Fitbit, except your child will wear it around their waist, and take it off any time they sleep or do any water activities (swimming or shower). Your child will be asked to wear this belt for 7 days, and to return it at their next study visit.

Visits #2-5 will take about 2.5 hours each to complete. We will book these visits at least 4 days apart. Each visit will be identical except for the type of exercise your child will do:

1) When your child comes into the lab, we will get them to lie down for 10 minutes. Then we will collect a resting blood sample. To do this we will put in a small, flexible plastic tube in their vein – this is called a catheter, and it will help us collect blood samples throughout your child's visit without having to poke your child with a needle each time.
2) After their resting sample, we will get your child set up on our bike and they will be asked to perform one of 4 exercises. The order of these exercises will be determined by chance, like picking a random number out of a hat:
    a. High intensity all-out intermittent cycling, where your child will pedal as hard as they can for 15-seconds, then get a 1-minute break. Your child will be asked to repeat the15-sec cycling/1-minute break a total of 24 times.
    b. Moderate intensity all-out intermittent cycling, where your child will pedal as hard as they can for 15-seconds, then get a 1-minute break. Your child will be asked to repeat the15-sec cycling/1-minute break a total of 24 times. For this test, the workload on the bike will be lower than the high intensity session.
    c. High intensity, continuous cycling, where your child will pedal at a comfortable pace for 30 minutes. The workload on the bike will feel challenging, but it will stay the same throughout the test.

d. Moderate intensity, continuous cycling, where your child will pedal at a comfortable pace for 30 minutes. The workload on the bike will feel easier than the high intensity session, and it will stay the same throughout the test.

3) We will collect blood samples before your child exercises, right after they exercise, 30-minutes after they stop exercising, and again 1 hour after they stop exercising. We will collect a total of 80 mL of blood. This is about 5 tablespoons of blood.
4) We will ask your child to fill out some questionnaires that tell us about their physical activity. All answers will be kept confidential.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

i) Blood sampling. An experienced investigator will insert the small needle that will be used to place the small, flexible plastic tube. Getting a poke with a needle can hurt. If your child is worried about the needle, we can use a special cream to numb the area where the needle will go in so they do not feel any pain. A small bruise might appear where the needle goes through the skin. Because we will provide water on a regular basis throughout each visit, taking this amount of blood will have no negative effects. There is also a chance your child will feel light-headed after the blood sample. We will have snacks and water on hand to minimize the risk of this happening.

ii) Exercise testing. The VO_{2max} test requires you to give a maximal effort. This means that your child will feel quite tired after they are done the test. The other exercise your child will be asked to complete during visits 2 – 5 will be very similar to what they might do as part of their daily life and may make them sweat and feel tired. There is a small chance your child could feel dizzy or nauseous when your child exercises, but this feeling will go away pretty quickly. None of the exercise tests will pose any health concern. We will monitor your child's heart rate during each exercise session.

HOW MANY PEOPLE WILL BE IN THIS STUDY?

We are asking 12 pre-pubertal and 12 pubertal boys and girls to participate in this study. Your participation is voluntary.

WHAT ARE THE POSSIBLE BENEFITS FOR MYSELF MY AND/OR FOR SOCIETY?

We cannot promise any personal benefits to your child from participation in this study. We will make each exercise session fun and enjoyable. Your child's participation will be very important for us to learn how natural killer cells work and will provide insight into how we can best utilize exercise to help strengthen the immune system. We will provide your child with a report card at the end of the study letting them know how they did.

WHAT INFORMATION WILL BE KEPT PRIVATE?

All of your child's information will be stored in locked filing cabinets or a password protected computer under the supervision of Dr. Joyce Obeid for 10 years. No one outside of our research team will have access to your information. Your child will be assigned a participant number, and this number will be used to identify them. Records identifying your child will be kept confidential. If the results of the study are published, your child's identity will remain confidential.

CAN PARTICIPATION IN THE STUDY END EARLY?

If your child volunteers to be in this study, they may withdraw at any time with no judgement by informing the investigator, Inna Ushcatz, at 647-688-6708 or by email at ushcati@mcmaster.ca. The investigator may withdraw your child from this research if circumstances arise which warrant doing so.

WILL I BE PAID TO PARTICIPATE IN THIS STUDY?

We will provide your child $100 as reimbursement for their participation in this study. If your child quits the study for personal reasons, we will change the amount for the time completed. If you and your child choose to quit because of a complication from the study, we will give your child the full amount.

IF I HAVE ANY QUESTIONS OR PROBLEMS, WHOM CAN I CALL?

If you have any questions about the research now or later, or if you think you may have a research-related injury, Inna Ushcatz at 647-688-6708 or Dr. Joyce Obeid directly at 905-521-2100 extension 75865 (Daytime) or 905-928-5538 (Nighttime). If you have any questions regarding your rights as a research participant, you may contact the Office of the Chair of the Hamilton Integrated Research Ethics Board at 905-521-2100 extension 42013.

CONSENT STATEMENT

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to allow my child to participate in this study entitled: "Characterizing the effects of **EX**ercise **i**ntensity and **D**uration on natural killer cell response at distinct stages of growth and development **(EXiD Study)"**. I understand that I will receive a signed copy of this form.

Would you like to be contacted by Dr. Obeid or a member of the Child Health & Exercise Medicine Program research team with information about future studies other than the one described in this consent form? Any future studies would be approved by the Research Ethics Board, and would require you to sign a new consent form. **Please note we will only contact you if you are eligible for a maximum of 2 times per year.**

☐ Yes, please contact me. ☐ No, please do not contact me.

_____
Name of Participant (child's name)

_____
Name of Legally Authorized Representative

_____ _____
Signature of Legally Authorized Representative Date

Consent form administered and explained in person by:

_____
Name and title

_____ _____
Signature Date

SIGNATURE OF INVESTIGATOR:

In my judgement, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to participate in this research study.

_____ _____
Signature of Investigator Date

FUTURE RESEARCH

At the end of the study, we may wish to store leftover blood sample for use in future studies which will aim to understand how exercise influences the immune system. We will not store your child's sample longer than 10 years. All records identifying your child will remain confidential. Information about your child will not be released. If the results of the study are published, your child's identity will remain confidential. No genetic testing will be performed on these samples. Hamilton integrated Research Ethics Board approval will be obtained for these future studies.

CONSENT STATEMENT FOR STORAGE OF SAMPLES (BLOOD)

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to have my child's blood stored so it can be used in future research studies approved by the Hamilton Integrated Research Ethics Board other than the one described in this information form.

_____
Name of Participant (child's name)

_____
Name of Legally Authorized Representative

_____ _____
Signature of Legally Authorized Representative Date

Consent form administered and explained in person by:

_____
Name and title

_____ _____
Signature Date

SIGNATURE OF INVESTIGATOR:

In my judgement, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to have their child's blood stored so it can be used in future research studies approved by the Hamilton Integrated Research Ethics Board other than the one described in this information form.

_____

Name and title

Signature of Investigator | Date

PHOTO, AUDIO AND VIDEO RELEASE FORM (OPTIONAL)

I, _____, hereby give McMaster University's Faculty of Health Sciences my permission to take and use any photographs, movie films, audio or video tapes taken on (date) _____, and I consent to the reproduction of same in any proper manner whatsoever for possible publication and I hereby waive any rights that I may have in such photographs, movie films and audio or video tapes or reproductions of same.

I hereby release McMaster University's Faculty of Health Sciences, its employees, agents, servants and attending physicians from all actions, causes of actions, claims and demands arising out of such consent.

Notice of collection of personal information

By taking my photograph, whether by still photograph, film or video and/or taping my voice, I acknowledge that McMaster University is collecting my personal information as defined by the Freedom of Information and Protection of Privacy Act of Ontario (RSO 1990).

The personal information is collected under the authority of The McMaster University Act, (1976). The information is used for public relations purposes of the Faculty of Health Sciences including, but not limited to, publications, websites and materials promoting McMaster University. Personal information will not be used for any unrelated purpose without prior consent. This information is protected and is being collected pursuant to section 39(2) and section 42 of the Freedom of Information and Protection of Privacy Act of Ontario (RSO 1990). Questions regarding the collection or use of this personal information should be directed to the Manager, Public Relations, Faculty of Health Sciences.

Name of Participant	Signature of Participant	Date
Name of Witness	Signature of Witness	Date

CHILD ASSENT FORM

Title of Study:	Characterizing the Effects of Exercise Intensity and Duration on Natural Killer Cell Response at Distinct Stages of Growth and Development
Local Principal Investigator:	Dr. Joyce Obeid, PhD (Department of Pediatrics)
Principal Investigator:	Inna Ushcatz, MSc Candidate (Faculty of Health Sciences)
Funding Source:	Natural Sciences and Engineering Research Council of Canada

Why are we doing this study?
A research study is a way to learn more about people. We are doing a research study to learn about how exercise changes your immune system. Your immune system is made up of different cells that help protect you from getting sick, one type of cell is called a natural killer cell. A natural killer cell is one of the first cells to protect your body from a virus or an invader. When you exercise, signals are sent natural killer cells that help them move around your body to look for invaders. We want to see if how different types of exercise help these cells do their job.

Why am I being asked to be in the study?
We are inviting you to be in the study because you are a healthy, young person.

What if I have questions?
You can ask questions if you do not understand any part of the study. If you have questions later that you don't think of now, you can talk to me again or ask your mom or dad to call Inna at 647-688-6708 or email Inna ushcati@mcmaster.ca; or contact Dr. Joyce Obeid at 905-521-2100 ext. 75865.

If I am in the study what will happen to me?
If you decide that you want to be part of this study, you will be asked to come visit our exercise lab 5 times. During each visit, we will ask you to ride a special bicycle. At your first visit, we will do a bike ride that feels like riding up a hill, you will also wear a face mask so we can measure how much oxygen your body is using. We will give you a small belt to wear at home to find out how active you are. When you come back for the rest of your study visits, we'll ask you to do different cycling activities. We will take a little bit of blood from a vein in your arm at these visits so that we can measure the natural killer cells. We will also write down how fast your heart is beating and how much air you are breathing when you are exercising.

Will I be hurt if I am in the study?
To take some blood, we need to put a special tiny plastic tube in your arm. We use a needle to make sure the tube goes in the right spot, but the needle part does not stay in your arm. The special tiny plastic tube will not hurt when it is in your arm. There is a small chance that you will get a bruise on

your arm where the needle first went in, but this will go away after a few days. The exercise you will do might make you feel a little tired, and some kids feel a bit dizzy, but it will not hurt you.

Will the study help me?
If you are in the study, it may not benefit you directly. You will learn about your fitness and physical activity level. We will also learn how exercise affects your immune system.

Do I have to be in this study?
You do not have to be in this study, if you do not want to be. If you decide that you don't want to be in the study after we begin, that's OK too. You can tell me, and nobody will be angry or upset. It is your choice. We are discussing the study with your parents and you should talk to them about it too.

What happens after the study?
When we are finished this study, we will write a report about what we learned. This report will not include your name or that you were in the study.

Assent:
If you decide you want to be in this study, please print/write your name in the spot below. If you decide that you don't want to be in the study, even if you have started in the study, then all you have to do is tell Dr. Obeid or Inna that you don't want to be in the study anymore. You can call Dr. Obeid at 905-521-2100 extension 75865 or Inna at 647-688-6708 at any time.

If you have questions regarding your rights as research participant, you may contact office of the Chair of Hamilton Integrated Research Ethics Board at 905-521-2100 ext. 42013.

I, _____ (Print your name) would like to be in this research study.

_____ (Date of assent)

_____ (Name of person who obtained assent)

_____ (Signature of person who obtained assent and Date)

_____ (Local Principal Investigator name)

_____ (LPI signature and Date)

Appendix C: Medical and Physical Activity Questionnaire

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

EXiD Study
MEDICAL QUESTIONNAIRE

Date of Birth: _____

1. Does your child have any of the following conditions (circle each of the appropriate):
   a) Heart disease
   b) High blood pressure
   c) Loss of consciousness
   d) Asthma
   e) Intestinal disease
   f) Surgery or fractures
   g) Allergies
   h) Diabetes
   i) Epilepsy
   j) Others: _____
   k) None

2. Present health:
   a) Good
   b) Complaints: _____

3. Is your child currently taking any medications? If yes, how frequently?

Medication	Frequency

☐ My child is not currently taking any medications

4. Has your child taken any medications in the past month? If yes, how frequently?

Medication	Frequency

☐ My child has not taken any medications in the past month

5. When thinking of prior exercise involvement, has your child experienced (circle the appropriate):
   a) An inability to keep up with other children
   b) Chest pain
   c) Fainting
   d) Dizziness
   e) Irregular heart beat
   f) Wheezing
   g) Other: _____
   h) None of the above

6. Has your physician ever suggested that your child restrict their levels of physical activity?
   ☐ Yes
   ☐ No

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

EXiD Study
MEDICAL QUESTIONNAIRE

7. Do you know of any medical reason that would prevent your child from participating in physical activity?

   ☐ Yes. Please specify: _____
   ☐ No

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

EXiD Study
MEDICAL QUESTIONNAIRE

Menstrul Cycle Questionnaire

1. Have you had your first period yet?
   - ☐ Yes (please complete questions #2-6)
   - ☐ No (you do not need to complete the rest of the questionnaire)

2. Approximately how old were you when you had your first period?

3. When is the last time you had your period?
   - ☐ I can't remember
   - ☐ The first day of my last period was on: _ _ - _ _ _ - _ _ _ _ (day – month – year)
   - ☐ I don't remember the first day, but I know I had my period on: _ _ - _ _ _ - _ _ _ (day – month – year)

4. On average, how many days does your period last?
   - ☐ 1 – 2 days
   - ☐ 3 – 4 days
   - ☐ 5 – 7 days
   - ☐ More than 7 days
   - ☐ It's different each time
   - ☐ I'm not sure

5. Do you get your period every month? If not, how often do you think you get your period?
   - ☐ Yes
   - ☐ No, I get my period: _____

6. Are you currently taking an oral contraceptive (birth control)?
   - ☐ Yes, name (if you remember): _____; I have been taking it for: _____
   - ☐ No, I never have
   - ☐ No, but I used to. I stopped taking it: _____.

Child Health & Exercise Medicine Program

Study ID: EXiD - _ _
Date: _ _ - _ _ _ - _ _

ACTIVITY QUESTIONNAIRE

Dear Parent:
The purpose of the following questionnaire is to help us evaluate the activity habits of your child. Please be as accurate as possible in your answers. Feel free to add any details that seem relevant.

1. How would you compare the physical activity of your child to that of her/his friends?
   - ☐ My child is as active as her/his friends
   - ☐ My child is more active than her/his friends
   - ☐ My child is less active than her/his friends
   - ☐ Not sure

2. How would you compare the activity of your child with that of her/his sibling(s)?
   - ☐ My child is as active as her/his sibling(s)
   - ☐ My child is more active than her/his sibling(s)
   - ☐ My child is less active than her/his sibling(s)
   - ☐ Not applicable (no siblings)
   - ☐ Not sure

3. How many hours in a typical day is your child engaged in the following activities:

Activity	Less than 1 hour	1-2 hours	2-3 hours	3-4 hours	4-5 hours	More than 5 hours
TV						
Video games						
Computer						
Phone						

4. Which mode of transportation does your child use to travel to and from school?
   - ☐ Car / bus
   - ☐ Walking
   - ☐ Biking
   - ☐ Other:_____

   If biking or walking; what is the total time spent actively travelling per day?
   - ☐ 0-10 minutes
   - ☐ 10-20 minutes
   - ☐ 20-30 minutes
   - ☐ More than 30 minutes

5. In your opinion, is your child as active as she/he should be?
   - ☐ Yes
   - ☐ My child is too active
   - ☐ My child is not sufficiently active
   - ☐ Not sure how much physical activity she/he needs

6. If your child is not as active as she/he should be, what, in your opinion, is the reason? (you can select more than one answer)
   - ☐ Lack of interest
   - ☐ Disease
   - ☐ Lack of suitable conditions
   - ☐ Other: _____
   - ☐ I don't know

7. In a typical week, how many days a week does your child participate in organized sport outside of school?
   - ☐ Once a week
   - ☐ 2 – 3 times a week
   - ☐ 4 – 5 times a week
   - ☐ More than 5 times a week
   - ☐ My child does not participate in sport outside of school
   - ☐ I don't know

8. How many hours a day is your child involved in organized sport?
   - ☐ Less than 1 hour a day
   - ☐ 1 – 2 hours a day
   - ☐ More than 2 hours a day
   - ☐ Not applicable

9. If your child participates in organized sport outside of school, please list the sport(s) below:

   _____

   _____

   _____

   _____

10. In a typical week, how many days a week does your child engage in spontaneous physical activity (e.g. walking the dog, going to the park, riding their bike, etc.)?
    - ☐ Once a week
    - ☐ 2 – 3 times a week
    - ☐ 4 – 5 times a week
    - ☐ 6 – 7 times a week
    - ☐ My child does not engage in spontaneous activity
    - ☐ I don't know

11. How many hours a day does your child engage in spontaneous physical activity?
    - ☐ Less than 1 hour a day
    - ☐ 1 – 2 hours a day
    - ☐ 3 – 4 hours a day
    - ☐ More than 4 hours a day
    - ☐ I don't know

Appendix D: Physical Activity Log

Child Health & Exercise Medicine Program

ACCELEROMETER DIARY:
In addition to wearing the accelerometer for one week, we ask that you keep this log to monitor the times the accelerometer was put on or taken off, and the activities that you participated in when wearing the accelerometer. This will help us to understand your regular physical activity. Please bring this form along with your accelerometer to your next study visit.

Event	Example	DAY 1 Day: _____ Date: _____	DAY 2 Day: _____ Date: _____	DAY 3 Day: _____ Date: _____	DAY 4 Day: _____ Date: _____	DAY 5 Day: _____ Date: _____	DAY 6 Day: _____ Date: _____	DAY 7 Day: _____ Date: _____
Time the device was put on	8:02 AM							
Times the device may have been taken off and put back on and reason(s) (e.g. nap, swimming, shower, etc)	4:45 pm – 5:27 pm (Nap)							
	7:10 pm – 7:37 pm (Shower)							
	-							
	-							
Time the device was taken off before bed	10:19 pm							
Activities (e.g. soccer, camp, game)	- Walked to school - Played outside - Soccer practice - Bike ride							

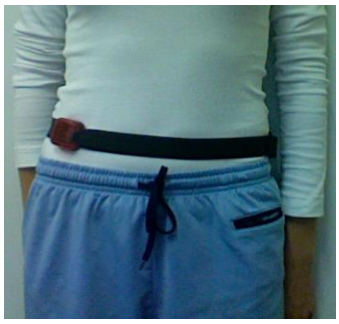
ID: _____
ACCELEROMETER #: _____

PLEASE SEE THE NEXT PAGE FOR DETAILED INSTRUCTIONS

Child Health & Exercise Medicine Program

HOW DO I WEAR THE ACCELEROMETER?

The accelerometer must be worn around your waist, over your **RIGHT HIP BONE**, like in the picture below. You can wear the accelerometer on top or underneath your clothes.




WHEN DO I WEAR THE ACCELEROMETER?

You should wear the accelerometer at **all times when you are awake**. You can wear the accelerometer when playing sports, even contact sports like hockey and football. You should **NOT** wear the accelerometer during:

1. Water activities (shower, bath, swimming, etc.)
2. Sleep (overnight and daytime naps)

HOW DO I KNOW THE ACCELEROMETER IS WORKING?

Remember when we showed you the flashing light on the accelerometer? **This light will NOT be flashing when the accelerometer starts recording**. If you notice that the light is flashing when you are wearing the belt, please call Joyce at 905-521-2100, ext. 73517.

WHAT DO I RECORD IN THE ACCELEROMETER DIARY?

1. The time you put the belt on when you wake up
2. The time you took the belt off before going to sleep at night
3. Any activities you did on each of the days you wear the accelerometer.

If you have to take the accelerometer off during the day for any reason, we ask that you record:

1. The time you took the belt off
2. The time you put the belt back on
3. The reason you took the belt off

If you have any questions or concerns, please do not hesitate to contact Joyce at 905-521-2100 ext. 73517. Thank you.