THE RELATIONSHIP BETWEEN AEROBIC FITNESS AND NATURAL KILLER CELL CYTOTOXICITY IN HEALTHY CHILDREN AND ADOLESCENTS

By

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Public Abstract

Natural killer (NK) cells are an important part of our innate immune system because they can target infected or cancerous cells without having encountered them before. Since these cells are so important, it is vital to know how they are modulated and what improves their function. NK cells are recruited into circulation during exercise, which causes an increase in their ability to kill harmful cells, also known as cytotoxicity (NKCA). It is uncertain whether this increase in cytotoxicity is due to more NK cells present (total NKCA) or whether each NK cell is becoming more cytotoxic (NKCA/cell). Interestingly, adults with higher levels of fitness demonstrate higher NKCA/cell, but research on NKCA/cell in children is limited. This thesis examined (1) the change in NKCA/cell following exercise in children, and (2) the relationship between fitness, pubertal status, and NKCA/cell at rest and after exercise. I predicted an increase in NKCA/cell following exercise and a positive relationship between fitness, puberty and NKCA/cell. To test this, seven healthy pre- and post-pubertal children completed a fitness test on a stationary bicycle. At a second visit, participants completed 30 minutes of high intensity cycling in intervals. Blood samples were collected before exercise, immediately after exercise, and after 30- and 60-minutes of recovery. NKCA was tested by determining how many cancer cells NK cells from blood samples were able to kill. The exercise protocol significantly affected NKCA/cell with NKCA/cell increasing significantly from post-exercise to 30-minutes recovery. Neither fitness nor pubertal status predicted NKCA/cell at baseline (before exercise) or

post-exercise. The trends observed were expected, but limitations including small sample size and exclusion of elite athletes may have impacted results. This study provides a basis for future research and suggests exercise can alter NKCA/cell in children; however, the importance of fitness for NK function remains unclear.

Abstract

Natural killer (NK) cells are lymphocytes that can identify and destroy infected or cancerous cells without prior sensitization. Acute exercise increases total NK cytotoxic activity (NKCA) in peripheral blood. However, this may be due to the increase in NK cells in circulation post-exercise, not an improvement in the function of each cell. Interestingly, adults with higher aerobic fitness demonstrate higher NKCA/cell. The only available study in children reported lower total NKCA at baseline, but a greater increase in total NKCA with exercise in swimmers vs. non-swimmers. This study examined (1) the change in NKCA/cell following exercise in children, and (2) the relationship between aerobic fitness, pubertal status, and NKCA/cell at rest and post-exercise. Seven healthy pre- and post-pubertal children completed an aerobic fitness test on a cycle ergometer. At a second visit, participants completed a high-intensity interval cycling protocol. Blood samples were collected at rest, immediately after exercise, and after 30- and 60-minutes recovery. NKCA against a cancer cell line (K562) was quantified using flow cytometry. There was a main effect of time for NKCA/cell with a significant increase occurring between post-exercise (0.07±0.05 lysed K562 per NK) and 30-minutes recovery (0.16±0.08, $p=0.014$). Neither fitness nor pubertal status predicted NKCA/cell at baseline or post-exercise. The trends observed were expected, but limitations including small sample size and exclusion of elite athletes may have impacted results. This study provides a basis for future

research and suggests exercise can alter NKCA/cell in children; however, the importance of aerobic fitness for NK function remains unclear.

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1. Introduction

1.1 Natural Killer Cells

Natural killer (NK) cells are lymphocytes that play an important role in the innate immune system.[1] They can recognize and target tumours and virally infected cells without prior sensitization, as well as secrete various cytokines to promote an adaptive immune response.[1] They make up about 10 to 15% of lymphocytes in circulation.[1–4] NK cells can be recognized by their cell surface markers; they lack expression of cluster of differentiation (CD)3 and express CD56.[3] NK cell activation relies on a balance of activating and inhibitory receptors that bind to ligands present on the target cell, indicating whether the cell is healthy or damaged.[5] This can be explained by "induced-self" recognition, where ligands regulated by cellular abuse like infection, transformation and stress are recognized by NK cells, leading to activation.[5] This may be accomplished by recognizing receptors like major histocompatibility complex (MHC) class I chain-related protein (MIC)-A and MIC-B, which are markers of stressed cells.[1] Activation may also be accomplished through antibody-dependent cell-mediated cytotoxicity (ADCC), wherein the Fcγ receptor (FcγR) on NK cells recognizes the Fc region of an IgG antibody bound to a tumour.[6] CD16, also known as FcγRIIIA, is essential for ADCC as it binds antibodies coating tumour cells.[7] NK activation, alternatively, can be explained by "missing-self" recognition; NK cells are activated by lower expression of MHC since

healthy cells tend to have higher MHC expression and cancer cells often have lower MHC expression.[5,8]

Two major subpopulations of NK cells exist based on their expression of CD56. These are $CD56^{bright}$ cells and $CD56^{dim}$ cells, which make up about 10% and 90% of total NK cells, respectively.[9] $CD56^{bright}$ cells express high levels of CD56, have low or no expression of CD16, and lack expression of CD57, a marker of NK cell maturation and cytotoxicity.[10] In contrast, $CD56^{dim}$ cells express low levels of CD56, high levels of CD16, and high levels of CD57.[9] $CD56^{bright}$ cells are mainly responsible for producing inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ).[11] These cytokines promote apoptosis, recruit other immune cells, and possibly promote the cytotoxicity of other NK cells.[12–14] $CD56^{dim}$ cells are thought to be more mature than $CD56^{bright}$ cells, which can be explained by the linear differentiation model.[15–17] $CD56^{dim}$ cells are primarily responsible for cytotoxicity.[18]

1.2 Cytotoxicity and $CD56^{dim}$ cells

There are several methods by which NK cells exert their cytotoxic effects on target cells. One of these is the release of proteins called perforin and granzyme. Perforin forms large pores in the target cell membrane, allowing granzyme to enter the cell and initiate apoptosis.[19] NK cells can also use proteins expressed on their surface, such as FasL/Fas, to ligate apoptosis-inducing receptors on target cells.[20] After stimulation with cytokines interleukin (IL)-2 and IL-15 that promote growth and function of NK cells, TNF-related apoptosis-inducing ligand

(TRAIL) is induced on NK cells and also contributes to cytotoxicity against infected cells.[21]

Cytotoxicity can be measured by observing the amount of target cell death caused when NK cells are incubated with target cells.[22] For example, K562 cells, which are a human erythroleukemic cell line, are commonly used as target cancer cells in this assay as they are particularly susceptible to NK cell-mediated cytotoxicity. This is because K562 cells have reduced expression of human leukocyte antigen (HLA) class I, the human version of MHC, and have elevated expression of ligands for activating NK receptors. [22] These ligands include MIC-A, intracellular adhesion molecule (ICAM)-1, and CD155.[23]

NK cells have an important function within our body's immune system due to their potent cytotoxicity against infected or cancerous cells. People lacking NK cells are at a much higher risk of developing viral infections and dying prematurely,[24] and dysfunctional NK cells are unable to prevent tumours from growing uncontrollably, leading to reduced overall survival in cancer patients.[25] It is clearly important for NK cells to be performing optimally to be able to fend off infections, cancers, and other threats. Thus, it is important to understand how NK cells are modulated and which stimuli promote their function. Previous research has shown that NK cells are responsive to exercise, suggesting this avenue should be further explored with respect to the potential impacts on NK cell function.

1.3 Effect of exercise on NK recruitment in adults

NK cells are known to have a strong, transient response to exercise in adults. This is thought to be caused by catecholamines norepinephrine and epinephrine being released during exercise[26] and binding to β-adrenergic receptors on NK cells to activate them.[27] This activation then promotes NK cell recruitment into circulation.[28] Blood flow-induced shear stress may also contribute to recruitment of NK cells during exercise.[29] Many studies done in adults have shown that the number of circulating NK cells increases immediately after an acute bout of exercise.[30–32] Oftentimes, the number of NK cells will subsequently drop to levels near or below their baseline during a recovery period.[30,33,34] Typically, $CD56^{bright}$ cells show a less marked response to exercise than cytotoxic $CD56^{dim}$ cells,[33,35] possibly due to higher expression of β-adrenergic receptors on $CD56^{dim}$ cells.[36,37] Exercise intensity also affects NK cell recruitment. NK cell number has been shown to increase to a greater degree after high-intensity exercise compared to moderate- or low-intensity.[33,38] In summary, we see that NK cells, especially the $CD56^{dim}$ subset, are recruited in response to exercise, especially at a high intensity, in adults.

While the number of cells recruited into circulation is an important measure for understanding how responsive NK cells are to exercise, it is not entirely representative of NK cell function once they are in circulation. Therefore, it is essential to look at natural killer cytotoxic activity (NKCA) as a measure of the functional response of NK cells to exercise.

1.4 Effect of exercise on NKCA in adults

1.4.1 Effect of exercise on total NKCA in adults

As previously mentioned, the number of NK cells in circulation tends to increase after exercise, but it is unclear if the function of these cells follows the same trend. Most studies have observed an increase in total NKCA immediately following exercise.[32,39,40] For example, Moyna et al. found a significant increase in total NKCA in healthy adult males and females after continuous, incremental exercise. However, total NKCA increased to a lesser degree than the proportion of circulating NK cells, possibly because the recruited cells were less mature.[32] On the contrary, Millard et al. observed that there was no change in specific lysis following running up and down 150 stair-steps in healthy adults.[41] The intensity of exercise performed may also impact total NKCA: total NKCA is increased to a greater degree after high- vs. moderate-intensity exercise,[39,42,43] though no differences are observed when total NKCA is normalized to the number of NK cells recruited.[43] Total NKCA seems to increase to a similar degree following continuous and intermittent exercise.[44] Overall, total NKCA generally seems to increase following exercise, but this does not necessarily explain the function of each NK cell. Therefore, we must look at NKCA per cell.

1.4.2 Effect of exercise on NKCA per cell in adults

Although an increase in total NKCA is often seen following exercise, the effects on NKCA per cell are less clear, with evidence to suggest NKCA per cell may not change.[40,43] For example, while Brenner et al. observed both an

increase in NK cell (defined as the $CD16^{+}CD56^{+}$ population here) number and total NKCA in healthy males after two 30-minute bouts of moderate exercise, NKCA per cell was not significantly changed as a result of exercise.[34] Contrary to the above results, some studies have shown that NKCA per cell can increase with exercise. For example, Bouillon et al. observed an increase in NKCA per cell following one hour of moderate exercise in triathletes and recreationally active participants.[45] Similarly, Field et al. saw an increased lytic activity per cell in healthy, fit young males exercising at 80% of maximum workload.[46] Bigley et al. also found a 1.6-fold increase in NKCA per cell one hour after three 30-minute bouts of cycling in healthy cyclists.[47] Like total NKCA, NKCA per cell may be intensity-dependent; Nieman et al. found a significant increase in NKCA per cell after high- but not moderate-intensity exercise in well-conditioned young males.[43]

Thus, it has been established that in adults total NKCA increases after exercise due to the recruitment of NK cells. NKCA per cell likely increases as well, but some results are inconsistent. In some situations, such as in people with impaired NK function, it may be desirable to be able to promote NK cells to have a further elevated cytotoxic capacity. It is unclear whether one bout of exercise is sufficient to do this. Since an acute bout of exercise seems to result in a moderate increase in NKCA and possibly an increase in NKCA per cell, it can be hypothesized that repeated exposure to exercise may increase baseline NKCA per cell. Repeated exposure to exercise is also associated with increased fitness, so this suggests fitness may be proportional to NKCA per cell.

1.5 Effect of fitness on NKCA in adults

1.5.1 Effect of fitness on total NKCA in adults

Most existing research investigating the relationship between fitness and NKCA has looked at total NKCA only. For example, young, healthy males participating in a four-week hypoxic exercise training program developed an increase in both total NKCA and fitness as measured by VO_{2max} (the maximum rate of oxygen uptake during exhaustive exercise).[48] Another study showed that highly trained young, male cyclists had elevated total NKCA at baseline compared to untrained individuals.[49] Additionally, VO_{2max} was significantly positively correlated with total NKCA (measured in lytic units) in marathon runners and sedentary controls.[50] Similar results have been shown in elderly populations.[51–54] For example, a significant positive correlation was shown between VO_{2max} and total NKCA (lytic units) at baseline in highly conditioned and sedentary elderly women.[53] However, not all studies in adults have demonstrated a trend towards increasing NKCA with increased fitness.[55–59] For example, Watson et al. found a decrease in total NKCA after 15 weeks of aerobic training in previously inactive young men.[57] Overall, it seems as though fitness and total NKCA are related, but findings are inconsistent.

1.5.2 Effect of fitness on NKCA per cell in adults

There has been very little research studying NKCA per cell as it relates to fitness. However, we can investigate markers of activation, such as CD69, and markers of degranulation capacity, such as CD107a, to see whether there is a

difference between trained and untrained individuals. Indeed, Moro-García et al. found that after NK cells were challenged with K562 cells, levels of activation and degranulation were significantly higher in athletes compared to non-athletes, indicating there may be a larger change on a per cell basis with increasing fitness.[60] Based on this minimal evidence, it remains unclear whether a relationship between fitness and NKCA per cell exists in adults, so further research is required.

It is vital to understand the mechanism that may result in increased NKCA per cell in individuals with higher levels of fitness. Wang and Weng found that perforin and granzyme contents in NK cells were increased at rest and after exercise in groups that had completed hypoxic exercise training for four weeks.[48] This means each of the NK cells had more of the components needed to induce apoptosis in target cells. Muscular IL-15 was also shown to be increased in healthy males who had completed 12 weeks of endurance training,[61] which may help to activate NK cells.[62] IL-2, another cytokine that can enhance NK activity,[63] increased following a four-week training program in young, male athletes, as well.[64] It is likely a combination of these factors that explains the potential correlation between fitness and NKCA per cell.

While there have been some promising results thus far, it is clear that further research is required to confirm whether or not NKCA per cell can be increased by exercising frequently. There has been limited research in this field in children, but

the following section will investigate the existing research on exercise and NK cells in children.

1.6 NK response to exercise in children

The studies that have been done in children to date show that children's NK cell response to exercise exhibits a similar trend to adults, but to a smaller degree. For example, NK cells were the most responsive cells to exercise in healthy children with a 442% increase in NK cell number following a graded maximal exercise test, and values returning to baseline by one hour following exercise.[65] Another study also observed this trend of an increase in NK cell number following ten two-minute bouts of exercise above the anaerobic threshold with levels returning to below baseline by two hours after exercise in children aged 8 to 17.[66] However, Timmons et al. demonstrated that while the proportion of NK cells after 60 minutes of moderate intensity exercise increased significantly in boys and men, it increased to a smaller extent in pre-pubertal or early pubertal boys compared to men.[67] Both boys and girls (12 years old) have also exhibited an increased $CD56^{bright}$: $CD56^{dim}$ ratio during recovery from exercise, which means the proportions of cytotoxic NK cells are lower during recovery than immediately after exercise.[68,69]

There are a few studies that have investigated the effect of exercise on NKCA in children, and most follow similar trends to adults, as well. Specifically, an increase in total NKCA occurs immediately following exercise, but it is not increased when normalized to the number of NK cells recruited.[65,70] Similarly,

Timmons and Bar-Or examined CD69 expression as a marker of NK cytotoxicity and found that NK cell function was unchanged in young girls after exercise.[68] Therefore, we see that total NKCA is increased as a result of exercise in children, but it is unclear whether or not they experience an increase in NKCA per cell.

Some results about the relationship between fitness and NKCA have also been demonstrated in children. For example, trained swimmers aged 9-17 had a stronger NK response than non-swimmers aged 9-17 after performing a Wingate test; $CD56^{dim}$ cell number increased by 490% in swimmers vs. 300% in non-swimmers and total NKCA increased by 154% in swimmers vs. 61% in non-swimmers.[70] Interestingly, the swimmers had lower total NKCA at baseline in this study. Chamorro-Viña et al. observed that the NKCA ratio (post-training/pre-training) in children undergoing allogenic hematopoietic stem cell transplantation (HSCT) was eight times greater in the group that had completed exercise regimens (three 60-minute sessions per week for ten weeks), compared to a non-exercise control group.[71] It is important to note that this is not a healthy population, which limits the generalizability of the results to healthy children.

It is clear that children have a strong NK response to exercise, but it is a smaller response than that typically observed in adults. We must understand what is causing this difference and whether it is a continuum across maturity levels, so the following section will outline how puberty impacts the relationship between NK cells and exercise.

1.7 Impact of puberty on NK response to exercise

Children's NK response to exercise may be a function of maturity. This may be demonstrated by Boas et al., who observed a trend towards increasing NK cell numbers after a Wingate test (exercising to maximum anaerobic capacity) in 9- to 17-year-old males. Specifically, they observed a 263%, 463% and 495% increase in $CD3^{-}CD56^{+}CD16^{+}$ NK cell number in pre-, peri- and post-pubertal children, respectively.[70] This demonstrates that children in earlier developmental stages have a dampened NK recruitment response. A similar trend can be seen for total NKCA: Boas et al. observed an 84%, 106% and 143% increase in total NKCA in pre-, peri- and post-pubertal children, respectively.[70] Thus, it appears as though puberty may impact the response of NK cells to exercise.

These differences in response may be due to the recruitment potential of the NK cells. There seems to be no difference between children and adults in the catecholamine response to exercise,[72] but β-adrenergic receptor density on lymphocytes is lower in childhood compared to adulthood.[73] This leads to reduced recruitment of NK cells into circulation. While this could impact recruitment and total NKCA, it does not explain NKCA per cell.

Although there are no studies on NKCA per cell in children, there are several reasons one might expect NKCA per cell post-exercise to differ between children and adults. For example, cytokines released with exercise could influence this difference. IL-2 and IL-15 are known to promote NK cell maturation, but they are produced at similar levels in adults and children.[74] Furthermore, perforin content

in CD56+ cells, which primarily consists of NK cells, appears to be similar between children and adults.[75] Levels of IFN-γ may be lower in childhood, and preincubation of NK cells with IFN-γ has been shown to facilitate their cytolysis of target cells.[14] Therefore, reduced quantities of IFN-γ may result in lower cytotoxic ability of NK cells. Another explanation could be the capacity of NK cells to upregulate CD69, whose activation triggers the cytolytic machinery of NK cells, since there seems to be a progressive trend towards increasing CD69 upregulation with age in response to stimulation with phorbol myristate acetate (PMA) and ionomycin.[76] Evidently, there are many possible explanations for the differences seen between children and adults, but the conclusive mechanism(s) remains unknown.

2. Gaps in the literature

Although many aspects of the NK response to exercise are well understood, there are many knowledge gaps remaining. First, there is considerably less research on exercise immunology in children. We know that children generally follow the same trend in the NK cell number response to exercise as adults, with NK cell number increasing after exercise and decreasing during recovery, but NK function is less well understood. Also less understood is the comparative change in NK cell function between pubertal groups and the factors that lead to this effect. Moreover, there is a lack of studies investigating baseline NKCA and NKCA following a bout of exercise in children, and a lack of studies determining whether changes are occurring on a per cell basis. Thus, there is a clear need for

more research that investigates the relationship between fitness and NKCA per cell in children and adolescents.

3. Objectives

This thesis aimed to fill the gaps in the literature previously outlined. Specifically, it was designed to answer the questions:

a) Is there a difference in NKCA per cell pre- and post-exercise in pre- and post-pubertal children?
b) What is the relationship between fitness and NKCA per cell in pre- vs. post-pubertal children at baseline?
c) What is the relationship between fitness and NKCA per cell in pre- vs. post-pubertal children after high-intensity aerobic exercise?

4. Hypotheses

For objective a), I hypothesized that there would be an increase in NKCA per cell following exercise in both pre- and post-pubertal children as this has been demonstrated in adults and mechanisms like CD69 upregulation have been proposed to explain this.[45,46] For objectives b) and c), I hypothesized that higher levels of fitness would be associated with increased NKCA per cell compared to individuals with lower levels of fitness both at rest and after exercise. Although per cell increases in NKCA have not been frequently studied, previous results demonstrate that athletes or highly trained individuals have elevated total NKCA at baseline or following a bout of exercise.[49,50] Furthermore, frequent participation

in exercise is associated with upregulation of activating factors on NK cells and increased degranulation.[60] I also predicted that the relationship between fitness and NKCA would be stronger in post-pubertal adolescents than pre-pubertal children. This would align with previous results showing reduced responses to exercise in children compared to adults and adolescents, as well as the data showing CD69 upregulation and cytokine secretions contributing to cytotoxicity are different between children and adults.[70,76]

5. Methods

This project is part of a larger study, "Determining the effects of **Ex**ercise **I**ntensity and **D**uration on NK cell response at distinct stages of growth and development" (ExID), in the Child Health & Exercise Medicine Program (CHEMP). The following methods comprise only the portion of the ExID study methods that are relevant to this project.

5.1 Participant recruitment

Healthy children aged 8 to 11 years and adolescents aged 15 to 18 years were eligible to participate. They were recruited from the Hamilton area via posters, online advertisements and word of mouth. The participants' eligibility based on physical activity and medical questionnaires was confirmed in a telephone call with the children and/or parents of children under the age of 16. After recruitment, they were screened to determine their developmental status using the Tanner stages. If participants were Tanner stage 1 (pre-pubertal) or

Tanner stage 4/5 (post-pubertal), they were eligible. This was to account for the potential impact of sex hormones and to avoid difficulty controlling for different rates of development. Participants were also recreationally active, meaning they participate in organized sport 2-3 times per week, and of a normal/healthy weight, meaning they had a Body Mass Index (BMI; weight in kg divided by height in metres squared) between the 5th and 85th percentile. This was to avoid effects of systemic inflammation that can be present in individuals that are overweight/obese, which may cause NK cells to have impaired functionality.[77,78] Elite athletes were excluded as part of the ExID criteria due to the potential impact this could have on NKCA, NK proliferation, and NK responsiveness to cytokines.[49,79,80] Children with a family history of inflammatory dysregulation (i.e., asthma or allergies) were excluded because they may have increased NKCA, which could interfere with the impacts of exercise on NKCA.[81–83] Additionally, children taking any medications were excluded since these may impact NK cells. For example, contraceptives have been found to reduce NKCA in women.[84]

5.2 Study visits

Participants each attended two study visits. Each visit took place at the CHEMP laboratory in the McMaster Children's Hospital. Adolescents over the age of 16 and parents of younger children provided consent for participation, and younger participants provided assent. All participants were confirmed to be eligible based on the CHEMP lab activity and medical questionnaires.

5.2.1 Visit 1: Fitness assessment

The first visit involved screening, including the Tanner screening previously mentioned. This consisted of showing children pictures of sequential pubertal development (breast development for females and pubic hair growth for males) so they could identify the stage that looked most like them. Anthropometric measurements, such as standing height (cm), sitting height (cm), weight in minimal clothing (kg), and waist and hip circumference (cm), were also taken. All anthropometric measurements were performed a minimum of two times or until at least two measurements were within 0.5 units of each other to acquire accurate measurements. Height, weight, and BMI percentiles were calculated using the Centre for Disease Control and Prevention (CDC) calculator for each participant based on their age and sex.

Following eligibility confirmation and the measurements described above, participants completed an aerobic fitness test (i.e., maximal oxygen uptake or VO_{2max} test) using the McMaster All Out Continuous Cycling Protocol on a cycle ergometer (Lode, Groningen, The Netherlands) to measure the level of fitness in all participants. Participants wore a BioHarness to measure heart rate (HR) continuously and a mouthpiece connected to a calibrated metabolic cart (Vmax29, Sensor Medics) to measure inspired/expired gases (O_2 uptake and CO_2 output) which are used to determine VO_{2max}. The test consisted of a two-minute rest period to gather baseline HR and gas data, followed by a warm-up (cycling at a workload of 10 watts), then an increase in workload every two

minutes until exhaustion (usually around 8-12 minutes). Workload was increased by a constant increment based on participants' body weight. Pediatric specific criteria were used to determine when peak exercise had been reached. These criteria included a heart rate greater than 195 bpm, a respiratory exchange rate greater than 1.0, and/or inability to maintain a pedaling cadence of 60 to 80 revolutions per minute in spite of strong verbal encouragement. VO_{2max} was defined as the highest volume of oxygen uptake over a 20-second period, normalized to body weight (mL/kg/min). Ventilatory threshold (VT) was defined as the point during exercise when VCO_2 starts to increase at a faster rate than VO_2, and is expressed as the VO_2 at this point, normalized to body weight (mL/kg/min). These values were used to standardize the intensity of exercise at subsequent visits.

5.2.2 Visit 2: High-intensity interval exercise

At the second visit, participants completed a high-intensity interval cycling protocol. This protocol was chosen because it is the most likely to elicit the strongest response from NK cells (compared to moderate exercise), thereby determining the largest extent to which the NK cells can respond to exercise. Intermittent exercise is also more characteristic of the natural activity patterns of children,[85] so using this protocol better represents how activity in their daily lives may impact their immune function.

Several potentially confounding factors were controlled in this study visit. Participants were told to abstain from eating and drinking anything except water

for four hours prior to the exercise visit and abstain from strenuous physical activity the day before and the day of the visit. Additionally, participants were confirmed to have no signs of illness or infection. All visits were completed in the afternoon (after school) to minimize effects of diurnal fluctuations in stress hormones, which can impact NK cells.[86–88]

Prior to completing the exercise protocols, participants were asked to rest in a supine position so an indwelling catheter could be placed in the antecubital region of the arm for blood draws. Blood draws were taken before exercise, immediately after completing the exercise protocol, and after 30 and 60 minutes of recovery from exercise. A plasma (EDTA) and serum tube were collected at each draw and the indwelling catheter was flushed with saline to prevent clotting and sample contamination from previous time-points. These blood samples were later used for analysis of NK cell populations.

The high-intensity interval protocol consisted of 20 intervals of 15 seconds of cycling with one minute of rest between each interval. Therefore, participants exercised for a total of five minutes over 30 minutes. The intensity of each exercise interval to be completed was calculated using the participant's VO_{2max} and VT data from the first visit and was set at a VO_2 that was 25% of the way between the participant's VT and VO_{2max}. This was calculated using **Equation 1.**

$$VO_2 \text{ at VT} + 0.25(VO_2 \text{ at } VO_{2max} - VO_2 \text{ at VT}) \quad \textbf{(1)}$$

Similar to the first visit, participants wore a HR monitor so that HR could be continuously recorded. Participants were also asked for their rating of exertion on

a scale of 6 (no exertion at all) to 20 (maximal exertion) using the Borg's Rating of Perceived Exhaustion (RPE) every five minutes throughout the exercise. HR and RPE were both used to verify exercise intensity.

5.3 Fitness analysis

The data for the fitness analyses was taken from the participants' first visit where they completed the VO_{2max} test. We chose to use VT, or the point in exercise where a person begins relying primarily on anaerobic metabolism to sustain their exercise, as a measure of aerobic fitness. It can be more informative than VO_{2max} because it describes the highest level of exercise a person could sustain for a long period of time, rather than the maximal exercise they can reach for a very short time.[89]

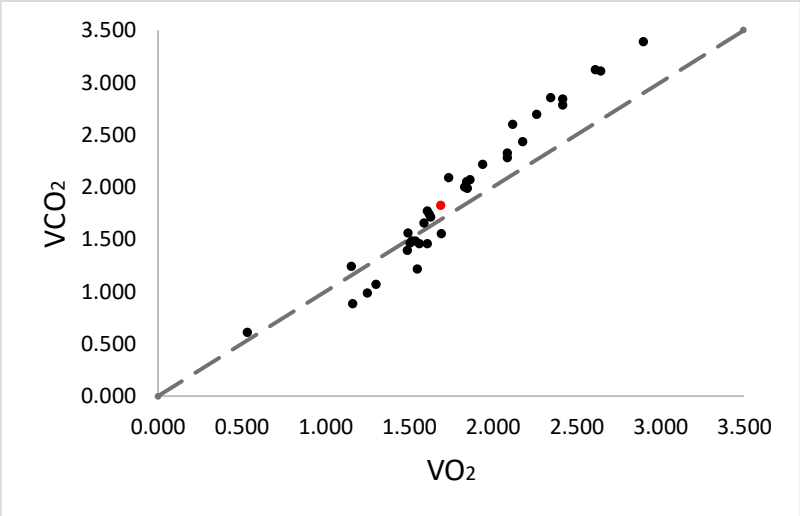

Figure 1: *Example plot demonstrating how to calculate VT based on a participant's plot of VO_2 vs. VCO_2 taken during their VO_{2max} test. The red point indicates the VT as it is the first point after which all points are above the trendline of x=y.*

VT is determined using V-slope methodology,[90] wherein VO_2 and VCO_2 from the participant's VO_{2max} test are plotted against each other, and the VO_2 at the

point where the data deviates from an x=y trend toward increasing CO_2 output is considered the VT (**Figure 1**). This indicates generation of excess CO_2 as increased lactate is produced due to reliance on anaerobic metabolism.[90,91]

5.4 Post-visit Blood Work

5.4.1 PBMC Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from each of the blood samples from the participants in a laminar flow hood using a protocol modified from the Bowdish lab at McMaster University.[92] Blood samples from EDTA tubes from each time-point were transferred into a pre-prepared Leucosep tube (Grenier, BioOne) containing Histopaque-1077 (a sterile solution of polysucrose and sodium diatrizoate with a density of 1.077 g/mL). Samples were then centrifuged to isolate PBMC. PBMC number and viability from each time point were quantified using a Cell Countess (Fisher Scientific). They were then cryopreserved in liquid nitrogen as 1 x 10^7 PMBC/mL using 12.5% FBS solution and 20% dimethyl sulfoxide (DMSO) solution, aliquoted into 1.5 mL cryovials.

5.4.2 Set up for cytotoxicity assay

The chronic myelogenous leukemia cell line K562 cells (ATCC, CCL-243) were used as target cells due to their lack of MHC and relative resistance to mutations. This means only NK cells will be responsible for target cell lysis when K562 cells are incubated with PBMC, which also consists of other lymphocytes and monocytes.

K562 cells were aliquoted and cryopreserved in liquid nitrogen. Based on previous methods,[93,94] target cells were thawed in a 37˚C water bath and transferred into pre-warmed K562 media (RPMI +10% FBS, 1% antibiotic, and 1% L-glutamine) in preparation for cytotoxicity experiments. K562 cells were then washed and stored in a 37˚C incubator (5% CO_2) to promote optimal growth. Cell growth was monitored daily using the Cell Countess to ensure that the concentration in each flask did not exceed 1 x 10^6 cells/mL. Cells were passaged and provided with new media every 2-3 days until needed for cytotoxic experiments since cell number doubles approximately every 24 hours.

One day before running the cytotoxic assay, PMBC from all time-points of one participant's exercise visit were removed from the nitrogen tank and washed using pre-warmed PBMC media (RPMI + 10% FBS, 1% antibiotic). PBMC were then aliquoted into a T25 and stored in the 37˚C incubator for 12-18 hours.

5.4.3 Cytotoxicity assay

NK cytotoxicity was measured by co-culturing K562 cells labelled with DiO, a fluorescent probe that binds the lipid bilayer of live cells, and PBMC at 37˚C and 5% CO_2 in a 1:50 target: effector ratio. This ratio was chosen based on previous optimization assays completed in the CHEMP lab. Each tube contained 1 x 10^4 K562 cells and 5 x 10^5 PBMC. Tubes were centrifuged and incubated as a pellet for four hours at 37˚C. The amount of target cell lysis after the four-hour incubation period was measured by staining K562 cells with Fixable Viability Dye (FD) eFluor™ 780 (Thermo Fisher Scientific), where only dead cells would stain

positive. This was compared to spontaneous cell death in a sample consisting of only 1 x 10^4 K562 cells that was incubated in the same conditions.

5.4.4 Flow cytometry analysis of cytotoxicity assay

NK cells and target cell death were quantified on a pre-calibrated and compensated MACSQuant 7-colour volumetric digital flow cytometer following the incubation. NK cells were identified as CD14- (PerCP-Vio), CD3- (VI), and CD56+ (PE). CD14 was used to ensure other cells that may be found in PBMC, such as monocytes, would be excluded from analyses. $CD56^{dim}$ cells were gated using the CD56 marker. **Figure 2** illustrates how $CD56^{dim}$ cells were gated in FlowJo. Target cells were identified as DiO+ (FITC), and lysed cells were identified as events that were positive for eFluor780 (**Figure 3**).

Outputs were analyzed using FlowJo software. Fluorescence minus one tubes (FMOs) were prepared for DiO-FITC and eFluor780. This consisted of all stains except DiO-FITC or eFluor780 being used to account for any interference of stain colours. The FMOs for these stains were used to place gates in FlowJo every time DiO or eFluor780 was gated, with the exception of spontaneous target death which used only the DiO-FITC FMO. Other gates were placed manually based on visual interpretation.

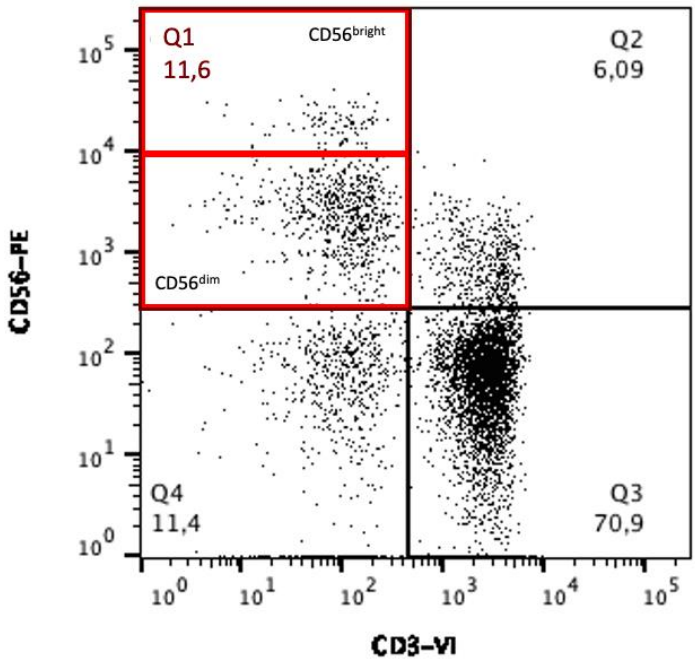

Figure 2: *Gating of NK cell subsets. Q1 (outlined by the dark red box) represents all NK cells (CD56+ CD3-) and the smaller red boxes divide NK cells into CD56^{bright} and CD56^{dim} subsets, with CD56^{dim} having lower expression of CD56.*

Total NKCA and NKCA per cell were analyzed at baseline (before exercise), after high-intensity interval exercise, and after 30- and 60-minutes of recovery. Total NKCA or specific lysis was calculated using **Equation 2.**

% of lysed K562 target cells - % spontaneous target cell death **(2)**

In order to calculate NKCA per cell, the number of K562 cells killed by NK cells was first quantified by taking the number of dead K562 cells from the K562 and PBMC tube and subtracting the number of cells that would have spontaneously died. The number of cells that would have spontaneously died is represented by **Equation 3**.

Total # K562 cells in sample x % spontaneous target death in K562 only tube **(3)**

This number was then divided by the number of CD56^{dim} cells present in the sample to achieve NKCA per cell (**Equation 4**).

K562 cells killed by NK cells/# CD56^{dim} cells present **(4)**

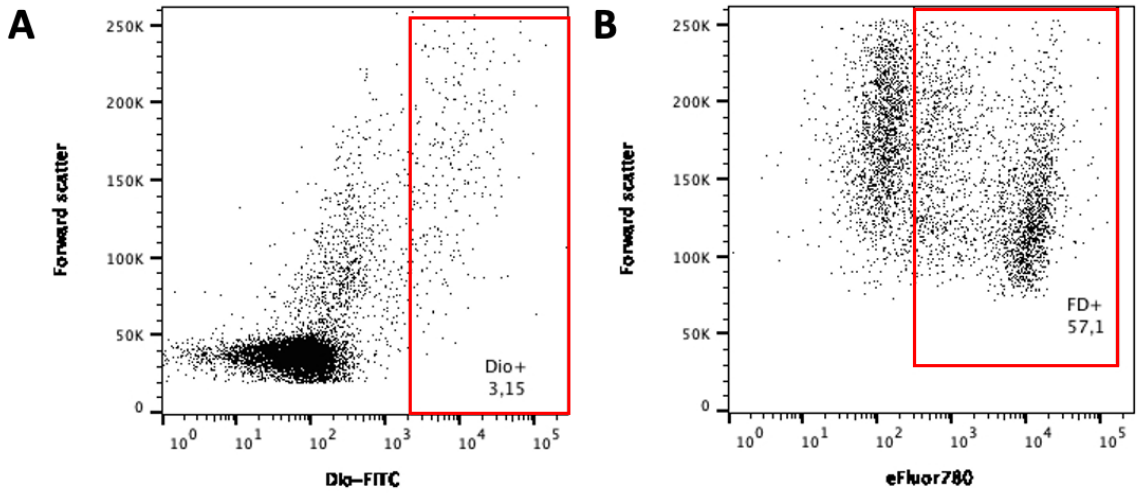

Figure 3: *Gated cells of interest for K562 lysis analysis.* ***A****. Live K562 cells stained with DiO conjugated to FITC are gated in the box on the right.* ***B.*** *Lysed K562 cells stained with eFluor780 (FD) are gated in the box on the right.*

5.5 Statistical Analysis

All data are displayed as mean ± standard deviation. The first objective of my thesis was to determine if there was an effect of exercise on NKCA per cell in children. I used a one-way repeated measures analysis of variance (ANOVA) to test whether there were differences in NKCA per cell at each time point (rest, post-exercise, 30-min recovery, and 60-min recovery). Post hoc tests using the Bonferroni correction were done when ANOVA tests showed significant differences. Effect sizes were also tested, where 0.2 was a small effect, 0.5 was a medium effect, and 0.8 was a large effect.[95] Since there were no observable differences in NKCA per cell between rest (pre-exercise) and 30- or 60-min

recovery, only the pre- and post-exercise timepoints were used for other analyses. The other objectives of my thesis were to examine the relationship between fitness and NKCA per cell in pre- vs. post-pubertal children both at baseline at after exercise. This was assessed with a multiple regression (independent variables: fitness [VT, mL/kg/min] and pubertal status [pre- vs. post-pubertal]; dependent variable: NKCA per cell). The multiple regression was completed for each relevant time point of the high-intensity interval exercise protocol, as described above, as well as for the change in NKCA per cell between time points. The regression was completed stepwise, where only VT was assessed as an independent variable first, then both VT and pubertal status were included in the model. All analyses were completed in SPSS (Version 27) and statistical significance was set at $p \leq 0.05$.

6. Results

6.1 Participant Characteristics

A total of seven participants were included in this study. Descriptive participant characteristics are displayed in **Table 1**. There was a fairly even split of females and males within both pre- and post-pubertal groups. Both pre- and post-pubertal children were near the median in their weight and height percentiles for their sex and age. All pre-pubertal children self-identified as Tanner stage 1, whereas three post-pubertal participants self-identified as Tanner stage 5 and one self-identified as Tanner stage 4.

Table 1: Descriptive Participant Characteristics

Participant characteristic	Pre-pubertal	Post-pubertal	All Participants	Min.	Max.
Sex	1F, 2M	2F, 2M	-	-	-
Age (years)	10.1 ± 1.3	17.1 ± 1.0	14.1 ± 3.9	8.6	17.8
Tanner	Tanner 1	Tanner 4 (n=1), Tanner 5 (n=3)	-	-	-
Height percentile	53.7 ± 13.4	43.2 ± 31.7	47.7 ± 24.4	1.9	77.0
Weight percentile	48.6 ± 22.2	65.3 ± 20.5	58.2 ± 21.3	23	89.4

Values reported as mean ± standard deviation.

6.2 Fitness outcomes

Absolute VO_{2max} and VT were significantly higher for post-pubertal as compared to pre-pubertal participants (p=0.040 and p=0.026, respectively). When these parameters were normalized to body weight, however, pre-pubertal children displayed slightly higher levels of fitness (VT p=0.057, VO_{2max} p=0.100). Both groups had similar VT values when expressed as a percentage of their VO_{2max} (p>0.05). A summary of fitness characteristics can be found in **Table 2**.

Table 2: Participant Fitness Descriptions

Fitness Characteristic	Pre-pubertal	Post-pubertal	All Participants	Min.	Max.
Absolute VO_{2max} (L/min)	1.5 ± 0.1	2.7 ± 0.7*	2.2 ± 0.8	1.3	3.6
Relative VO_{2max} (mL/kg/min)	47.1 ± 7.0	39.7 ± 2.6	42.9 ± 6.0	36.5	53.1
Absolute VT (L/min)	0.9 ± 0.1	1.5 ± 0.3*	1.3 ± 0.4	0.9	1.8
Relative VT (mL/kg/min)	30.1 ± 4.2	23.1 ± 3.3	26.1 ± 5.1	19.4	34.8
VT as percent VO_{2max}	64.4 ± 8.7	58.4 ± 8.6	61.0 ± 8.6	51.4	71.3

*Values reported as mean ± standard deviation. *p<0.05 between pre- and post-pubertal.*

6.3 Objective 1: Impact of high-intensity interval exercise on NKCA per cell

A repeated measures ANOVA determined that NKCA per cell demonstrated a significant main effect for time ($F(3,15) = 4.806$, $p=0.015$). Post hoc tests using the Bonferroni correction showed that NKCA per cell increased significantly between post-exercise (0.07 ± 0.05 lysed K562 per NK cell) and 30-minutes recovery (0.16 ± 0.08, $p=0.014$), while other pairwise comparisons were not significant. Importantly, the partial eta squared, or effect size, was 0.490.

Table 3: NKCA per cell at each time point of high-intensity interval exercise and between pubertal groups

Time point	Average NKCA per $CD56^{dim}$ cell	Pre-pubertal	Post-pubertal
PRE	0.13 ± 0.08	0.11 ± 0.05	0.15 ± 0.10
POST	0.07 ± 0.04	0.09 ± 0.07	0.05 ± 0.01
REC1	0.16 ± 0.08	0.17 ± 0.11	0.15 ± 0.05
REC2	0.25 ± 0.19	0.19 ± 0.18	0.30 ±0.22

Values reported as mean ± standard deviation

Table 3 shows the average NKCA per cell at each time point. The average NKCA per cell between pubertal groups can also be seen in **Table 3**, where no significant differences between pubertal groups were observed ($p>0.05$). **Figure 4** shows the NKCA per cell values from each participant alongside the average values for each time point.

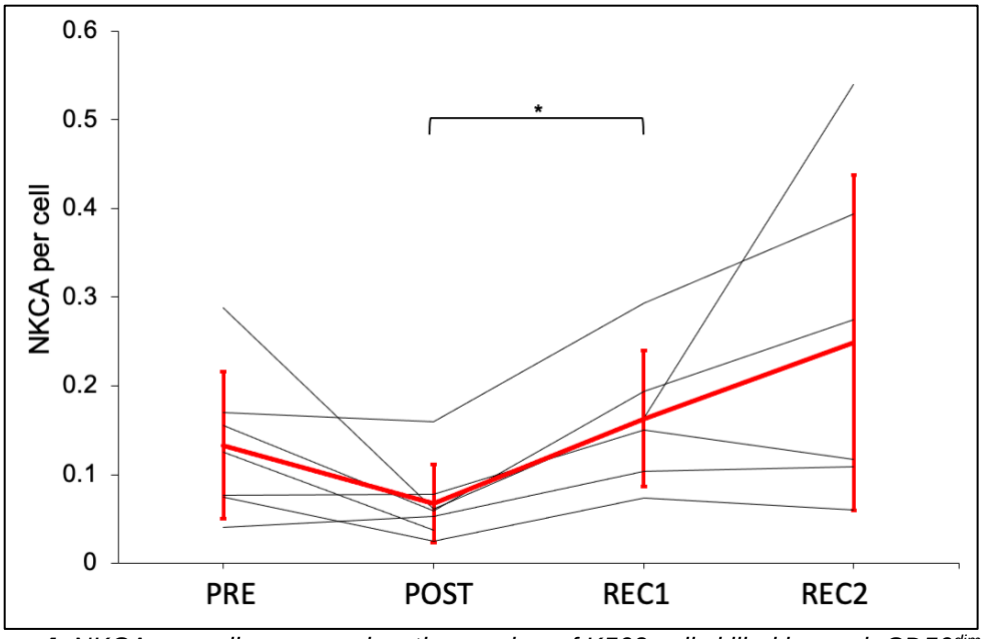

Figure 4: *NKCA per cell expressed as the number of K562 cells killed by each CD56^{dim} cell before exercise (PRE), immediately after exercise (POST), after 30-minutes of recovery (REC1), and after 60-minutes of recovery (REC2). Thin black lines represent individual participant data, and the thicker red line is the average of all participants. Error bars represent the standard deviation of the data. *p<0.05.*

Interestingly, these trends differed greatly from total NKCA (i.e., not normalized to the number of cells present) or specific lysis. **Figure 5** shows the specific lysis of each participant, as well as the average values for each time point. Specific lysis also had a main effect of time ($F(3,15) = 6.485$, $p=0.005$), but no significant differences were seen between any of the time points. A trend towards a decrease between 30- and 60-minutes recovery was observed, however ($p=0.057$). Specific lysis also had a medium effect size of 0.565.

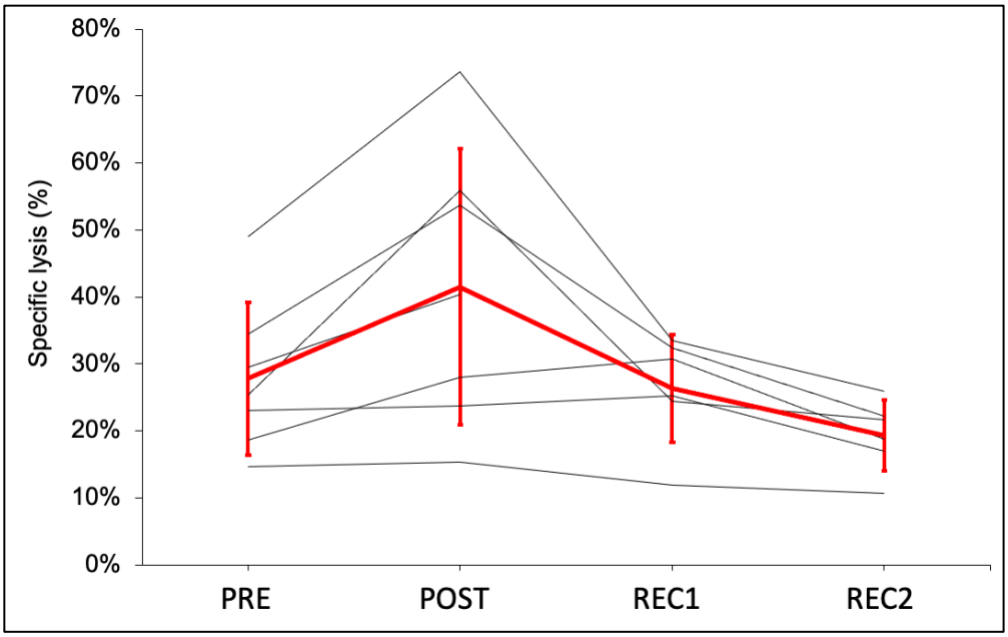

Figure 5: *Specific lysis expressed as the percentage of K562 cells killed by all NK cells before exercise (PRE), immediately after exercise (POST), after 30-minutes of recovery (REC1), and after 60-minutes of recovery (REC2). Thin black lines represent individual participant data, and the thicker red line is the average of all participants. Error bars represent the standard deviation of the data.*

6.4 Objective 2: Relationship between fitness, puberty and NKCA at baseline

A multiple regression was run to predict NKCA per cell from relative VT (mL/kg/min) and pubertal status (pre- vs. post-pubertal). Neither variable significantly predicted NKCA per cell, $F(2,4)=0.196$, $p>0.05$, $R^2=0.089$ (**Tables 4** and **5**). **Figure 6** shows the distribution of data points when plotting VT against NKCA per $CD56^{dim}$ cell at baseline.

Table 4: Summary of hierarchical linear regression results at baseline

Model	R^2	Adjusted R^2	Std. Error	F	Df1	Df2	Sig.	SS	MS
Model 1: Fitness (VT)	0.061	-0.127	0.088	0.324	1	5	0.594	0.003	0.003
Model 2: Fitness (VT) Puberty	0.089	-0.366	0.097	0.196	2	4	0.829	0.004	0.002

Table 5: Coefficients table from hierarchical linear regression at baseline

	Unstandardized Coefficients		Standardized Coefficients			95% Confidence Interval	
Model	**B**	**Std. Error**	**Beta**	**t**	**Sig**	**Lower Bound**	**Upper Bound**
Model 1:							
(Constant)	0.218	0.154		1.421	0.214	-0.176	0.613
Fitness (VT)	-0.003	0.005	-0.247	-0.569	0.594	-0.017	0.011
Model 2:							
(Constant)	0.140	0.279		0.501	0.643	-0.635	0.914
Fitness (VT)	-0.001	0.008	-0.079	-0.117	0.912	-0.024	0.022
Puberty	-0.038	0.107	0.238	0.354	0.741	-0.259	0.334

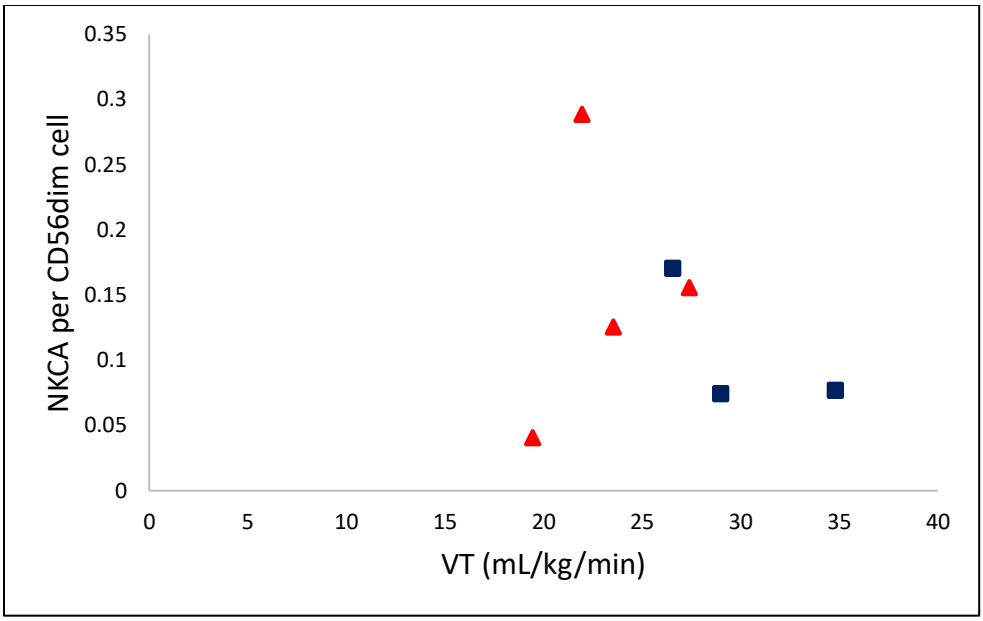

Figure 6: *Distribution of data points when plotting VT (ml/kg/min) against NKCA per cell at baseline. Navy blue squares are pre-pubertal participants and red triangles are post-pubertal participants.*

6.5 Objective 3: Relationship between fitness, puberty and NKCA after exercise

Similar to baseline or before exercise, a stepwise multiple regression predicting NKCA per cell from VT and pubertal status showed that neither variable significantly predicted NKCA per cell after exercise, $F(2,4)=0.560$, $p>0.05$, $R^2=0.219$. No visible trend can be seen when VT is plotted against NKCA per cell after high-intensity interval exercise (**Figure 7**).

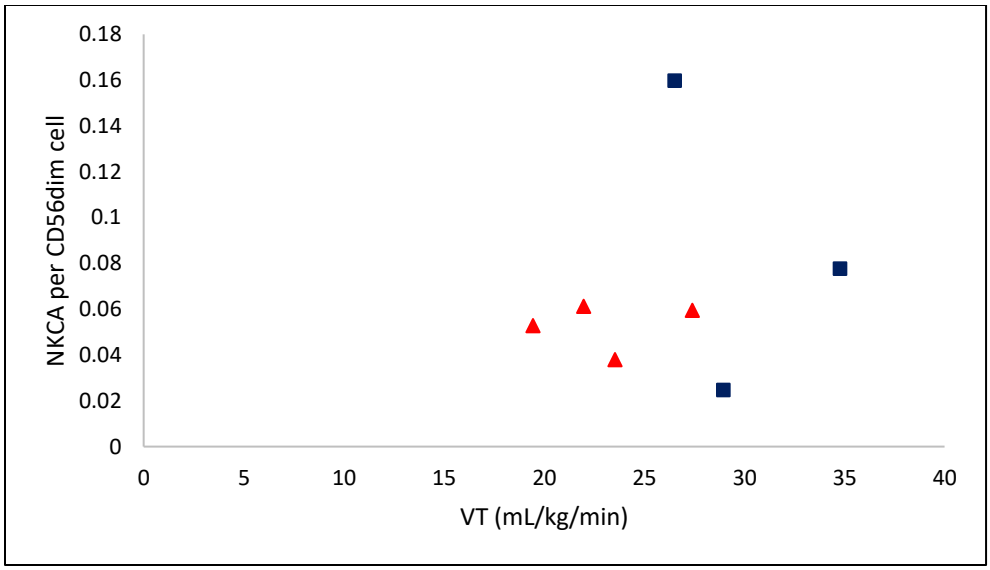

Figure 7: *Distribution of data points when plotting VT (ml/kg/min) against NKCA per cell immediately after high-intensity interval exercise. Navy blue squares are pre-pubertal participants and red triangles are post-pubertal participants.*

The NKCA per cell post-exercise is not necessarily a meaningful measurement because it does not take initial NKCA per cell into account. Therefore, we studied the change in NKCA with exercise. The change in NKCA per cell between time points was also not predicted by VT or pubertal status in linear regressions. Specifically, the change from PRE to POST, POST to REC1, REC1 to REC2, PRE to REC1, POST to REC2, and PRE to REC2 were all analyzed, and none were predicted by VT or pubertal status (all $p>0.05$). The distribution of data points in the change from PRE to POST time points can be seen in **Figure 8.** Results were not significant ($F(2,4)=0.734$, $p>0.05$, $R^2=0.268$). A summary of the model for the change PRE to POST is displayed in **Tables 6** and **7**.

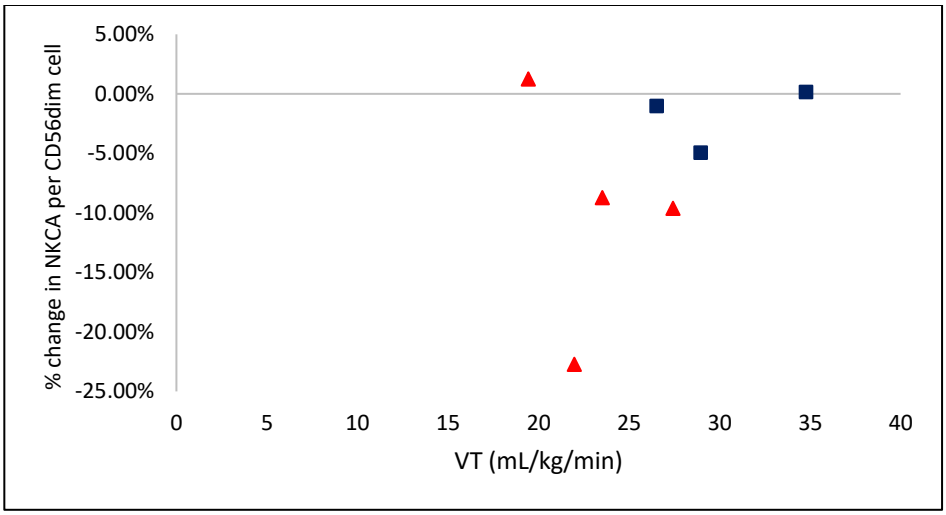

Figure 8: *Distribution of data points when plotting VT (ml/kg/min) against percent change in NKCA per cell from before to immediately after high-intensity interval exercise. Navy blue squares are pre-pubertal participants and red triangles are post-pubertal participants.*

Table 6: Summary of hierarchical linear regression results for the change pre- to post-exercise

Model	R^2	Adjusted R^2	Std. Error	F	Df1	Df2	Sig.	SS	MS
Model 1: Fitness (VT)	0.105	-0.074	0.086	0.587	1	5	0.478	0.004	0.004
Model 2: Fitness (VT) Puberty	0.268	-0.097	0.087	0.734	2	4	0.398	0.011	0.006

Table 7: Coefficients table from hierarchical linear regression for the change pre- to post-exercise

	Unstandardized Coefficients		Standardized Coefficients			95% Confidence Interval	
Model	B	Std. Error	Beta	t	Sig	Lower Bound	Upper Bound
Model 1:							
(Constant)	-0.178	0.150		-1.185	0.289	-0.563	0.208
Fitness (VT)	0.004	0.005	0.324	0.766	0.478	-0.009	0.017
Model 2:							
(Constant)	0.010	0.250		0.042	0.969	-0.684	0.705
Fitness (VT)	-0.001	0.007	-0.077	-0.128	0.905	-0.021	0.019
Puberty	-0.090	0.096	-0.569	-0.945	0.398	-0.356	0.175

7. Discussion

7.1 NKCA response to high-intensity interval exercise

The overall trends observed for the total NKCA response to exercise are very similar to what would have been expected based on the literature. Many other studies have shown that total NKCA increases directly following exercise, then subsequently decreases to baseline or slightly below baseline in a period of recovery.[32,39,40] This appears visually to be similar to what we observed in the current study, but although a main effect of time was noted, differences between time points were not significant. This could be because children are known to have a smaller NK responses to exercise than adults,[67] and therefore any changes observed may not have been large enough to reach significance. It may also be explained by the large amount of variability observed between participants, making trends harder to detect. Furthermore, there were only seven participants, which may have limited the ability to detect significant changes. We observed a medium effect size of 0.565, which suggests the potential of a meaningful change in total NKCA, as this means the effect is large enough to be visible to the naked eye.[95]

Although several studies have previously found that NKCA per cell increases after exercise in adults, they did not all find increases at the same timepoints. Some have found an increase directly after exercise,[45,46] while others observed an increase in NKCA per cell during recovery from exercise.[43,47] Our results align more closely with the latter, as we saw no change from pre- to post-exercise,

followed by a significant increase from post-exercise to 30-minutes recovery. The effect size for NKCA per cell was also medium, again suggesting the potential of a meaningful change. With that said, there are some differences in protocols between our study and previous literature observing an increase immediately after exercise that should be noted. For example, Bouillon et al. normalized NKCA to all NK cells, not $CD56^{dim}$ cells only.[45] This could have impacted measurements because exercise preferentially recruits $CD56^{dim}$ cells, causing our results to be different. Field et al. normalized NKCA to NK cells defined only by expression of CD16, which also differs from our methods and may partially explain the disparity in results.[46] An increase in NKCA per cell directly after exercise may have been expected because more mature subsets of NK cells that express killer immunoglobulin-like receptors (KIR) are recruited into circulation,[47] and expression of KIR is proportional to NKCA against HLA-deficient cell lines like K562.[96] However, the decrease or maintenance of NKCA per cell following exercise that we observed does line up with some other studies, who proposed that this may occur because catecholamines produced by exercise suppress NK function.[32] For example, we know that epinephrine is produced during exercise, and this hormone may suppress NKCA.[97]

As previously mentioned, the increase in NKCA per cell in recovery aligns with results observed by Nieman et al. and Bigley et al., who found that NKCA per cell increased in adults after a period of recovery.[43,47] Importantly, Bigley et al. only found NKCA per cell increases in HLA-expressing cell lines such as

U266 cells, so their results may not be comparable to this study.[47] This increase during recovery may be attributed to cytokines produced by CD56^{bright} NK cells. It is well known that proportions of CD56^{bright} cells increase during recovery from exercise,[68,69,98] and these cells are primarily responsible for producing cytokines such as TNF-α and IFN-γ.[9] Cytokines TNF-α and IFN-γ have been shown to improve the cytolytic function of NK cells,[14] although this was tested in cell lines that express HLA, unlike K562 which was used here, and concentrations of cytokines used were much higher than would naturally be produced in response to exercise. Thus, this may not be a complete explanation for the increase in NKCA observed. However, these are only a few cytokines that may affect NK function after exercise. Further research is required to determine the effects of other exercise-induced cytokines in children.

The increase in NKCA during recovery could also be attributed to differential redeployment of NK cells to tissues. It is well known that NK cell numbers in circulation decrease during recovery after exercise because the cells are being redeployed to other tissues like the lungs for functions like tissue repair.[99] However, not all NK cells are redeployed equally. Within the CD56^{dim} subset, cells express different activating and inhibitory receptors. NK cells expressing the activating receptor NKG2C are not distributed to other tissues as readily as other NK cells, possibly because they are less responsive to catecholamine stimulation.[100] This means there are more NKG2C+ NK cells remaining in circulation during recovery from exercise.[47] These cells are highly cytotoxic.

Since more cytotoxic cells remain in circulation, this may explain the increase in NKCA per cell seen after a short recovery period. However, NKG2C mainly acts by binding to type I HLA,[47] which is not present on the K562 cells we used, so this may not be a complete explanation for the increase in NKCA per cell during recovery.

It is also important to note that while there was a significant increase in NKCA per cell from post-exercise to 30-minutes recovery, NKCA per cell during recovery did not differ significantly from NKCA per cell before exercise. This may mean that exercise had little effect on improving NKCA per cell from baseline values, at least towards K562 cells, an HLA-deficient cell line. This aligns with results observed by Bigley et al., who noted adults' NKCA per cell towards K562 cells was not altered after a 30-minute bout of exercise at 15% above participants' lactate threshold.[47]

7.2 Relationship between aerobic fitness, pubertal status, and NKCA per cell

This is the first study to my knowledge that assesses the relationship between the fitness of children, pubertal status and NKCA on a per cell basis. No significant relationships were observed, but the findings still form a good basis for comparison for future work.

At baseline, there was no consistent trend between points when VT was plotted against NKCA per cell, but notably four points do seem to follow a positive linear trend and three of these points are from the post-pubertal group.

This may suggest that pre-pubertal participants have more variation in the relationship between VT and NKCA per cell than do post-pubertal participants. However, since the results were not significant and we had a limited number of participants, nothing can be concluded. In the plot of VT vs. the change in NKCA per cell from pre- to post-exercise, it appears as though there is potential for a linear trend with increasing VT correlating with a smaller decrease in NKCA per cell. However, results again were not significant so nothing can be concluded.

The lack of a relationship between puberty and NKCA per cell may be explained by previous studies showing similarity between pre- and post-pubertal children's NK cells. Mahapatra et al. showed that overall differences in NK phenotypes were mainly observed between children and adults, with much less variability occurring between pediatric groups (5-10 years old, 11-15 years old, 16-20 years old).[76] This would align with our results as they may indicate pre- and post-pubertal children do not respond significantly differently to exercise. However, this group also found that CD69 expression upon stimulation with PMA and ionomycin progressively increases with age, which would suggest that NKCA per cell should follow the same trend.[76] It is possible, however, that this trend was not significant enough or the stimulation with exercise (as opposed to PMA and ionomycin which are known to maximally activate NK cells) did not cause enough of a change to be detected. Timmons and Bar-Or found CD69 levels remained constant in children after exercise,[68] which may have occurred here, too. Other studies have also suggested total NKCA should progressively

increase with age following stimulation such as exercise,[67,70] so it is possible that the results obtained here are not entirely accurate due to limitations of the study, which will be further discussed later.

The lack of a relationship between aerobic fitness and NKCA per cell may be attributed to the smaller response children have to exercise compared to adults. Each bout of exercise seems to cause a small, possibly insignificant as in the case of this study, change in NK numbers and NKCA in children. Thus, repeated bouts of exercise leading to increased aerobic fitness may not have an added effect if there is no significant effect of exercise on NK cells in children in the first place. On the contrary, Ronsen et al. showed that CD69 expression on NK cells was decreased after more than one bout of exercise was completed on the same day.[101] Although this is not equivalent to increased fitness, it might indicate that more frequent training does not improve the NKCA of each NK cell.

Another interesting point that may have confounded results is the levels of fitness in each population. The pre-pubertal group had marginally higher fitness than the post-pubertal group on average ($p=0.057$ for relative VT) (**Table 2**). This means that for our participants, with increasing pubertal status, fitness generally decreases. We expected to see an increase in NKCA with increasing fitness and pubertal status, but this is very difficult to assess when the increase in one variable is correlated with a decrease in another. Consequently, this may explain why no significant correlation was seen. With that said, we performed a stepwise multiple regression, and even when only VT was included as an independent

variable no relationship was observed. Thus, this inverse relationship between pubertal status and fitness was likely not the main reason we did not observe a correlation, and possible limitations of the study likely played a larger role.

7.3 Limitations

Several factors relating to this study may have hindered our ability to assess whether a relationship exists between the studied variables. In particular, the small number of participants was an important limitation as only seven participants were included in these analyses. Even if trends could be observed in the data, they were underpowered to detect significance and not completely representative of the population because of the very small sample size. Moreover, some participants were missing data, which further hindered our ability to assess trends. Specifically, one participant only had blood samples collected at the PRE and POST timepoints, meaning they could not be included in any analyses that investigated the recovery timepoints. As well, if one participant was an outlier, this would have a much greater impact on the results because of such a small dataset. For example, one participant seemed to be the farthest from the trends seen with other points; they had relatively low VT but the highest NKCA per $CD56^{dim}$ cell at baseline and the largest decrease in NKCA per cell between pre- and post-exercise. Although we cannot be certain how this impacted our data, we know that having a very small number of participants limits our ability to see the complete picture.

Participants included in this study were also quite homogenous in their levels of fitness. This study was completed as part of a larger study that excluded elite athletes. This limited the range of fitness values present in our dataset because elite athletes are likely to have higher levels of fitness than recreationally active children.[102] All participants were deemed to be recreationally active, meaning they participated in exercise several times each week. This also excludes a sedentary population of children that likely would have had much lower levels of fitness. Thus, the majority of participants included had similar fitness levels, with VT values ranging from 19.4 to 34.8 mL/kg/min. The narrow range of fitness values likely limited our ability to accurately determine whether individuals with different levels of fitness have different NKCA per cell. Previous studies that have shown relationships between aerobic fitness and NKCA have often included elite athletes,[49,50,60] including the sole study in children which compared swimmers to non-swimmers.[70] This further reinforces the importance of including individuals with broad ranges of fitness in these analyses.

Finally, sex differences were not accounted for in this analysis. Some studies[84,98] have shown that males and females have different NK responses to exercise, so our results obtained by grouping males and females together may not have accurately represented these differences. Post-pubertal females were also not tested at a consistent phase of their menstrual cycle, which may have impacted results as women in the luteal phase may have lower NKCA.[103]

7.4 Future directions

The results found in this study are somewhat limited, but they provide an excellent platform on which to base future research. This section will outline future directions that should be investigated to better understand the impact of a bout of exercise on NKCA per cell in children, and whether fitness or pubertal status impact this.

First, one of the major limitations of this study was the small number of participants and low level of diversity in fitness of these participants. In order to accurately determine whether any relationships between fitness and NKCA per cell are present, we would have needed a much larger sample size and larger variability. Therefore, future studies should include more participants as well as participants with a wide range of fitness values and lifestyles, from sedentary children to elite athletes.

It could also be valuable to see how the results of this study compare to results that may be found in other populations. For example, future studies could investigate how the NK response of healthy children compares to those with chronic conditions or cancer. This may be especially useful for cancer patients as increased NKCA could be a desirable mechanism for reducing tumour volume. Some research in this area has already been done as cancer patients have been proven to develop increased total NKCA following exercise training.[104–107] Our study provided a greater understanding of how NKCA per cell can be modulated and may make research into potential treatments that target tumours more

precise and thorough by demonstrating which factors may or may not influence a person's ability to improve their NKCA per cell. It would also be interesting to determine how these results compare to adults. Although previous studies have shown adults have stronger NK responses to exercise than children,[67] these studies did not specifically determine how NKCA per cell was affected. This would also put the impact of puberty into perspective by examining how NKCA per cell differs along the continuum from childhood to adulthood. Therefore, we could better understand how development affects the immune system.

An alternative way to measure the connection between aerobic fitness and NKCA per cell would be to complete a longitudinal study. Many of the longitudinal studies that have already been done looked at the change in NKCA after completing a training program over a period of time to improve their fitness.[48,51,57] This would be a great way to assess whether improvement in each individual's fitness would affect their NKCA per cell and whether an individual's NKCA per cell changes as they develop. It would also allow us to test whether implementing training programs for children would be feasible. This would be beneficial if treatment plans for improving NK function were required, as we could demonstrate that exercise training is a feasible option.

Additionally, we were limited in this study by the use of only one cell line. NK cells respond differently to different cell lines,[47] so it is unclear whether the trends observed here would also hold true against other types of cells. Future research should use cell lines that express HLA, unlike K562 cells, because NK cells may

be able to target them differently after exercise. This would be especially relevant in determining if NKCA per cell increases following exercise because Bigley et al. have suggested that the cells remaining in circulation have increased NKCA against HLA-expressing cell lines, but not HLA-null lines, due to their receptor expression.[47]

It might also be useful to gate NK cell subsets by expression of both the CD56 surface marker and the CD16 surface marker. $CD56^{dim}$ cells typically express CD16 while $CD56^{bright}$ cells do not,[9,108] so gating using both markers could make results more accurate in future analyses.

Based on the results found here, we can only hypothesize the mechanisms behind our observations. It would therefore be important to have future studies investigate the specific mechanisms of NKCA to determine how NK cells are changed following a bout of exercise, and whether they are different in more mature or more fit individuals. Factors to investigate could include CD69 expression, CD107a expression, perforin and granzyme contents in cells, and activating or inhibitory receptor expression on cells at different time points. It would also be interesting to look into the maximum activation of NK cells from each time point, which could be determined by exposing them to PMA and ionomycin.

Finally, many children and adolescents do not achieve an appropriate amount of physical activity in their daily lives.[109,110] Although no significant correlation between NKCA and fitness was observed here, more studies should be done to

determine whether a relationship does exist. If these future studies show that benefits to the immune system may ensue from higher levels of fitness, it may be possible to develop more effective and evidence-based initiatives to promote physical activity and health among youth since increased physical activity leads to higher levels of fitness.

8. Conclusion

We found that there was a significant effect of time for NKCA, both as total specific lysis and on a per NK cell basis, following a bout of high-intensity interval exercise in pre- and post-pubertal children. No correlation was observed between aerobic fitness, pubertal status and NKCA per cell, but limitations like small sample size and exclusion of elite athletes may have influenced this. This work acts as a baseline for future studies as it opens up possibilities for more research into NKCA per cell in children, as well as which variables affect it.

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