EFFECTS OF EXERCISE ON NATURAL KILLER CELLS IN CHILDREN WITH LEUKEMIA

EXAMINING THE EFFECTS OF ACUTE EXERCISE ON NATURAL KILLER CELLS IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

By MILA BJELICA, HON. B.Sc.

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AUTHOR:	Mila Bjelica, Hon. B.Sc.
SUPERVISOR:	Dr. Joyce Obeid
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LAY ABSTRACT

Children treated for leukemia have weak immune systems, making them more susceptible to developing infections and cancer recurrence. Natural killer cells are a special immune cell that is very effective at combatting cancer and infections; however, children treated for leukemia have very low amounts of natural killer cells and they do not function well. Exercise is a simple way to boost the immune system in healthy adults and children, by increasing the number and function of natural killer cells. We don't know what effect exercise has on natural killer cells in children with leukemia. Previous studies looking at the effects of exercise on the immune system of children with cancer have not been able to recruit enough children to participate. Therefore, it is also important to investigate why children with cancer may not want to participate in exercise studies looking at immune function. The main goals of this thesis were to assess how likely we are to recruit enough children being treated for leukemia to participate in a study looking at how exercise changes natural killer cells, if our participants enjoyed being part of this study, and how safe exercise is for children being treated for leukemia. We also wanted to learn about how natural killer cells respond to exercise in children being treated for leukemia.

We found that most of the children and families that decided not to participate in our study felt they did not have time, and the second most common reason for not participating was because the children experienced anxiety surrounding blood draws for the study. The children that decided to participate in

the study enjoyed the exercise and being in the study. We also found that the exercise was safe. Finally, we saw that exercise was able to increase natural killer cell numbers and function in some, but not all, children treated for leukemia. The results of this study suggest that exercise may be a realistic and safe way to improve immune function in some children with leukemia.

ABSTRACT

Children treated for acute lymphoblastic leukemia (ALL) are immunodeficient and therefore at an increased risk of infection and cancer recurrence. Natural killer (NK) cells are a subset of lymphocytes that are very efficient at combatting infections and cancer; however, children treated for ALL have impaired NK cell number and function. Exercise has the potential to bolster NK cell number and function, at least in healthy children and adults. Limited evidence suggests exercise may also have beneficial effects on NK cells in children treated for cancer. However, these previous exercise immunology studies in children with cancer have yielded low sample sizes. Therefore, the aim of this study was to assess the: 1a) feasibility, 1b) acceptability and 1c) safety of performing an exercise intervention in children with ALL. The secondary objectives were to assess the 2a) effects of acute exercise on NK cell number, function and receptor expression in children receiving maintenance therapy for ALL compared to healthy children, as well as to 2b) assess how the NK response changes over 4 months of therapy, and to 2c) assess the link between physical activity and NK cell number and function at rest in children receiving maintenance therapy for ALL.

Children undergoing maintenance therapy for ALL (n=4) were recruited from McMaster Children's Hospital, and healthy sex and pubertal-status matched children (n=4) were recruited from the Hamilton community. ALL patients completed a total of 3 exercise visits, occurring monthly after their regularly

scheduled chemotherapy session. At each exercise visit, children were asked to complete 30 minutes of continuous biking, followed by 1 hour of rest. Blood samples were drawn at rest prior to exercise (PRE), immediately after exercise (POST) and 1 hour into recovery (REC). Healthy children only completed one exercise visit. During recovery, participants were asked to complete a physical activity enjoyment scale (PACES) questionnaire and a structured interview in order to assess exercise acceptability and to gauge participant feedback on study components, respectively. Participants were outfitted with an accelerometer to track physical activity levels between visits. Feasibility was assessed by tracking recruitment statistics, study completion rates and exercise completion rates. Acceptability of accelerometer wear was assessed by tracking accelerometer wear and log rates. Safety was assessed by tracking adverse events. All parameters were reported using descriptive statistics.

We approached 22 patients to participate, and 4 children completed the study (100% completion rate) out of a goal of 15. Primary deterrents to participation were that patients and families did not want to extend time spent at the hospital or had time restrictions and that patients were uncomfortable with blood collection methods. Exercise was feasible (94% exercise completion rate), acceptable (4.2 ± 0.38 out of 5 PACES score), and safe. Accelerometer wear rates (61.9% (range 3.7-100.0%)) and log completion rates (69.0% (25.9-100.0)) were moderate. Exercise transiently increased NK cell number and function in healthy children and some children with ALL. There were no patterns in the

change of the NK cell response to acute exercise over time. We were unable to assess the link between physical activity and NK cells due to a paucity of data. This study cautiously suggests that exercise is a feasible, acceptable and safe intervention that may increase NK cell number and function in children treated for ALL.

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LIST OF ABBREVIATIONS

%ile	Percentile
CD	Cluster of differentiation
CXCL	CXC chemokine ligand
DNAM-1	DNAX accessory molecule 1
FMO	Fluorescence minus one
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
KIR	Killer-cell immunoglobulin like receptor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
NK	Natural killer
NKG	Natural Killer Group
PA	Physical activity
PACES	Physical activity enjoyment scale
PMA	Phorbol 12-myristate 13-acetate
S1P	Sphingosine-1-phosphate
TGF-β1	Transforming growth factor beta 1
TGIT	T cell immunoglobulin and ITIM domain
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
YPHV	Years to and from peak height velocity

FORMATTING AND ORGANIZATION OF THIS DOCUMENT

This thesis was prepared in a “sandwich thesis” format, as defined by McMaster University School of Graduate Studies Guide for the Preparation of Master’s and Doctoral Theses. Chapter 1 will serve to introduce the background and context for the research performed. Chapters 2 and 3 are two separate original research manuscripts, which are prepared for publication. Chapter 4 shows findings addressing thesis objectives that were not included in the manuscripts prepared for publication. Chapter 6 provides a detailed discussion of study findings and directions for future research. Finally, Supplementary Chapter A contains additional relevant methodological details not included in the manuscripts due to journal-specific restrictions. Supplementary Chapter B includes the Parent Study Consent Form, followed by the Child Assent Form.

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CHAPTER 1: INTRODUCTION

1.1 A BRIEF OVERVIEW OF PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer. [1,2] Approximately 400 children aged 0 to 14 years are diagnosed with cancer in Ontario each year, of these cases about 25% are attributed to ALL.[1,2] Diagnosis is most common in children 1 to 4 years of age and affects only marginally (1.2 fold) more males than females.[3] ALL is a quickly progressing cancer of the blood and bone marrow, that results in the overproduction of immature lymphoid cells. Diagnosis is classified based on the affected lymphoid precursor cell. In children, B-cell ALL is the most common (~85% of diagnosed children), followed by T-cell ALL (~15% of diagnosed children), and lastly, ALLs with a highly undifferentiated phenotype or of NK cell origin are extremely rare (<1% of diagnosed children).[3] These classifications can further be stratified by cytogenic analysis to determine the genetic mutations present in the blasts.[3]

As of yet, the concrete etiology of ALL, like many cancers, remains unknown. Certain genetic disorders such as trisomy 21 or ataxia telangiectasia seem to predispose children to developing ALL, although this only accounts for a small proportion of ALL patients. Environmental factors are thought to be strongly linked to ALL incidence.[4] Most prominently, it seems that infections at certain

points during immune system development may increase or decrease the risk of childhood ALL development.[4]

1.1.1 TREATMENT OVERVIEW

Currently, multidrug chemotherapy is the leading treatment option for children with newly diagnosed ALL. This regimen consists of 4 main components as detailed in **Table 1.1.1**.[5] Over the last decade, the 5-year survival rate with this mode of treatment has steadily increased and is almost at 90% in developed countries.[2] While it is important not to undermine this success, it is also critical point out the shortcomings of multi-drug chemotherapy. Notably, the agents used are non-specific, and therefore target any rapidly-proliferating cells in the body. Indeed, it is noxious to malignant cells but also healthy dividing cells.[6] Among these are: mucosal cells, hair follicles, and the bone marrow, which in turn lead to mucositis, hair loss, and myelosuppression, respectively.[6] The latter, by extension, causes a vast immunosuppression.

Table 1.1.1. Multi-drug chemotherapy treatment for newly diagnosed leukemia

Therapy blocks	**Duration**	**Goal**	**General agents**
Remission induction	4-6 weeks	Induce remission (eliminate leukemic cells)	• Vincristine • Corticosteroids • Asparaginase • Anthracycline (may be omitted in low risk)
Consolidation	6-9 months	Eliminate submicroscopic residual disease (stray or remaining leukemic cells)	• Purine analogue • Methotrexate • Cyclophosphamide • Etoposide • Cytarabine

Maintenance	2-3 years	Low dose chemotherapy to maintain remission (prevent relapse)	• Corticosteroids • Methotrexate • Mercaptopurine • Vincristine
Central nervous system directed therapy	In tandem with whole treatment or may omit during maintenance therapy	Induce CNS remission or prophylaxis	Option 1: Intrathecal administration of methotrexate +/- cytarabine and hydrocortisone Option 2: systemic administration of chemotherapy agents that can penetrate the blood-brain barrier (dexamethasone, high-dose methotrexate, cytarabine, and asparaginase) Option 3: Cranial radiation. (Uncommon, employed for very high-risk CNS relapse)

Specific sub-groups of patients such as very high-risk cases, those that do not respond to treatment or relapsed cases may be treated using different approaches (i.e., hematopoietic transplant, biologic therapies) that are not outlined here. Corticosteroids are typically prednisone or dexamethasone; Anthracyclines are typically doxorubicin or daunorubicin; Asparaginase also includes pegylated asparaginase and Erwinia asparaginase; Purine analogues include mercaptopurine or thioguanine. CNS= central nervous system. Table information from Cooper.[5]

Currently, clinicians employ risk-based stratification to help determine the least toxic but still sufficiently effective treatment option for each patient.[5] That is, based on specific clinical features (i.e., white blood cell counts at diagnosis, age, cytogenic profile, involvement of sanctuary sites), the risk of treatment failure can be estimated so that higher risk patients receive more aggressive (toxic) treatment regimens while patients with a lower risk profile receive less toxic

regimens.[5] This is an attempt to reduce the negative treatment side-effects where possible; however, even low risk groups with less aggressive interventions suffer from notable side-effects.

The most common acute side effects of treatment include fatigue, nausea, vomiting, diarrhea, abdominal pain, hair loss, mouth sores, and flu-like symptoms, to name a few. Ultimately, the organ-specific and systemic damage from this harsh treatment leads to long term side effects, evident well beyond the completion of therapy.[7] Long-term side effects may include, but are not limited to, an increased risk of developing secondary malignancies, cardiac complications, endocrine and metabolic disorders, muscular weakness, neurosensory impairments, cognitive impairments, and poor fitness.[7] It is important to underscore the length of this therapy and the accompanying long terms side effects, both of which may warrant that ALL be re-classified as a chronic disease. Therefore, it is important for key decision-makers in pediatric oncology to continually identify and implement methods to manage and alleviate these life-altering side effects.

1.1.2. EFFECTS OF ALL AND ALL TREATMENT ON THE IMMUNE SYSTEM

In healthy children, red bone marrow is the site of hematopoiesis: hematopoietic stem cells are produced, and a portion give rise to myeloid lineage cells (erythrocytes, thrombocytes, granulocytes, monocytes) or lymphoid lineage cells (B lymphocytes, T lymphocytes, Natural Killer cells). ALL develops as the

result of two or more genetic or epigenetic mutations that cause a committed lymphoid precursor cell (lymphoblast) to reproduce without maturing.[8] Clonal expansion of the malignant cell ensues, and as lymphoblasts proliferate uncontrollably, they compete with normally developing hematopoietic cells for space and resources in the bone marrow. ALL-related alterations in the bone marrow microenvironment can promote survival and proliferation of ALL cells (i.e., asparaginase production by stromal cells, increased vascularization, survival and proliferation signals from stromal cell soluble factors and direct cell-cell signaling), but also the disruption of hematopoietic stem cell niches (induced cell quiescence, competition for favorable bone marrow niches).[9] In fact, the induction of an abnormal bone marrow environment in leukemia-free mice is sufficient to induce poor hematopoiesis and the development of cancer.[9] Unlike mature lymphocytes, leukemic lymphoblasts do not function properly and are unable to respond to pathogens. From the bone marrow, leukemic lymphoblasts intravasate, circulate through the blood, and may infiltrate in organs such as the lymph nodes, spleen, liver, skin, testicles, ovaries and spinal fluid. The latter three sites are also known as sanctuary sites, where leukemic blasts can avoid eradication by chemotherapy and cause cancer re lapse.[10] Leukemic blasts can exert an immunosuppressive effect on healthy cells and evade targeting using direct and indirect methods such as T cell co-inhibition, induction of regulatory immune cells, and/or evasion of phagocytosis.[11–13]

After successful induction therapy, patients are considered to be in remission and therefore have sub-microscopic levels of malignant cells.[14] However, the chemotherapies used to treat ALL are primarily cytotoxic drugs that do not target cancer-specific pathways, and instead broadly affect proliferating cells. Cytotoxic drugs interfere with the cell cycle by damaging DNA and microtubules, and exposure to many cytotoxic drugs at corresponding cell cycle stages induces cell death or quiescence.[15] As such, cells types with higher rates of proliferation are more chemosensitive.[16,17] While it is true that a hallmark of malignant cells is quick, uncontrolled proliferation, many cells in the body actually have a shorter doubling times than leukemic cells. Most notably, immune cells in the human body can proliferate at the same or even a faster rate than leukemic cells.[16] By the same token, the bone marrow microenvironment is further ravaged by chemotherapy leading to a decrease in the amount of hematopoietic stems cells, as well as a reduced ability to give rise to progenitor cells.[18] Chemotherapy for ALL may also dysregulate bone marrow stromal cell function, inducing higher environmental concentrations of transforming growth factor beta 1 (TGF-β1).[19] In the context of the bone marrow, high levels of TGF-β1 may mediate immune function by inducing quiescence and preventing proliferation of hematopoietic stem cells, lymphoid, and myeloid progenitors.[19,20] Therefore, immune impairments persist, if not worsen, during ALL therapy.

Children treated for ALL continue to face immune impairments after the termination of therapy. Even 6 months post-therapy children treated for leukemia

present with some immune dysfunction, characterized by poor vaccine response, reduced lymphocyte proliferation, dysfunctional NK cells, leukopenia, neutropenia, and lymphopenia.[21–23] These effects seem to be linked to treatment intensity, whereby high-risk groups, that received more intensive treatment, suffer more immune impairments.[24] Interestingly, the perturbation in immune function due to childhood cancer and associated treatment may even persist well into adulthood. Indeed, adult survivors of childhood ALL have a higher incidence of infectious disease than healthy sex- and age-matched controls (RR=3.00 CI= 2.68-3.35).[25] Similarly, survivors of childhood ALL are at an increased risk of cancer recurrence, which is possibly attributable to a weakened immune system, reduced immunosurveillance, and genotoxic drug exposure. The mortality rate due to cancer recurrence in adult survivors of pediatric leukemia is second only to survivors of central nervous system tumors. In fact, 73% (0.8% per year) of mortalities among adult survivors of pediatric leukemia were due to cancer recurrence.[26]

Clearly, it is critical to prioritize patient immune health during and after therapy for ALL. A potential area for focus may be bolstering NK cells, a subset of lymphocyte that is particularly effective at cancer immunosurveillance, combatting hematological cancers, and many infections.

1.2 NATURAL KILLER CELLS

1.2.1 OVERVIEW OF NK CELL MATURATION, PHENOTYPE AND FUNCTION

Natural killer (NK) cells are a subset of innate cytotoxic lymphocytes that develop primarily in the bone marrow, but also in secondary lymphoid tissue.[27] Unlike cytotoxic T cells, NK cells are able to eliminate target cells without prior antigenic exposure and in the absence of major histocompatibility complex (MHC).[27] On average, NK cells make up about 5-20% of the circulating lymphocyte population in healthy children aged 1 to 18 years old. However, populations of human NK cells are also located in the bone marrow, lymph nodes, spleen, liver, lungs, gut, and skin.[28]

Broadly, circulating NK cell function changes with maturation and functional capabilities can be subdivided based into two principal functional types: immunomodulatory NK cells and cytotoxic NK cells. Each functional subset can be crudely characterized based on the density of surface marker CD56 expression: $CD56^{DIM}$ cells are cytotoxic and release cytolytic granules and cytokines upon activation, while $CD56^{BRIGHT}$ cells are immunomodulatory and primarily produce cytokines upon activation.[27] In healthy people, $CD56^{DIM}$ cells account for 90% of circulating NK cells and $CD56^{BRIGHT}$ account for the remaining 10%. $CD56^{BRIGHT}$ NK cells are a less mature precursor to $CD56^{DIM}$ cells. As $CD56^{BRIGHT}$ mature, they downregulate CD56 expression (to eventually become $CD56^{DIM}$), gain CD16 expression, and acquire cytotoxic capabilities.[27] There is an

extensive list of changes in receptor expression as NK cells undergo development, as illustrated in **Figure 1.2.1**.

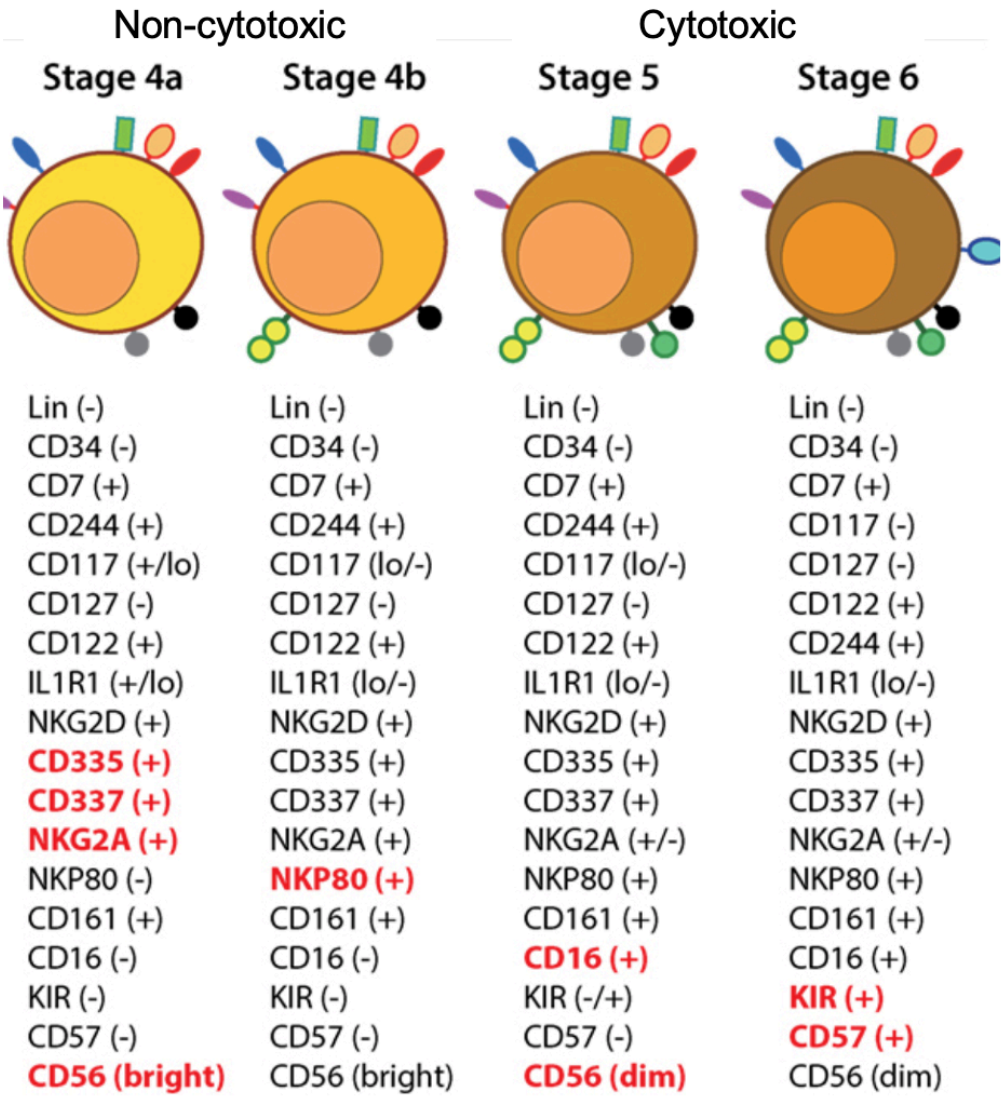

Figure 1.2.1. NK cell receptor expression at different developmental stages. Stages 1-3 (not pictured) are primarily focused on the progenitor cell's commitment to the NK lineage. Stages 4a-6 depict the stages of the committed NK cell's functional maturation. Changes in the markers highlighted in red are defining features of each stage. Adapted from Abel.[27]

Emerging evidence supports the existence of memory NK cells, which may be either immunomodulatory or cytotoxic, arising as a result of certain infections,

pregnancy, cytokine exposure and hapten exposure.[29,30] Interestingly, some, but not all, NK memory responses may even be antigen-specific, a hallmark of the adaptive immune response. There are still many gaps in our knowledge of NK memory abilities; however, the available evidence supports that these NK cells can generate a rapid and enhanced response upon re-exposure to a stimulus – a defining feature of all immune memory responses. [29,30]

It is important to acknowledge that many classically held notions are being challenged by recent discoveries in NK cell biology, and the gray area surrounding once well-defined concepts is growing. For instance, the NK cell phenotype or surface markers may not always correspond to their functional ability and maturational status.[31] NK phenotype is subject to change in a non-linear matter (i.e., NK cells with low CD56 expression that are typical of more mature cells may upregulate CD56 expression in response to cytokine stimulation).[31]This is not to say that the standard classifications of NK cells are incorrect, but rather to point out that there are exceptions to these standards, and that multiple factors can influence NK marker expression. Nevertheless, broad classifications of phenotype and function remain extremely useful for general knowledge translation.

1.2.2 NK CELL ACTIVATION

NK cell activation is primarily modulated via stimulation of activating and inhibitory receptors expressed on the surface of the NK cell. The receptors

recognize cognate ligands expressed on target cells and upon binding, may transmit activation or inhibition signals, respectively.[27] These signals are integrated, and their balance determines whether the cell will become activated or remain inactive. Activating receptors (i.e., NKp46) generally recognize a variety of ligands associated with viral infection, malignancies, and antibody-coated cells. Moreover, a subset of activating receptors may recognize proteins regularly expressed on healthy cells (e.g., Qa1 recognized by NKG2C; see **Table 1.2.2** for examples**).**[27] Activation receptors contain charged transmembrane residues. Upon ligand interaction, these residues associate with other transmembrane proteins (e.g., CD3ζ, FcεRIγ) containing immune tyrosine-based activation motifs that transduce activation signals. Meanwhile, inhibitory receptors (e.g. inhibitory KIRs) typically recognize self-molecules and proteins expressed on healthy cells such as classical MHC **(Table 1.2.2.)**.[27] Unlike the previous receptors, inhibitory receptors contain immunoreceptor-based tyrosine inhibitory motifs in their cytoplasmic portion.[27] In the absence of self-ligand induced inhibitory signals, NK cells can be activated by stimulation from activating receptors.[27] Typically, inhibitory receptor signals are dominant: in the event that both types of receptors are stimulated, the inhibitory receptor will prevent activation. However, under certain circumstances, such as infection, upregulated stress ligands that engage activating receptors can overcome inhibitory signals to induce activation.[32]

Table 1.2.2. Non-exhaustive list of the major activating and inhibitory human NK cells receptors, and examples of corresponding ligands.

Receptor	Function	Ligand
CD16	Activation	IgG (Fc portion)
NKp30	Activation and Inhibition	Gal-3, BAG6
NKp44	Activation and Inhibition	Hemagglutinin, heparan sulfate, glycosaminoglycans
NKp46	Activation	Hemagglutinin, heparan sulfate, glycosaminoglycans
KIR2DS	Activation	HLA-C
NKG2C/CD94	Activation	HLA-E
NKG2D		MICA/B, ULBP1-6
DNAM-1	Activation	CD155, CD112
KIR2DL	Inhibition	HLA-C
KIR3DL	Inhibition	HLA-B, HLA-A
NKG2A/CD94	Inhibition	HLA-E
CD85j,d	Inhibition	HLA class I
TGIT	Inhibition	CD155, CD112

NK cells express a variety of activating and inhibitory receptors. Some ligands can stimulate both inhibitory and activating receptors. Information from Lanier[33] and Chan[34].

NK cells also express cytokine and chemokine receptors that contribute to regulating NK function, maturation and trafficking.[27,35] Indeed, the presence of specific cytokines (i.e., interleukin (IL)-12, IL-15, and IL-18) can induce NK activation, or significantly lower the threshold for activation. NK^{BRIGHT} cells respond more readily to cytokine stimulation and tend to produce more cytokines than their dim counterpart.[35]

Despite being labelled as the cytotoxic subset, NK^{DIM} cells are potent producers of pro-inflammatory cytokines, especially in response to target cell activation. Indeed, when stimulated with target cells, NK^{DIM} cells produce pro-inflammatory cytokines faster than NK^{BRIGHT} cells.[35] Overall NK production of

cytokines seems to be dependent on the NK receptors engaged.[35] Both subsets prominently secrete IFN-γ and TNF-α, which serve to initiate a Th1 immune response cells and inhibit proliferation of virally infected and malignant cells.[27,35] There is evidence that NK cell also produce chemokines and chemotactic factors (i.e., CCL_3, CXCL8), regulatory cytokines (i.e., L-5, IL-10, IL-13), and growth factor (i.e., GM-CSF).[27,35]

NK^{DIM} cells store abundant pre-formed lytic granules (i.e., perforin, granzymes) in their secretory lysosome.[36] Upon recognition of a target cell, an immunological synapse forms between the NK cell and the target cell, and the secretary lysosome of the NK cell is polarized towards the synapse. The secretory lysosome then docks with the plasma membrane at the synapse prior to releasing these cytotoxic molecules.[36] In this manner, killing is limited to the targeted cell and does not damage nearby healthy cells. Interestingly, an NK cell is able to perform serial killing with the help of another death-inducing mechanism: death ligands (FAS ligand or TRAIL).[37] It seems that NK cells start killing via degranulation and will switch to using death ligands as they as they continue executing target cells.[37]

It is important to highlight that NK cell activation is mediated in an almost complimentary fashion to CD8+ T cell activation. More specifically, cytotoxic T cells require their target cells to express antigens in the context of MHC class I in order to identify them. Conversely, NK cells receive activating signals from targets with downregulated or no MHC class I, thus allowing for activation and

cytotoxic function.[27,36] Cancerous cells may downregulate MHC class I expression in order to evade T cell recognition all together, thereby making them more susceptible to NK cell killing (see NK cells in children with ALL, below).[38] Another notable receptor present on NK cells that should be emphasized is CD16 or FcγRIII. It is through this receptor that NK cells may recognize target cells coated in Immunoglobulin G (IgG) and participate in antibody-dependent cytotoxicity.[27]Binding of the FcγR induces NK cell degranulation and secretion of pro-inflammatory cytokines. [27]

1.2.3. NK CELLS IN PEDIATRIC ALL PATIENTS

At diagnosis, children with ALL are already deficient in circulating NK cells, and the existing NK cells are hypofunctional. Compared with healthy counterparts, children with ALL at diagnosis were found to have significantly lower levels of circulating NK cells (mean ± SD for ALL vs. healthy, 3.3 ± 0.33% vs 6.2 ± 0.71% of lymphocytes, $p > 0.0001$, or 255 ± 48.9 vs 442 ± 48.3/μl of whole blood, $p = 0.016$).[23] Furthermore, NK cells of children with ALL seem to present a more inhibitory phenotype than those of healthy children, exhibiting higher levels of the inhibitory receptor NKG2A (30.1 ± 2.7% vs 16.6 ± 3.5% of total NK, $p = 0.007$), but also lower levels of activating receptors NKp46 (23.3 ± 3.2% vs 55.3 ± 7.1% of total NK, $p = 0.0001$). This study reported similar expression of NKp30, NKp44, NKG2D and KIRs in children with ALL and healthy children.[23] Finally, children with ALL also tended to have a higher proportion of

NK $CD56^{BRIGHT}$ cells, the immunoregulatory phenotype, compared with healthy controls.[23] Effects of this inhibitory phenotype were reflected in NK function assays. As expected, when incubated with target K562 cells, NK cell function was impaired as evidenced by the lower levels of target cell lysis by NK cells in ALL compared to healthy children.[23] Similarly, children with ALL had lower IFN-γ secretion ($CD56^{+}/CD3^{-}/IFN\text{-}\gamma^{+}$ 1.5 ± 0.4% vs 4.2 ± 0.7%, p = 0.0005) as well as lower levels of degranulation as measured by CD107a (mean $CD56^{+}/CD3^{-}/CD107a^{+}$ 5.8±1.2% vs 13.3±2.4%, p=0.0027), although TNF-α production was comparable to values in healthy controls.[23] Interestingly, another study has suggested that NK cell impairment in children diagnosed with ALL may vary by patient age. Patients aged 1 to 9 years had lower proportions of NK cells (as a percentage of lymphocytes) than older pediatric patients, while patients aged 10 to 17 years had noticeably lower NK function on average than younger patients.[39]

Since ALL patients receive a cocktail of therapeutic agents at each timepoint, it is difficult to delineate the individual effects each drug on NK cells without using an experimental approach. Reviews of a combination of trials performed in vitro, in animal models, and in different clinical populations allows to gain some insight into how specific drugs may interact with NK cells and NK cell killing of tumor targets (outlined in **Table 1.2.3**).

Table 1.2.3. Non-exhaustive summary of the effects of ALL drugs on NK cells and cancer cell susceptibility to NK cells.

Drug class	Effects on NK cells	Effect on cancer cell susceptibility to NK cells
Glucocorticoids	Methylprednisolone: • Suppressed NKp30 and NKp44 expression [40] • Suppressed (IL-2 cultured) NK proliferation and survival [40] • Suppressed cytotoxic function mediated by NKp46 and NKG2D[40] Dexamethasone: • Effects may be dependent on cytokine milieu.[41,42] • Suppressed NK inflammatory cytokine production, cytotoxic function, in absence of IL-2 and IL-12[41] • Increased NK cytokine production, cytotoxic function, proliferation,CD16, DNAM-1 and NKG2A expression when supplemented with IL-2 and IL-12.[42] Prednisolone: • Suppressed NK cytotoxic function[43]	Unknown
Nitrogen mustards	Cyclophosphamide: • Restored NK cytotoxic function (to levels comparable to healthy controls)[44] Chlorambucil: • Suppressed NK cytotoxic function [45]	Cyclophosphamide: Increased tumor infiltration by NK cells[46]
Anthracycline	Doxorubicin: • Increased proportion of NK cells [47] • Permitted efficient NK cytotoxic function [45]	Doxorubicin: Upregulated TRAIL receptor expression on tumor cells [47]
Purine analogue	Mercaptopurine: • Induced NK cell apoptosis [45,48]	Unknown

	• Permitted efficient NK cytotoxic function [45] Cladribine: • Inhibited NK cytotoxic function [45]	
Folic acid analogue	Methotrexate: • Low doses increased NK cell cytotoxic function[48] • Toxic to NK cells[45]	Methotrexate: Upregulated FAS and TRAIL receptor expression on tumor cells[49]

Information was supplemented with drugs within the same class when data for ALL-specific drugs (mentioned in Table 1.1.1) were not available.

Overall, it is well established that children receiving therapy for ALL have reduced NK concentrations and impaired NK function.[22,23] And while understanding the individual effects of medications on NK cells is important, it is also necessary to account for the cumulative effects of these agents, which are likely best illustrated by assessing the state of NK cells in children with ALL at various time points throughout and beyond treatment. Perhaps the most detailed assessment of the kinetics of NK cell function comes from preliminary evidence from tracking NK cell activity in 5 Japanese children at multiple time points throughout induction therapy.[50] Within 2-3 weeks of the start of chemotherapy, NK function plummeted. However, at the end of induction therapy, NK cytotoxic function exceeded functional values measured at diagnosis in 4 of the 5 children (values only reported in graphs).[50] In this study, induction therapy lasted a total of 10 weeks, whereby patients receive prednisolone daily and vincristine weekly for the first 5 weeks, followed by weekly methotrexate and cranial radiation in weeks 6 to 10.[50] Glucocorticoids tend to reduce NK function, which aligns with findings at the onset of induction therapy. [41,42] Multiple factors may account for the

increased cytotoxic function seen after induction, including: a rebound effect after the cessation of corticosteroids, low dose radiation and methotrexate that may potentiate NK cytotoxicity, coupled with the elimination of immunosuppressive cancerous blasts.[48,51] It is important to note that current treatment protocols differ from those reported by Tanaka et al., therefore, the same pattern may not emerge in patients treated for ALL today (refer to *section 1.1.1)*. A more contemporary study performed in North America demonstrated that at the *end* of induction therapy, children treated for ALL showed significantly impaired NK activation and cytokine production compared to children in maintenance and healthy controls, they also demonstrated slightly lower values than children at diagnosis.[23]

In maintenance therapy, children are considered in remission, with no to minimal detectable residual cancer cells. NK cell impairments in maintenance therapy are likely due to cytotoxic drugs, as well as residual ALL- and treatment-induced changes to the bone marrow (refer to *section 1.1.2)*, though it is difficult to distinguish their exact contributions to overall NK status. The most recent and most well-powered study maintains that NK activation, as measured by CD107a expression, was low at diagnosis, lowest at induction, and comparable to healthy children during maintenance.[23] It is important to note that this study had much fewer healthy controls than children with ALL in maintenance (9 controls vs. 23 ALL).[23] Moreover, while there was no statistically significant difference in NK cell activation (%$CD107a^+$ of NK) or cytokine production between children in

maintenance and healthy children (average % $CD107a^+$ NK in ALL vs healthy children: 10.3 ± 2.19% vs 13.3 ± 2.38%, p=0.49; average IFN-γ^+ NK 2.88 ± 0.50% vs 4.23 ± 0.67%, p=0.16; average TNF-α^+ NK 3.91 ± 0.71% vs 3.43 ± 0.55%, p = 0.59), children in maintenance had a much wider range of activation, with many more falling below the lowest value reported in healthy children.[23] However, when NK cells were cultured and expanded ex-vivo, the cells of healthy children were able to lyse autologous blasts (isolated from children with ALL) and K562 cells better than those of patients in maintenance therapy (exact values not reported).[23] The inhibitory phenotype observed at diagnosis in the patient group was partially corrected in maintenance therapy.[23]

Deficiencies in NK cell function seem to persist well after therapy is completed. NK cell activity in off-therapy patients was 62.5 ± 19.9% of healthy controls.[22] When former ALL patients were stratified by off-therapy period (2-6 months, 7-12 months, 12+ months), all groups showed impaired NK function compared with healthy controls. [22] Similarly Williams et al. found that impairments in NK numbers were abnormally low at 6 months post-therapy, but comparable to healthy NK counts at 12 months post-therapy.[52] Altogether, residual negative effects of disease and therapy plague patients well beyond the end of treatment, however there is no consensus on how long impairments in number and function last.

The NK cell impairments reported in children being treated for ALL may have additional negative clinical implications. Jarosz et al. examined NK cell

levels in children with ALL and non-Hodgkin's lymphoma up to 12 months after completing consolidation therapy.[53] The authors reported that lower levels of cytotoxic NK cells tended to correlate with increased number of hospitalizations due to infection ($r = –0.4609$; $p = 0.0617$).[53] They also found that all NK cell levels remained significantly suppressed even 12 months after the cessation of intense chemotherapy in children with ALL. [53] Interestingly, this is longer than the period of immunosuppression observed in other types of hematological cancers such as non-Hodgkin's lymphoma. [53] In adults with hematological malignancies, reduced NK cell function correlated with a higher incidence of cancer recurrence after treatment.[54] Taken together, these findings suggest that without intervention, circulating NK cells in children with ALL may not be as effective at immunosurveillance, which is linked to increased health issues. Therefore, augmenting the number and function of NK cells in children with ALL may have important implications for immune health both during and beyond cancer therapy.[55]

1.3. EXERCISE AND NK CELLS

Exercise may represent one simple but potent strategy to boost NK cell number and function. In fact, NK cells are the most responsive immune cells to exercise.[56]The majority of our understanding of the effects of exercise on NK cells comes from studies conducted in adults or animals. In the section below, I highlight our current understanding of the effects of exercise on NK cells, relying on evidence from adult and animal literature, supplementing this with pediatric

data when available. Finally, I will also summarize the scarce studies that discuss the effects of exercise on NK cells in pediatric oncology patients.

1.3.1. NK CELLS AND ACUTE EXERCISE

It is widely accepted that a single bout of exercise induces a biphasic NK cell response. This phenomenon has been observed across people of all ages, sexes, and even in multiple clinical conditions.[57] During acute exercise, there is a significant increase in circulating NK cells (up to 10-fold). Upon the cessation of exercise, levels of circulating NK cells start to decrease and at 1-2 hours post-exercise, NK cell levels may even plunge below baseline levels, at least in adults. [56] However, this decrease is transient and NK cell levels typically return to baseline within 24 hours. [56] It appears that maximal NK cell deployment is achieved within 30 minutes of moderate intensity aerobic exercise and anything over 3 hours of exercise typically causes a decrease in NK cell levels. [56] Even NK cells of female breast cancer patients who had completed therapy for 3 to 6 months are responsive to acute exercise.[58] Thirty minutes of moderate intensity intermittent exercise induced an increase in circulating NK cells (70.3 ± 37.9 to 172.8 ± 118.8 cells/ μL), although NK cell values in healthy counterparts were higher at both pre- and post-exercise (108.9 ± 51.7 to 286.8 ± 134.2 cells/ μL). [58] Despite these differences, overall, the cancer group experienced a 2.46 fold increase in NK cells, while the control group experienced a comparable 2.62 fold increase in NK cells with exercise. [58]

Exercise is a form of physiological stress that causes an increase in circulating catecholamines (epinephrine, norepinephrine).[57] NK cells express the highest density of β_2-adrenergic receptors of any lymphocyte, thereby making them very responsive to changes in catecholamines.[57] In fact, as little as 70 seconds of stair climbing is sufficient to elicit a 6-fold increase in NK cell levels.[59] Interaction with catecholamines downregulates adhesion molecules on the NK cells, which allows NK cells to be deployed into the circulation, particularly from the vascular endothelium and spleen.[57] Exercise also increases cardiac output and, in turn, increases the shear stress of the flowing blood on the endothelial surface of blood vessels, leading to further NK cell recruitment.[57]

Interestingly, not all NK cell subpopulations are uniformly deployed with exercise. Rather, there is a preferential recruitment of more mature, $CD56^{DIM}$, cells with exercise. More mature $CD56^{DIM}$ cells may be particularly sensitive to exercise due to a higher density of β_2-adrenergic receptor expression compared with $CD56^{BRIGHT}$ counterparts.[57] Moreover, $CD56^{DIM}$ are primarily stored in the spleen and vascular endothelium while $CD56^{BRIGHT}$ cells reside predominantly in lymph nodes. With exercise, organs housing $CD56^{DIM}$ cells are engaged: there is an increase in shear stress on blood vessels, and the spleen contracts and expels contents of the red pulp, thus facilitating $CD56^{DIM}$ deployment.[57,60]

Acute exercise can also transiently increase NK cell function. Naturally, an increase in NK cell number would result in an increased ability to lyse target cells. Immediately after 30 minutes of moderate intensity cycling, NK cells extracted

from the peripheral blood of healthy adult cyclists showed increased lysis of target cancer cells ranging from 18.76% to 51.61%, depending on the target cell line.[61] This increase in lysis was primarily due to the increased number of NK cells circulating post-exercise. However, a second blood draw at 1 hour post-exercise revealed a 4.8-10.3% increase in cytotoxicity per NK cell, suggesting that exercise may also elicit an increase in each individual NK cell's function, independent of NK cell number. [61] Even in elderly, immunocompromised adults with poorly cytotoxic NK cells, acute aerobic exercise elicited an increase in cytotoxicity per NK cell that was comparable to that of healthy young adults.[62]

Only two available studies have assessed NK function in pediatric populations.[63,64] The first study demonstrated a similar exercise response between healthy children and children with CF after a maximal fitness test: with a 43.6% increase in cytotoxic function in healthy children and a 57.9% increase in cytotoxic function in children with cystic fibrosis.[63] Interestingly, when comparing the NK exercise response in trained and untrained children, trained children displayed lower NK cytolysis at baseline than untrained children (54± 6 vs 87± 10 cytolytic units, respectively).[64] However, NK function became comparable between groups in response to exercise; NK cytolytic activity increased on average 109% in trained children and 34% in untrained children.[64] Furthermore, NK cytotoxicity decreased to below baseline at 1-hour post-exercise in untrained children, while NK cells of trained children returned to baseline[64] This indicates a

potential role for exercise training in the modulation of the NK cell response as well as baseline NK values.

It is difficult to delineate whether exercise causes a deployment of selective NK cell subsets into the blood, or perhaps induces a change in the circulating NK cells thereby affecting their function, or a combination of the two. The leading hypothesis is that preferential recruitment of more mature NK cells induces a shift in the proportion of $CD56^{DIM}$:$CD56^{BRIGHT}$ cells in circulation, thereby also changing the NK receptor expression and cytotoxic ability of this population.[57] For instance, more mature $NKG2A^-/KIR^+$ and $NKG2A^+/KIR^+$ are mobilized with exercise more than less differentiated $NKG2A^+/KIR^-$ counterparts.[61] Incidentally, these licenced, more mature cells have more potent killing capacity.[27] It is important to note that NK cytotoxic capacity also differs based on the target cell; however, preliminary evidence indicated that NK cell activity increased with exercise when assessed with both HLA-bearing and HLA-deficient cell lines.[61,65] For instance, increases in the proportion of NK cells expressing NKG2C may increase lysis of HLA-E bearing targets. While selective NK recruitment may largely explain the increase in cytotoxicity with exercise, there is some evidence to suggest that exercise can also modulate NK cell phenotype via epigenetic modifications.[66] Zimmer et al have noted an increased histone acetylation post-exercise, which aligns with an increased expression in NKG2D activating receptor. .[66] There is still much work needed to decipher the full mechanisms of how exercise may modulate NK function.

It is not yet clear why NK levels drop in circulation post-exercise. The leading hypothesis is that they are redistributed into peripheral tissues to aid immunosurveillance. Studies in rodents have tracked T lymphocyte movement with exercise and found that similar to NK cells, there was increase in circulating T cells during exercise. These cells were then re-distributed to peripheral tissues such as the lungs, gut and bone marrow post-exercise.[67] If NK cells follow a similar trend or redeployment with exercise, an increase in cytotoxic NK cells in mucosal surfaces and the bone marrow could be particularity useful for children with ALL since mucosal surfaces are primary sites of infection and the bone marrow is the site of leukemogenesis. To this point, according to the homing receptors present on each NK cell subpopulation, NK^{DIM} cells home to sites of inflammation (i.e., sites with elevated concentrations of IL-6, S1P, IL-8, CXCL1), while NK^{BIGHT} cells home to lymph nodes.[68] Exercise-induced NK recirculation may be meaningful to individuals that received chemotherapy who are more susceptible to cancer recurrence, especially in light of the central role of NK cells in early tumor immunosurveillance.

1.3.2. NK CELLS AND CHRONIC EXERCISE

While results from acute exercise interventions are encouraging, it is unlikely that a single bout of exercise can induce a long-lasting, clinically meaningful change in NK cells for patients. However, regular repetitions of exercise, or chronic exercise in the form of exercise training, are thought to

enhance the acute effects of exercise and induce more lasting improvements in NK cells, even at rest. Examining the effects of acute exercise may provide an estimate of the effects of chronic exercise on NK cells before investing in a lengthy and resource-intensive training study.

Recent work based on tumour mouse models suggests that mice who exercise chronically for 6 weeks experienced a greater NK cell infiltration of subcutaneous and lung tumours than non-running control mice.[69] Importantly, mice with greater NK cell infiltrate showed a significantly lower tumour burden. [69] The effects of exercise training on tumours were observed even in athymic mice lacking functional T cells but not in NK cell-depleted mice, indicating that NK cells are likely responsible for the reduction in tumour burden. [69] When IL-6 was blocked during exercise, NK tumour infiltration was halted. [69] However, daily injections of exogenous IL-6 were not sufficient to elicit a decrease in tumor burden nor in NK recruitment in these mice. These findings indicate that the effects of exercise on NK cells are likely attributable to changes in a complex network of factors that include but are not limited to IL-6. [69] Tumor infiltration by NK cells has been shown to be a positive prognostic factor, therefore, chronic exercise may also be useful for children prior to maintenance therapy or even for patients with solid cancers

Similar to the acute effects of exercise, some exercise training interventions in adult cancer patients elicited a significant increase in NK cell

number and function, even at rest, compared to non-exercising cancer patient controls.[70–72] One study in breast cancer survivors
found that the exercising participants experienced a 6.34% larger increase in NK cytotoxic function than non-exercising participants.[72] This change was attributed to an increase in a proportion of NK cells numbers, and when normalized to function per cell, cytotoxicity changed -2.72 lytic units in the exercise group compared to controls from pre- to post-intervention. [72] In this instance lytic units were defined as the amount of cells required to lyse a set proportion of target cells- therefore a decrease in lytic units would be considered beneficial. [72] Contrarily, a 2-week intervention in post-operative cancer patients demonstrated that exercising patients had on average greater NK cytotoxicity (27.9%, variance not reported) than non-exercising counterparts (13.3%, variance not reported).[70] However, it is unclear whether these values were adjusted for changes in NK number. Finally, a 6-month training intervention in survivors of breast cancer reported that exercise increased cytotoxic function but not number.[71]

Taken together, the findings from adult and mouse studies suggests that acute exercise can transiently upregulate NK number and function, and chronic exercise exposure may elicit more lasting, positive effects on NK cells, even in those with poor immune function at baseline.

1.3.3. EXERCISE AND NK CELLS IN PEDITRIC ONCOLOGY PATIENTS

A very limited amount of literature is available on the effects of exercise on the immune system in children with cancer, including ALL. Of these, the available studies have very few children (3-6), and/or a cohort of mixed cancer diagnoses.[73–76] A single study observed the effects of an acute, 30-minute bout of treadmill exercise in children receiving maintenance therapy for ALL.[74] Although they did not measure NK cell subsets, they did note that there was a significant increase in overall lymphocyte counts (~46%) immediately post-exercise, followed by decrease towards baseline levels at 1-hour post-exercise. .[74] A very similar response pattern was seen in healthy controls who only experienced a 31% increase in lymphocyte counts but had much higher (>2-fold) lymphocyte concentrations at rest.[74] Similarly, a pioneering study in the field by Shore et al. reported that an acute bout of 30 minutes of high intensity cycling induced transient lymphocytosis in 6 children who were receiving therapy for different neoplasms (including ALL) or had recently completed chemotherapy.[75] Again, chemotherapy-treated children had overall lower levels of leukocytes and lymphocytes compared to healthy controls. [75]

Few studies have also explored chronic exercise interventions. In the same study by Shore et al., 3 children still receiving chemotherapy and 11 healthy children completed a high intensity (70-85% of maximum heart rate) aerobic training program 3 times per week for 12 weeks. [75] Interestingly, a slight decrease in leukocytes and $CD56^{+}$ cells was observed with training in both

patients and healthy children.[75] However, both groups maintained leukocytes concentrations within a healthy range (>2.4 × 10^9c/L). It is important to note that a leukocyte concentration of 2.4±0.7 × 10^9c/L is rather high for children receiving chemotherapy; however, the study did not specify the treatment protocols nor status of participants at the time of participation.[75] Furthermore, many cells aside from NK cells express CD56, therefore, these results may not be indicative of changes in the NK cell subset alone. Finally, children treated for cancer had lower levels of $CD56^+$ cell cytotoxicity at baseline compared to healthy children (3.9±3.2 vs 10.6±3.27 lytic units/10^6 PBMCs).[75] However, post-training there was a trend towards an increase in NK cytotoxic function in children treated for ALL (5.0±2.4 lytic units/10^6 PBMCs) but the opposite was true for healthy children (6.9±1.7 lytic units/10^6 PBMCs).[75] A 10-week aerobic and resistance exercise training intervention elicited a more long-lasting effect on NK cells in children who received a hematopoietic stem cell transplant.[73] The authors reported a higher ratio of $CD56^{DIM}$:$CD56^{BRIGHT}$ cells 36 hours after the last training session compared to non-exercising controls.[73] Moreover, although the training group initially displayed a lower NK cell cytotoxic ability compared to controls (~12 vs ~22% lysis), upon completion of the program the training group displayed a significantly higher cytotoxic ability (~32% vs ~20%).[73] Finally, Fiuza Luces et al. assessed the effects of an exercise training intervention starting and ending at the same time as chemotherapy (6-7 cycles) in children treated for solid tumors (11 non-exercising, 9 exercising).[76] There was a trend towards an increase in NK

cell numbers in exercising patients but not in non-exercising controls (0.5 (0.1) to 1.1(0.8) versus 1.0 (0.3) to 1.0 (0.4) x1000 c/ μL).[76] There were no clear patterns regarding changes in NK cell function with exercise training.

Given that higher NK function is associated with a lower risk of cancer relapse and rate of infection, interventions to improve NK status in children treated for ALL should be a priority. The evidence presented above suggests that exercise is a simple and effective intervention to improve NK number and function in many populations. Few studies have examined the effects of exercise on NK cells in children treated for ALL. The limited and small studies in pediatric ALL or mixed pediatric diagnoses provide some promising though inconsistent results. As of yet, no studies have assessed the effects of *acute* exercise on NK cell function and receptor expression in children with ALL. Examining NK number, function and receptor expression in response to exercise in tandem may provide a more complete picture and yield better insights into the mechanism mediating NK changes with exercise. Understanding these mechanisms may give more insight into where incorporating exercise interventions may be most beneficial for these children (i.e., at which time point, in combination with specific therapies etc).

1.4. IMPLEMENTING EXERCISE INTO PEDIATRIC ONCOLOGY

Exercise provides numerous physical and psychological health benefits for children treated for cancer; however, it is not yet a standard part of pediatric oncology care. Given the many challenges associated with a cancer diagnosis, it

is not altogether surprising that many children with cancer, including those with leukemia, are far less active than their healthy counterparts. Device-based measures of physical activity levels indicate that children undergoing treatment for leukemia are on average 66% less active than their healthy peers (2992 ±1994 steps per day versus 8096 ±2951 steps per day).[77] Even when active, pediatric leukemia patients performed more low intensity physical activity and less moderate-to-vigorous physical activity than healthy controls.[77] A qualitative study found that children spent less time performing physical activity during ALL treatment compared to prior to treatment (approximately 7-fold lower).[78] Furthermore, healthy children were over 4 times more active than children in therapy.[78] The bulk of this evidence suggests that without intervention, pediatric leukemia patients are likely not engaging in sufficient amounts of physical activity for overall health. The low levels of physical activity are not just limited to time in treatment, rather, they persist well past recovery and into adulthood. In fact, adult survivors of childhood ALL are more likely to be inactive and less likely to meet physical activity guidelines than the general population, when adjusted for sex and age.[79] Therefore, it is important to instill healthy physical activity habits early and consistently in children treated for ALL. An exercise intervention study or clinical program may be an effective method to promote a more active lifestyle and maximize the likelihood that children with ALL can reap the benefits of regular physical activity.

1.4.1 BARRIERS AND SAFETY OF EXERCISE AND PHYSICAL ACTIVITY IN CHILDREN WITH CANCER

In order to target low levels of physical activity and develop feasible and appropriate exercise interventions, it is important to understand why childhood cancer patients are less active than their healthy peers. In this context, physical activity is defined as any type of movement that is mediated by skeletal muscle and expends energy; while exercise is defined as activity that is planned and goal oriented, and represents a type of physical activity.[80] Broadly, barriers to being active in children with medical conditions can be classified as those that occur as a direct result of their medical condition and those that are incidental to their condition.[81] Barriers caused by disease or treatment that may limit physical ability differ by diagnosis. Barriers to physical activity applicable across different types of cancer include general fatigue, treatment side effects causing pain, nausea or disability, and medical equipment that may limit movement or fear of disturbing the medical device with too much movement (i.e., infusion pumps). [81] Common barriers incidental to a pediatric cancer diagnosis may include psycho-emotional implications, perceptions of physical activity, and social influences. [81]

Psycho-emotional barriers to physical activity are often more evident in older children diagnosed with cancer who report feelings of stress, anxiety, and fear related to their diagnosis, which cause them to feel much less interested in physical activity. [81] Perceptions of physical activity may vary greatly between patients, with some believing that physical activity may actually worsen their

condition by wasting already low energy levels, or by exposing them to infection. Furthermore, many participants are unaware of the detriments of being inactive and believe they should focus on resting to recover. In these instances, prioritizing exercise comes later, when they are “cured”. [81] Finally, social influences may act as barriers to physical activity in two forms: social isolation and discouragement from guardians, and in some cases, physicians. [81] Children may isolate themselves from social physical activities due to visible physical differences (i.e., hair loss), and fear of stigmatization due to their disease.[81,82] In addition to self-imposed barriers to physical activity, parents also play an important role in facilitating or limiting activity. [81,82] More specifically, many parents are uncertain about the safety of physical activity for children with cancer, they may believe that physical activity may lead to injuries or increase risk of infections. [81,82]

Next, is important to consider the actual safety and perceptions of the safety of physical activity for children with cancer. For example, Ross et al. reported that participants still tended to maintain views that exercise was unsafe, despite receiving physician recommendation to be physically active.[82] Interestingly, some children also noted that their attending physician did not speak to them about the importance of physical activity or encourage them to be active. [82] As a result, children reported they were under the impression that physical activity is not important or that it may even be hazardous. In light of this, a physician-led conversation about staying physically active may help reduce the

stigmatization of exercise by parents and patients to some extent; however, a more hands-on approach, such as a clinic-based exercise intervention or even physical activity counselling may be more effective. [82]

Despite the aforementioned barriers, it is important to note there are many successful exercise training studies that include children at different stages of cancer treatment. While these studies do not diminish the barriers to physical activity for children with cancer, they serve highlight that physical activity and/or exercise is actually possible for these children. Collectively, these studies suggest that taking time to address barriers to physical activity and providing guidance to support safe participation in physical activity are critical elements of pediatric oncology rehabilitation. Fortunately, there is ample evidence to support that exercise is safe and feasible specifically for pediatric patients with ALL. Children with leukemia safely and successfully completed both resistance and aerobic exercise at various intensities ranging from light stretching to high intensity (85%VO_2peak) treadmill running.[83] Not only does this provide evidence that children with cancer are able to be physically active, but it also supports the fact that these children can participate in a wide variety of exercises, even at challenging intensities, without adverse events. [82] Importantly, many interventions in this population have been longer-term supervised and/or home-based exercise training programs suggesting that even chronic or repeated exposure to exercise is safe and feasible for these youth. While these results are extremely positive, it should be noted that pediatric oncology patients tend to be less physically fit (i.e.,

diminished aerobic capacity, lower muscle strength, fatigue) than healthy counterparts.[84] Therefore, the physical capacity of each patient must be considered, and exercise should be tailored accordingly to reduce the chance of injury and maximize benefits of physical activity.

Taken together, the existing evidence suggests that it is safe and feasible for children with cancer to preform regular exercise for prolonged periods of time. Despite this, participation in exercise immunology studies remains low, which in turn limits our ability to develop evidence-based exercise interventions to improve immune outcomes in this population. Unfortunately, there is little to no discussion about the barriers to participation in such studies. Therefore, it is important to explore potential barriers to recruitment and participation that children with cancer face when asked to participate in a structured exercise immunology study.

1.4.2. BENEFITS OF EXERCISE FOR CHILDREN WITH CANCER

Aside from the potential immune benefits discussed throughout this document, habitual physical activity can improve a vast array of physiological and psychological parameters in children with cancer. This broad body of evidence is important to emphasize when advocating for incorporating physical activity prescription or exercise interventions into pediatric oncology care. Even in healthy children, physical activity is critical to building and maintaining cardiopulmonary health, musculoskeletal stability, weight status, and mental well-

being.[85] The following section will highlight some of the widely reported benefits of exercise for pediatric cancer patients.

Bone mineral content in children with ALL is severely weakened as a result of soluble cancer secretions, leukemic joint lesions, treatment (corticosteroids, methotrexate), and compounded by physical inactivity. Multiple studies have reported that a regular exercise regimen starting at diagnosis can significantly prevent reductions in bone mineral content, as compared to non-exercising patients.[86,87]

Muscle weakness, wasting, and reduced range of motion are another common feature in children with cancer due to inactivity and treatment.[88] A study found that an 8-week high intensity aerobic and resistance training intervention was able to significantly improve muscle strength.[88] Interestingly, this improvement was maintained even 20 weeks after the completion of training.[88]

Finally, chemotherapy agents, muscle weakness and wasting, and lack of physical activity can all have negative effects on aerobic fitness .[88] This finding is especially important because aerobic fitness is independently associated with a reduced risk of cardiovascular disease and all-cause mortality. San Juan et al. reported that average aerobic fitness, defined as maximum volume of oxygen uptake or VO_2peak, of children treated for leukemia was 24 $mL/kg^{-1}/min^{-1}$ compared with an average of 45 $mL/kg^{-1}/min^{-1}$ in healthy age-matched controls.[88] To further put this into perspective, consider the approximate aerobic requirement of daily activities relevant to children, such as getting dressed (9.45 $mL/kg^{-1}/min^{-}$

^{1}), climbing the stairs or playing basketball (24.5 mL/kg^{-1}/min^{-1}), or playing on the playground (17.5 mL/kg^{-1}/min^{-1}).[88] Given that deconditioned children with ALL must push themselves to nearly 100% of their peak capacity to perform the aforementioned tasks, it is not surprising that they are less likely to participate in common activities of daily living.[88] This, in turn, creates a feed forward cycle of inactivity. Fortunately, there is evidence that chronic exercise training can significantly increase aerobic fitness children treated for ALL. An 8-week moderate intensity aerobic and resistance training intervention elicited a ~17% increase in aerobic fitness in ALL patients, while no difference was seen in the control group. [88]

Clearly, exercise has broad spanning effects that extend beyond the immune system for children treated for ALL. These physiological benefits are relevant to health and quality of life for these patients. Therefore, efforts to promote physical activity and exercise for children with ALL are urgently needed and may have important implications for overall wellbeing both during and beyond therapy.

1.5. RATIONALE FOR A PILOT STUDY

While the evidence suggests that exercise is safe for children with ALL, a pilot study of the proposed protocol is still necessary. More specifically, the results of a pilot study will serve to provide participants, parents, the ethics board, and funding agencies with reassurance that our specific study plan is feasible, acceptable, and safe.

From a practical perspective, we must consider the unique burdens for patients and their families undergoing treatment for cancer that might limit their ability to participate in an exercise study. In recent decades, many children receiving cancer therapy are able to receive treatment primarily as out-patients, therefore there is an increased emphasis on home-based care for patients and their parents.[89] This demands a greater commitment from patients and their families to address physical, social, and emotional challenges associated with cancer care on their own. As researchers, these represent additional obstacles to participation that must be considered in our study design, which requires additional time and commitment from both parents and patients.

From an experimental perspective, few exercise immunology studies have been conducted on pediatric cancer patients; however, all studies to date have very few participants and/or mixed diagnosis cohorts. Importantly, these studies underreport barriers to recruitment and participation. In order for future research in this field to achieve a sufficient sample size, it is necessary to identify barriers to recruitment and provide plausible methods to address them. A pilot study will provide insights into recruitment and barriers to participation, while also supporting preliminary data collection. This initial wave of data collection will allow us to calculate effects sizes, and in turn, estimate samples sizes for future studies. Moreover, a pilot study will provide us with the preliminary data to support the design and resource allocation for a larger, definitive trial.

1.6. SUMMARY

Children treated for ALL are immunosuppressed and therefore at a higher risk of cancer recurrence and infection. There is increasing evidence of the beneficial effects of exercise for improving NK cell number and function in healthy people as well as certain adult clinical populations. However, little is known about the effects of acute exercise on the immune system of children with ALL. Currently available studies looking at the effects of exercise on children with cancer have small sample sizes and/or study a wide variety of cancer diagnoses- therefore it is difficult to build a consistent and robust base of evidence on the effects of exercise on NK cells in these populations. Despite low recruitment rates in these studies, barriers to recruitment and participation remain underreported. Reporting barriers to recruitment and participation as well as participant feedback on study components may be beneficial information to create more sustainable intervention and overcome low participation rates.

Furthermore, no studies have assessed the effects of acute exercise on NK cell function and receptor expression in children with ALL and how these values compare with healthy children. Examining transient effects of acute exercise may be a simpler preliminary step that is indicative of more enduring effects of chronic exercise. Furthermore, there is evidence to suggest that regular exercise may modulate NK number and function even at rest. Therefore, examining the link between habitual physical activity levels and NK number and function at rest in children with ALL may provide insight to create cohort specific

activity guidelines. Finally, there is evidence that NK number and function changes as children progress through therapy- this may also influence the NK cell response to exercise.

1.7 OBJECTIVES AND HYPOTHESES

1.7.1. GENERAL OBJECTIVES

The general objectives of this thesis were to examine the feasibility, acceptability, safety and NK cell response to acute exercise in children with ALL.

1.7.2. SPECIFIC OBJECTIVES

The primary objectives (Chapter 2) of this thesis are to assess the:

1a. feasibility of recruiting and testing children undergoing maintenance therapy for ALL

1b. acceptability of exercise and accelerometer wear for children undergoing maintenance therapy for ALL

1c. safety of exercise for children undergoing maintenance therapy for ALL

The secondary objectives of this study are to:

2a. Determine the effects of an acute bout of exercise on NK cell number, receptor expression and function in children with ALL compared to healthy children (Chapter 3)

2b. Assess changes in the NK cell response to acute exercise over 3 months of maintenance therapy (Chapter 4)

2c. Examine the link between habitual physical activity levels and NK cell number and function in children with ALL at rest (Chapter 4).

1.7.3. SPECIFIC HYPOTHESES

Based on the available evidence, we hypothesized that:

1a. Recruitment and testing goals will be feasible in this population. The exercise protocol will also be feasible for this population.

1b. Exercise and accelerometer wear will both be acceptable for this population.

1c. Exercise will be safe for this population.

2a. Acute exercise will induce an increase in circulating NK cell numbers and function in children with ALL. Accordingly, there will be an upregulation of activating receptor and a downregulation of inhibitory receptors with exercise. Healthy children will experience a more robust response to exercise than children treated for ALL.

2b. As children progress through therapy, their NK cell response will become more robust.

2c. Children who are consistently less active will have lower levels of circulating NK cells and lower NK cell function.

CHAPTER 2: USING EXERCISE TO BOOST THE IMMUNE SYSTEM OF CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA: A PILOT STUDY ASSESSING FEASIBILITY, ACCEPTABILITY, AND SAFETY

Prepared for publication as:

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2.1. ABSTRACT

Introduction: Exercise may improve immune function in healthy and clinical populations. Children treated for acute lymphoblastic leukemia (ALL) are immunodeficient and may benefit from an exercise intervention; however, little is known about the effects of exercise on the immune system in this population. The limited studies examining the effects of exercise on the immune system in children with cancer are underpowered and often include a variety of different cancer types. Importantly, these studies provide little to no insight into the barriers to recruitment and exercise testing in this population. As such, the aims of this study were to assess the feasibility, acceptability and safety of performing an acute exercise study in children treated for ALL. Methods: Children aged 7-18 years who were receiving maintenance chemotherapy for ALL were recruited

from McMaster Children's Hospital. Participants attended 3 separate study visits, each occurring after their monthly chemotherapy appointment. At each visit, participants performed a 30-minute continuous cycling exercise followed by 1 hour of rest. Blood samples were collected before and after exercise via the participant's central line. Participants were then outfitted with an accelerometer that was worn between study visits to estimate levels of sedentary time and physical activity. Feasibility of recruitment was assessed by tracking recruitment, enrollment, and withdrawal rates as well as reasons for refusal. Feasibility of retention was assessed by tracking visit completion rates. Feasibility of exercise was assessed by tracking exercise completion rates. Acceptability of accelerometer wear was assessed using wear and log completion rates. A physical activity enjoyment scale was to gauge the acceptability of exercise and a semi-structured questionnaire collected participant experience feedback. Exercise-related adverse events were tracked to assess safety. Results: Recruitment was not feasible for this site (22 patients approached, 4 out of intended 15 enrolled and completed study). Primary barriers to participation included extended hospital time and anxiety surrounding extended port access for blood draws. Participant testing (100% completion rate) and exercise (94% completion rate) were feasible. Participants enjoyed the exercise (4.2 ± 0.38 out of 5 on PACES). Accelerometer wear rates (61.9% (range 3.7-100.0%)) and log completion rates (69.0% (25.9-100.0)) were moderate. There were no adverse events associated with exercise. Conclusion: This study examining the effects of

exercise on immune health was feasible and safe for children with ALL. Future research may wish to incorporate methods to overcome the present barriers to recruitment.

2.2. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer but also among the most curable, with an approximate 5-year survival rate exceeding 90% in developed countries. Currently, multi-drug chemotherapy is the gold standard for treating pediatric ALL; however, the lengthy therapy causes many side effects.[2,3] Notably, the cytotoxic treatment results in the elimination of healthy dividing cells, such as immune cells. Diminished immunosurveillance and a decreased capacity to mount appropriate immune responses among patients leads to increased susceptibility to infection and even cancer recurrence.[4,5] Detrimental effects on the immune system may last well into adulthood, with adult survivors of childhood ALL showing higher incidences of infection and cancer than sex- and age-matched controls.[6,7]Altogether, these findings emphasize the need to improve immune health in children during and after treatment for ALL.

Exercise is a simple and accessible intervention that can be implemented during and beyond treatment to help boost components of the immune system. Recent literature outlines the physiological and psychological benefits of exercise for patients and survivors of childhood cancer;[8] however, the effects of exercise on the immune system in this population are still relatively understudied. This is

particularly true for NK cells, a subset of lymphocytes that play a key role in combatting malignancies and opportunistic infections that often plague children treated for ALL.[9,10] A single bout of exercise can transiently increase NK cell concentrations and NK cytotoxic function in healthy and chronically ill people.[11–15] Importantly, exercise training (i.e., repeated bouts of exercise) may lead to more long-lasting improvements in NK cell levels and cytotoxicitiy.[16,17]

Since pediatric patients treated for ALL present with low levels of circulating NK cells and impaired NK function, exploring exercise as a method to bolster NK function may be useful.[18–20] Despite the encouraging preliminary findings, a strong base of empirical research is needed to help advocate for the incorporation of exercise into standard clinical care for pediatric ALL. Therefore, before launching a large and costly randomized clinical trial, we designed this pilot project to assess the a) feasibility, b) acceptability, and c) safety of studying the effects of exercise on NK cell number and function in children undergoing maintenance therapy for ALL. This pilot study will explore the barriers to recruitment and testing, which are often underreported in the existing exercise-immunology studies in pediatric cancer patients. It is important to assess and report feasibility and acceptability of conducting such studies in an effort to inform and adapt future research designs to overcome the often-reported sample size limitations of studies published to date.

2.3. METHODS

Participant recruitment:

We aimed to recruit 15 children aged 7 to 18 years, undergoing maintenance chemotherapy for ALL at McMaster Children's Hospital. Eligible patients were cleared for exercise by a physician, had no conditions that would prevent understanding of or cooperation with the study, presented no evidence of disease relapse, and had at least 4 months of maintenance therapy remaining. There were no restrictions on medication usage, therapy protocol or progress in maintenance therapy; however, this information was collected for descriptive purposes. Before starting the study, all participants and/or parents/guardians provided informed assent or consent, as appropriate. This study was approved by the Hamilton Integrated Research Ethics Board.

Study overview:

It Is important to emphasize that this study was not designed as an exercise training study, rather we examined effects of acute exercise on a monthly basis over a 12-week period to gather relevant pilot data to inform the design of a larger trial.

Participants attended a total of 4 study visits each 4 weeks apart. Children in maintenance for ALL typically attend the oncology clinic every 4 weeks to receive a chemotherapy, and every 12 weeks they are also sedated to receive a lumbar puncture with intrathecal chemotherapy. To maximize convenience for participants, all 4 study visits corresponded with regularly scheduled clinic

appointments, whereby each exercise visit (3 in total) occurred after their regularly scheduled monthly chemotherapy session. Only a resting blood sample was taken on lumbar puncture visits (1 in total).

Each exercise study visit was identical. After receiving their chemotherapy, participants were accompanied to the exercise lab and height, weight, and body fat percent were measured. Next, participants completed a 30-minute moderate intensity continuous cycling protocol. After cycling, participants were asked to sit and rest for an hour while completing an exercise enjoyment questionnaire and a study experiences interview with researchers. Three blood samples were collected: a resting sample pre-exercise, a sample immediately post-exercise, and a sample after 60 minutes of rest. The participant's subcutaneous central venous catheter port was accessed to administer chemotherapy and was kept accessed throughout the visit for all blood draws. Blood samples were assessed for NK cell concentrations, function and receptor expression. After each visit, participants were given an accelerometer and asked to wear it between study visits (~ 4 weeks at a time) to assess habitual physical activity over the course of the study **(Supplementary figure 1).**

Exercise protocol:

Participants completed 30 minutes of continuous cycling on a cycle ergometer (Corival or Corival Pediatric, Lode, Groningen, Netherlands). Heart rate was recorded every minute using a Polar heart rate monitor worn around the chest (Polar Electro OY, Kempele, Finland). While cycling, participants were

asked to maintain a steady cadence of 60 to 80 revolutions per minute, and researchers adjusted the workload to elicit and sustain a heart rate between 140 and 160 beats per minute, which roughly corresponds to a moderate-to-high exercise intensity in children.[21,22] This intensity was selected to maximize completion rates among participants who may be deconditioned while also ensuring a sufficient stimulus to elicit NK cell deployment.[12] Participants watched a movie or television show of their choosing while cycling. Any breaks in cycling or modifications to workload were noted.

Habitual physical activity:

Participants wore an ActiGraph GT3X accelerometer (ActiGraph LLC, Pensacola, USA) to assess free-living, habitual physical activity and sedentary time over the course of the study (12 weeks). At the end of the first visit, participants were outfitted with the waist-worn unit, and instructed to only remove the device during water activities or while sleeping. Participants were also provided with a logbook to track when they removed and donned their accelerometers. Accelerometers were downloaded, charged, and reinitialized at each study visit. All accelerometer data were stored in 3-sec intervals, cleaned, and processed using ActiLife software.

Feasibility:

We determined the feasibility of recruitment, retention, and exercise completion. Feasibility of recruitment was evaluated by tracking recruitment, refusal and withdrawal rates over 2 years. Recruitment was deemed feasible if

we attained our goal of enrolling 15 participants over the course of 2 years. Study retention was determined by tracking study visit completion rates for each participant who began the study. The study visits were deemed feasible if all participants attended all 4 study visits. To assess the feasibility of exercise, we recorded any breaks or the termination of the cycling exercise prior to completion of the prescribed 30 minutes. The exercise was deemed feasible if all participant completed the prescribed 30 minutes of cycling without pauses.

Acceptability:

We examined the acceptability of the exercise and accelerometer wear. To evaluate the acceptability of exercise, participants completed the Physical activity enjoyment scale (PACES) during their rest period after cycling at each study visit.[23] The PACES questionnaire presents the participant with 16 statements about the exercise they just completed and asks the participant to rate these statements on a 5 point Likert scale ranging from “disagree a lot (1)” to “agree a lot (5)”. To calculate the score answers to negative statements were converted (i.e., negative perceptions had lower scores), and all values were averaged. The exercise was considered acceptable if participants found it *enjoyable*, defined as an average rating ≥4 out of a possible 5. Qualitative feedback was collected from the *study experiences interview* performed during recovery, where one member of the research team (MB) asked the participant open-ended questions about the exercise they performed, including: “If you had the equipment, is this exercise something you would do in your own time? Why or why not?”, and “How did you

feel about the exercise you just did?". Participant responses to these answers were recorded and reported as frequency of responses. Acceptability of accelerometer wear was assessed by tracking device wear rates, and logbook use rates. Device wear rates for each 4-week physical activity assessment period were calculated using the following equation (Eq1.):

Eq1. Wear rates = valid days per month ÷ total potential wear days × 100

Where a valid day was defined as any days with ≥6 hours of wear time. Accelerometer wear rates of ≥50% of potential wear days were considered acceptable. Logbook completion rates were calculated using the following equation (Eq2.):

Eq2. Logbook entry rates = days logged ÷ total potential log days × 100

Where "days logged" was defined as at least 2 recordings per day (times the device was put on after waking and removed for sleep). Logbook completion rates ≥50% of potential wear days were considered acceptable. Qualitative feedback on the participant's experience wearing the accelerometer was collected during the *study experiences interview*. Specific open-ended questions were asked, including: "What did you dislike about wearing an accelerometer over the last month?", and "Is there something that would make wearing this

accelerometer more enjoyable for you?". Participant responses to these answers were recorded and reported as frequency of responses.

Safety:

Safety was assessed by tracking any observed, participant- or parent-reported adverse events to exercise. The exercise was deemed safe if there were no reported major adverse events, as defined by our clinical team.

Statistics:

Feasibility, acceptability and safety results were all reported using descriptive statistics including mean, standard deviation, median, range, and frequencies for interview responses. De-identified participant-level data are presented where possible.

2.4. RESULTS

Feasibility of recruitment:

Participant recruitment began March 2018 and ended March 2020. During this time, 27 patients met the age, diagnosis, and treatment criteria; of these, 23 were also physically able to participate. In total, 22 patients were approached to participate. One eligible patient was not approached because all research activities were discontinued as a safety precaution for the COVID-19 pandemic. Of the patients approached, 10 consented to be contacted further about the study, while 12 refused to participate immediately. After follow-up, 5 patients enrolled in the study. One participant withdrew from the study after consenting to

participate, but before attending any study visits. Overall, a total of 4 participants completed the study (18% of those approached, as outlined in **Figure 1**). Our first participant visit was in May 2018, and the final participant visit was completed in October 2019. Overall, the goal of recruiting 15 children in 2 years was not feasible for our site.

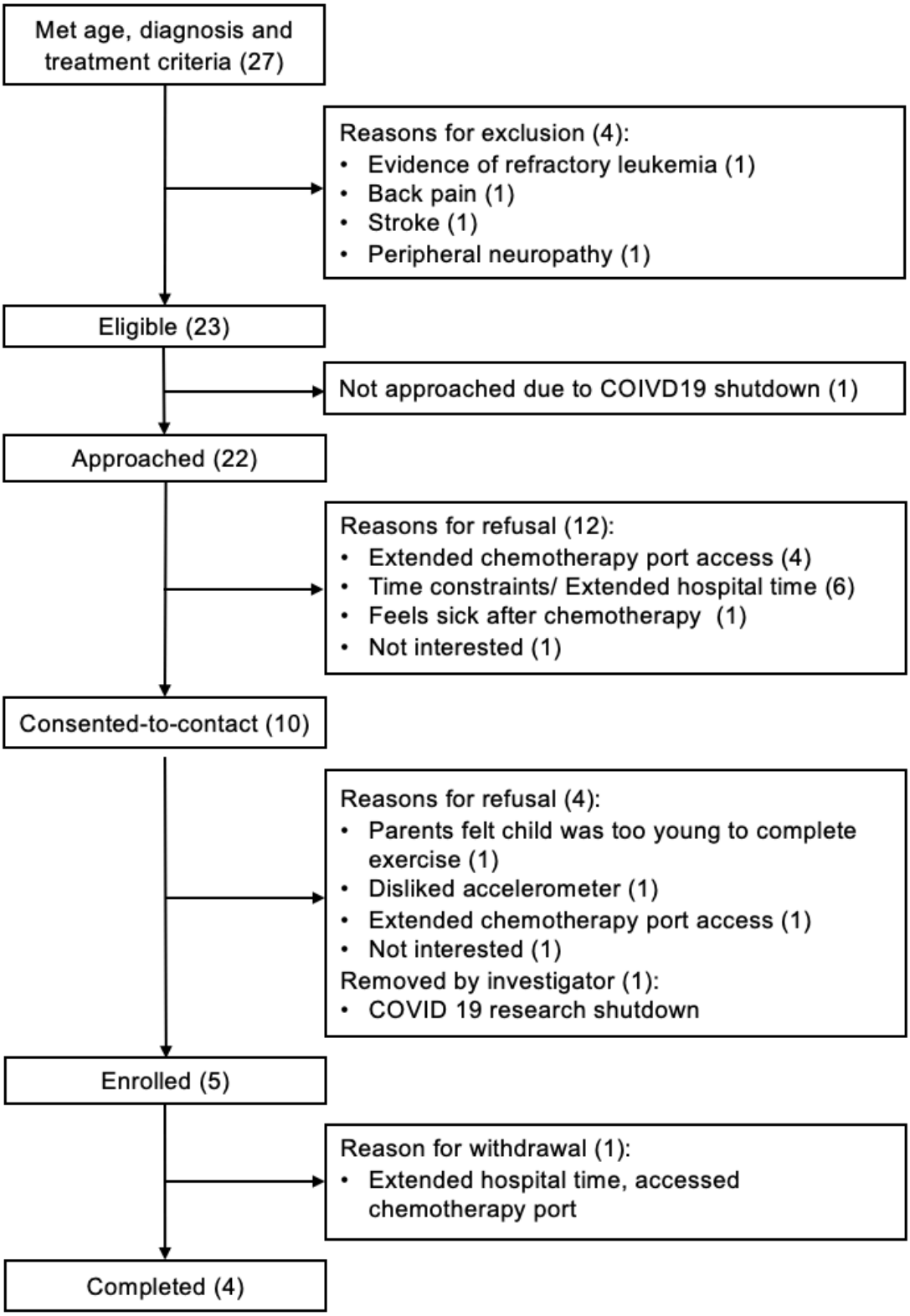

Figure 1. Recruitment flow chart

Our participants were on either end of the age eligibility criteria: two participants were pre-pubertal while two were post-pubertal. All of our participants were male and were already at least one year into maintenance therapy, which typically lasts 2-3 years **(Table 1).**

Table 1. Overall and individual participant characteristics at their first study visits.

	All participants	**B-01**	**B-02**	**B-04**	**B-05**
N (% male)	4 (100%)	M	M	M	M
Age (years)	13.4 ± 5.2	17.2	18.6	8.7	9.0
Height (m)	1.53 ± 0.24	1.65	1.81	1.35	1.30
Height %ile	45.2 ± 31.8	7.9	74.2	68.8	29.8
Weight (kg)	45.0 ± 20.2	62.7	61.8	31.4	23.9
Weight %ile	38.7 ± 27.7	41.3	25.5	76.1	11.9
BF %	16.4 ± 9.2	29.5	14.1	7.9	13.8
BF %ile	38.9 ± 35.0	83.9	35.6	1.92	30.5
Weeks of maintenance therapy	70 ± 11	56	69	82	76

Data are presented as Mean ± SD for all participants, as well as reported for each individual participant (ID = B-##). BF, body fat; %ile, weight and height percentiles based on normative data from the CDC growth charts[23], body fat percentiles based on normative data from Laurson et al.[24]

Feasibility of participant retention:

All 4 participants who started the study completed the 4 required visits (3 exercise visits and 1 resting blood sample visit). Three participants were able to complete the study in 3 months, in accordance with our study schedule. A single participant required an extra month (total 4 months) to complete the study. This participant missed a scheduled study visit because his oncology clinic appointments lasted significantly longer than expected and the participant did not have time to then attend his study visit. The missed exercise visit was easily

rescheduled for the following month. Chiefly, participant retention and testing were feasible.

Feasibility of exercise:

Three participants were able to complete 100% of the prescribed exercise. One child completed 94% of the prescribed exercise. At the first visit, the participant started to feel nauseous and stopped cycling with 5 minutes remaining. The participant completed all following exercise sessions without any issues. Since this participant was able to complete all subsequent exercise without any issues, the exercise protocol was deemed feasible for the children in our study.

Acceptability of exercise:

On average the participant found the cycling exercise to be enjoyable, rating it a 4.2 ± 0.38 out of a total score of 5 (most enjoyable) on the PACES questionnaires (**Figure 2)**. These ratings were consistent over the course of the 3 exercise study visits. Participants provided largely positive feedback in the semi-structured interviews on acceptability of exercise, as outlined in **Table 2**. Together, the PACES scores and qualitative feedback suggest the exercise protocol was acceptable.

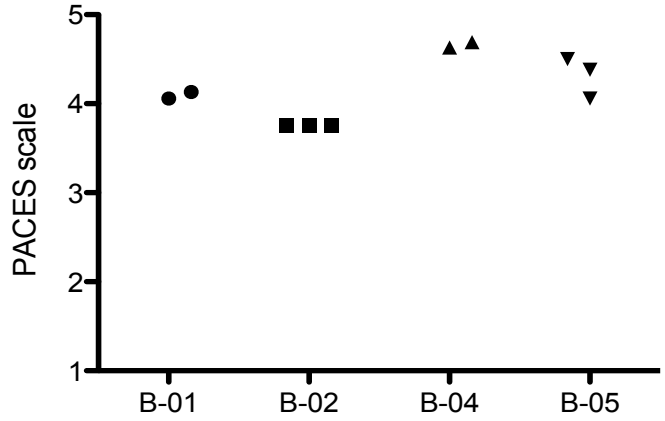

Figure 2. Participant enjoyment of the exercise protocol. Depicted are the participant responses to the PACES questionnaire. The PACES scale (Y-axis) spans from 1 (not enjoyable) to 5 (very enjoyable). Responses from each exercise study visit (total of 3) are represented for each participant on the graph. Participant B-01 and B-02 did not complete their PACES questionnaires at exercise visits 1 and 3, respectively.

Table 2. Participant responses (and frequencies) to semi-structured interview questions on the acceptability of the exercise.

Question: If you had the equipment, is this exercise something you would do in your own time? Why or why not?	
Yes (3)	• "Easy to do" (1) • "Good for your body" (1) • "I want to get back into shape" (1)
No (1)	• "Enjoyed the exercise but I would prefer an elliptical" (1)
Question: How did you feel about the exercise you just did?	
Respondents (4)	• "Good but tiring" and/or "challenging" (3) • "I feel proud for completing it" (1) • "I would do it again" (1) • "Motivated to exercise more" (1) • "I would prefer if the workload was kept consistent throughout" (1)

Acceptability of accelerometer wear:

Generally, accelerometer wear rates were moderate, with an average wear rate of 61.9% (range 3.7-100.0%; Table 4). One participant only wore the

accelerometer during 2 of 3 prescribed physical activity assessment periods, and another participant had a corrupted accelerometer file for 1 of the 3 prescribed physical activity assessment period. Overall, the average logbook completion rate was 69.0% (25.9-100.0; **Table 3**). One participant only returned 1 of the 3 logbooks. When asked what they disliked about wearing the accelerometer, participants identified relatively minor issues that would not preclude the use of this device in future studies**.** When asked what could make wearing the accelerometer more enjoyable, most participants agreed a wrist-worn accelerometer would be better **(Table 4).**

Table 3. Accelerometer wear rates reported as mean (range).

Participant	**Accelerometer wear rates**	**Logbook entry rates**
B-01	87.0% (74.1-100.0)	100% (100).0
B-02	8.6% (3.7-14.8)	29.6% (7.4-44.4)
B-04	69.0% (51.9-86.2)	75.3% (25.9-100.0)
B-05	82.8% (73.1-92.6)	69.2%[+]

[+] Only returned 1 of 3 logbooks.

Table 4. Frequency of participant responses to semi-structured interview questions on the acceptability of accelerometer wear.

Question: What did you dislike about wearing an accelerometer over the last month?	
Respondents (4)	• "Falls off" (1) • "Having to readjust it while wearing" (1) • "Putting it on every morning" (1) • "Covering it when going outside" (1) • "Nothing" (1)
Question: Is there something that would make wearing this accelerometer more enjoyable for you?	
Respondents (4)	• "Bracelet" (3) • "Not removing to sleep" (1) • "Waterproof" (1) • "Shorter wear periods" (1)

Study safety:

One participant had a minor incident while cycling. After 25 minutes of cycling during the first visit, a participant began to feel nauseous and had to stop cycling. After a brief rest, the participant felt well enough to go home. The participant indicated that they occasionally felt nauseous shortly after receiving chemotherapy. Therefore, it is difficult to attribute this event solely to exercise. The participant was able to complete all following exercise visits without any issues. All in all, the exercise protocol was deemed safe due to a lack of major adverse events.

2.5. DISCUSSION

This was the first study to explore the effects of acute exercise on NK cell number and function over 12 weeks of maintenance therapy in children being treated for ALL. In this pilot project, we found that our recruitment goal was not feasible for this site and timeline. However, participant retention and the exercise protocol were feasible. Both the exercise protocol and accelerometer wear were deemed acceptable and the exercise was safe for participants treated for ALL.

Feasibility of recruitment:

The most common reason for refusal to participate in our study was that patients did not want to prolong their hospital time and/or had time constraints **(Figure 1)**. Many patients were required to travel long distances and/or face long

travel times to and from the treatment site. This was specifically mentioned as a barrier to participation by 4 of the 16 families that declined the study invitation.

Once on-site, patients typically wait to see their physician, to get their prescription filled, and to receive therapy. Wait times are often unpredictable and depend on many moving factors within the clinic. Therefore, one might expect that on a particularly long clinic day, the patient and/or their family do not want to spend more time in hospital than is necessary for medical care. Concretely, 5 of the 16 families that declined to participate felt that the time needed for a research study takes time away from other commitments, including caring for another child, school and/or work. Parents suggested that incorporating the exercise visit into patient wait times might make the study more convenient; however, this may come at the cost of standardized and identical visits for every participant (i.e., experimental design vs. practical considerations).

The most unexpected deterrent to participation was that patients were uncomfortable with having their chemotherapy ports accessed for the duration of the exercise visit (for ease of blood draws), which was reported by 5 of the 16 patients that declined participation **(Figure 1)**. We did not anticipate this issue in light of the fact that a) port access was performed using topical anesthetics, b) maintaining the port opened did not cause any additional physical pain, and c) children already experienced keeping their port accessed for longer durations of time (consecutively for ~2 weeks) at the start of their therapy. This barrier to participation was primarily reported by younger patients, ages 11 years and

younger in our sample, who may experience anxiety surrounding port access.[25] This is particularly important when considering that ALL is most commonly diagnosed in children between 1 and 4 years of age.[26] Nonetheless, adequate blood sampling is critical for immune research, therefore, considerations should be made to minimize participant discomfort. This might include, but is not limited to, engaging the clinical team to ensure patients and families have access to and are using appropriate coping strategies to help manage fear and anxiety surrounding chemotherapy port access.[27] While our results focus on children with ALL, central venous catheter devices are used in the treatment of a large variety of pediatric cancers. As such, these observations may also be relevant to other patient populations. Finally, a single eligible patient cited that side effects of the treatment as a barrier to participation; however, no patients said they were deterred by the potential side effects or risks of exercising.

Overall, 18% of patients approached participated in our study. Our recruitment rate was lower than that of a similar acute exercise study in pediatric ALL patients which reported a 44% recruitment rate, however this study only required 2 visits.[28]

Based on our experience, we recommend recruitment from multiple sites to achieve a sufficient sample size within a reasonable timeline. This may not be a significant concern for hospitals with larger patient volumes. Despite consistently low recruitment rates in pediatric exercise immunology studies, the available exercise immunology studies in children with cancer underreport the

barriers to recruitment. We hope our recruitment information can help direct attempts to increase participation; however, study sites may wish to also examine potential site-specific barriers.

Feasibility of retention and participant testing:

All participants attended and completed all of the required study visits. It is important to note that participants enrolled in our study were particularly motivated to exercise. One of our participants bought a bicycle after their first visit so they could start biking to school, while another offered to perform additional exercise visits after completing the study. Moreover, the proximity of our laboratory to the oncology clinic and the availability of our staff allowed for swift rescheduling and participant accommodations. We recommend using a flexible study design that aligns with clinic visits to accommodate patient and family schedules.

Feasibility and acceptability of exercise:

All participants were able to complete the exercise and found it enjoyable. We chose stationary cycling for this study because it is familiar for most participants and the seated exercising position minimizes the risk of injury. Naturally, recruitment bias may favorably skew our results, as typically children who enjoy exercise, or are otherwise motivated to exercise, will participate in such studies. Thus, it is possible that while our small cohort found the exercise to be feasible and enjoyable, a random sampling of patients with ALL might not agree. Notwithstanding this, other exercise intervention studies also reported that

a moderate-to-vigorous intensity cycling exercise is feasible for children treated for ALL.[7] In our study, participants were able to watch a movie or show of their choice while cycling, which often helps distract participants from the exertion they feel during exercise.

It is still important to recognize that children treated for cancer face unique barriers that may influence the feasibility of performing exercise and being physically active. These include, but are not limited to, physical health barriers (e.g., nausea, lower fitness), psycho-social barriers (e.g., feeling self-conscious, no friends to be active with), organizational barriers (e.g., no time, lack of sports equipment).[29] While a structured exercise intervention study may assuage some of these barriers, many patients may still be reluctant or unable to exercise. Therefore, although this cycling intervention was appropriate for children motivated enough to participate in an exercise study, application to a clinical setting will likely require a more individualized approach.

Feasibility and acceptability of accelerometer wear:

Accelerometer wear and log completions rates in this study were acceptable. It is important to highlight that 3 of 4 participants had average wear rates above 82% and log completion rates above 69%; however, with our small sample size, a single participant (B-02) with extremely low wear and log completion rates was able to significantly impact the group averages **(Table 4).** When participant data was assessed for valid daily data (≥6 hours a day for ≥3 days per assessment period), one participant had only 1 valid physical activity

assessment period. Although accelerometer wear log rates met the acceptability goal, it is important to note that one participant did not return two of his logs, and another had 3 logs that were unusable.

Overall, it is possible that asking our participants to wear the accelerometer for such long periods of time was overwhelming or made them less likely to adhere to instructions. In healthy children, accelerometers are traditionally worn over a 7-day period to estimate habitual physical activity levels.[30,31] Given the cyclical nature of cancer treatments and treatment-related side effects, physical activity patterns in children with cancer may not be as consistent as those observed in healthy children. Therefore, we opted to measure free-living activity over a 12-week period (entire study duration). We recommend that wear periods that exceed the traditional 7-day wear period should explore an electronic interface to track accelerometer wear times. Such an interface might allow for real-time updates, and reminders to encourage completion and avoid the loss of physical logbooks. Similarly, exploring the use of research-grade or commercially available wrist-worn accelerometers, as suggested by our participants, may be less intrusive alternatives that are sufficient to provide an estimate of free-living movement profiles.

Safety:

Altogether, there were no adverse events associated with exercise. Therefore, the moderate intensity cycling exercise protocol was deemed to be safe for our participants. Despite our small sample size, this finding is in

agreement with previous exercise studies in pediatric ALL patients.[7] We recommend that clinicians and researchers consider how the patient feels on a day-to-day basis, and to adjust the exercise task accordingly.

A few noteworthy limitations to consider when interpreting our results are the small sample size, a lack of female participants, a large participant age range, and finally, the time frame our participants were followed for (12 weeks of a 2- to 3-year long therapy) **(Table 1).** Despite these limitations, a key strength is the pragmatic nature of our study, including our broad eligibility criteria and the flexible study visits scheduled together with clinic visits. We believe this design will provide information that is most practical for clinical use.

2.6. CONCLUSION

Overall, we found that participant recruitment was not feasible for this site and timeline, while retention of participating patients was feasible. The proposed cycling exercise protocol was found to be feasible, acceptable and safe for our participants. Likewise, accelerometer wear was found to be acceptable. When conducting an exercise immunology study in this population, we recommend that investigators consider strategies to address the recruitment barriers, as outlined above, as well as collaborate with multiple sites to achieve the required sample size to adequately address research objectives.

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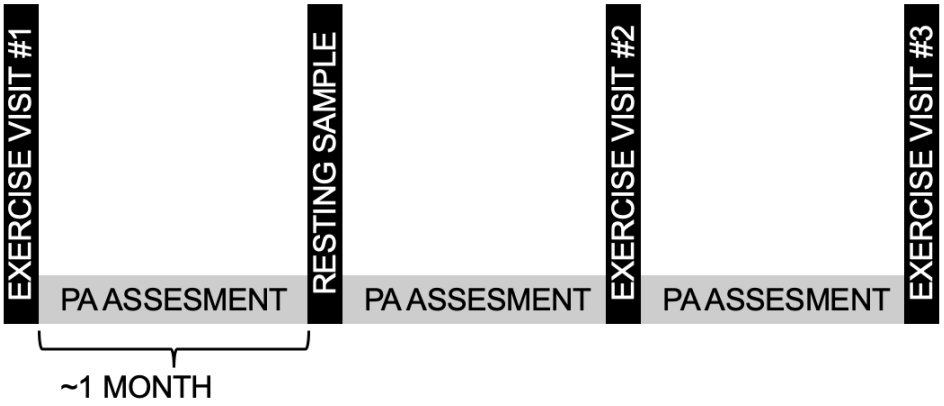
2.9 SUPPLEMENTARY MATERIALS



Supplementary figure 1. Study outline schematic.
Participants attend 4 study visits each a month apart: 3 exercise visits and 1 resting blood sample visit. After each visit (except exercise visit 3) participants were given an accelerometer to wear for ~1 month (PA [physical activity] assessment), until their next study visit.

CHAPTER 3: EXAMINING THE EFFECTS OF ACUTE EXERCISE ON NATURAL KILLER CELL QUANTITY, FUNCTION, AND RECEPTOR EXPRESSION IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA AND HEALTHY CONTROLS

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3.1. ABSTRACT

Introduction: Children treated for acute lymphoblastic leukemia (ALL) are immunocompromised and therefor at an increased risk of infection and cancer recurrence during treatment and beyond. A single bout of exercise can boost natural killer (NK) cell number and function in healthy people and other clinical populations. However, little is known about the effects of acute exercise on NK cells in children treated for ALL. The aims of this study were to assess the effects of acute exercise on NK cell 1) number 2) function and 3) receptor expression in children treated for ALL compared to healthy counterparts. Methods: Participants (4 children with ALL undergoing maintenance therapy and 4 healthy controls, CNT) completed 30 minutes of continuous cycling, followed by 1 hour of rest.

Blood was collected at 3 timepoints: at rest before exercise (PRE), immediately after exercise (POST), and after 1 hour of recovery (REC). Blood samples were used to quantify NK cells, NK cell activation in response to K562 and PMA/Ionomycin stimulation, and NK receptor expression. Descriptive statistics were used to report all data. Results: There was an increase in NK number (PRE to POST in ALL: 110% CNT: 138%) and activation (PRE to POST in ALL: 39%, CNT: 56%) with exercise in CNT and some children treated for ALL. PMA/Ionomycin-induced activation increased with exercise in children treated for ALL but showed a transient decrease in CNT. There were no clear patterns in receptor expression with exercise in either group. This study suggests that exercise may be able to favorably modulate the immune system of children undergoing ALL treatment.

3.2. INTRODUCTION

Between 2014 and 2018, approximately 539 children aged 14 and under survived ALL in Ontario (POGO). Today, treatment with multi-drug chemotherapy yields 5-year survival rates of >90%; however, patients remain burdened with side effects.[1] Most notably, the cytotoxic treatment can induce immunosuppression, thereby putting pediatric ALL patients at an increased risk of infection and cancer development.[2,3] Such side effects may persist even beyond treatment and into adulthood, with adult survivors of childhood ALL experiencing higher incidences of infection and cancer occurrence than sex- and age-matched

controls.[4,5] Therefore, it is important to consider interventions to bolster immune health in pediatric ALL patients during and after therapy.

Recent and on-going interventions are exploring methods to restore or amplify natural killer (NK) cell function for cancer patients, both as a curative treatment and adjunct therapy.[6,7] Indeed, NK cells, a subset of innate cytotoxic lymphocytes, have gained notoriety for their key role in immunosurveillance against cancerous and virally infected cells.[8] Most notably, NK cells therapies can target MHC void targets and have not displayed evidence of significant toxicity or an increased risk of infection.[9] NK cell activity is mediated by activating and inhibitory NK cell surface receptors, which convey secondary signals upon interaction with cognate ligands expressed on target cells.[8] Ultimately, an integration of these signals will either inhibit NK cell activation or induce degranulation of pre-formed cytolytic granules and/or cytokine production.[8] In this way, NK cells are able to detect and eliminate target cells expressing stress signals and potentiate the immune response without prior antigenic exposure.[8] Existing evidence suggests that children with ALL present with abnormally low NK numbers and function during therapy and beyond.[10–12] Identifying strategies to improve NK cell number and function in children with ALL may have important implications for overall health in this population.

Exercise is a simple and accessible intervention to improve NK number and function. More specifically, NK cells are the most responsive immune cell to exercise, likely due to their abundance of β_2-adernergic receptors. Numerous

studies in healthy adults and children have demonstrated that a single bout of exercise can transiently (up to ~1 hour) increase NK cell number.[13] Likewise, various studies in adults and a limited number of studies in healthy children have demonstrated that acute exercise may temporarily increase NK cytotoxic activity.[14–17] In a recent exercise training intervention in children treated with hematopoietic stem cell transplant for malignancies and immunodeficiencies, Chamorro-Viña reported that children in the exercise group had 8 times greater NK cell cytotoxicity at rest than non-exercising counterparts.[18] Taken together, it appears that while the effects of an acute bout of exercise are transient, repeated exposures to the exercise stimulus can lead to enduring improvements in NK number and function. The extent to which these promising findings also apply to children with ALL remains unknown.

As such, the objectives of this study were to assess the effects of acute exercise on NK cell number, function and receptor expression in pediatric ALL patients compared to healthy controls. An exploratory objective was to assess the relationships between receptor expression and NK function with exercise. Given the resource intensive nature of exercise training studies, we opted to first investigate acute exercise as a preliminary model to gain insight into the potential benefits of chronic exercise for children with ALL.

3.3. METHODS

Participant recruitment:

Children aged 7 to 18 years undergoing maintenance chemotherapy for ALL (hereafter referred to as ALL) were recruited from McMaster Children's Hospital to participate in a pilot study. Eligible patients presented no evidence of disease relapse, were cleared by a physician for exercise, had no conditions that would prevent understanding of or cooperation with the study, and had at least 4 months of maintenance therapy remaining. All participants and/or their parent/guardians provided informed assent and/or consent upon enrolling in this study, which was approved by the Hamilton Integrated Research Ethics Board. Healthy children aged 8-11 or 15-18 years were recruited from the Hamilton community as part of a concurrent study also examining NK number and function is response to exercise. Eligible healthy controls were recreationally active (defined as participating in organized sport 2-3 times a week), with a healthy weight status (defined as a body mass index within the 5^{th}-85^{th} percentiles), were not taking any medication, had no diagnosed or suspected medical conditions, and no acute illness for at least 2 weeks prior to participation. Healthy participants (hereafter referred to as CNT) were matched by sex and pubertal status to the ALL group.

Study overview:

During maintenance therapy, patients being treated for ALL attend the oncology clinic every 4 weeks to receive a chemotherapy infusion through their central

venous catheter. To minimize participant burden, study visits were scheduled to happen immediately after their morning clinic visit, which took place between 9:00-11:30 AM. Participants were escorted to the exercise lab where standing and seated height, weight and body fat were assessed using a wall-mounted stadiometer (Harpenden Stadiometer 2109, CMS Weighing Equipment Ltd, London, UK), digital scale (Tanita BWB-800, Tokyo, Japan), and bioelectrical impedance analyzer (InBody 570 Cerritos, California, USA), respectively. Next, participants completed 30 minutes of continuous, moderate intensity cycling on a stationary bicycle, followed by a seated rest period of 60 minutes. Blood samples were collected at 3 time points: pre-exercise at rest (PRE), immediately post-exercise (POST), and after 60 minutes of recovery (REC). CNT participants completed a similar protocol, with the exception of the timing of study visits, which were all completed immediately after school. Blood samples from both groups were analyzed for NK cell concentrations, function, and receptor expression, as described below.

Exercise protocol:

ALL and CNT completed 30 minutes of continuous cycling on a height-appropriate cycle ergometer (Corival and Corival Pediatric, Lode, Groningen, Netherlands). Children were asked to maintain a cadence of 60 to 80 revolutions per minute, and workload was adjusted to maintain a moderate-to-high exercise intensity, with a heart rate between 140 and 160 beats per minute (bpm).[19,20] This exercise intensity was chosen to ensure that participants who may be

deconditioned would be able to complete the exercise, while also providing a sufficient physiological stimulus to elicit NK cell recruitment.[13] Heart rate was monitored and recorded every minute using a chest-worn Polar heart rate monitor (Polar Electro OY, Kempele, Finland). The exercise was timed and any adjustments to workload or breaks throughout the 30-min cycling period were recorded. Children were allowed to watch a movie or TV show of their choice while cycling and during recovery.

Blood analysis:

At each time point, 2 × 10-mL (1 × 10-mL for CNT) blood samples were collected in EDTA-coated vacutainers and kept on ice until further processing (~3-4 hours from PRE). NK cell concentrations were measured from fresh peripheral blood mononuclear cells (PBMCs) for ALL and from fresh whole blood for CNT. PBMCs were isolated from blood samples using a density gradient cell separation medium (Ficoll Histopaque, Sigma-Aldrich Co., Darmstadt, Germany), as directed by the manufacturer. PBMCs not used for NK quantification were cryopreserved until assessment of NK receptor expression and function at a later date. For ALL, an additional 1 × 2-mL blood sample was collected in EDTA-coated vacutainers at each time point and sent to the McMaster Children's Hospital Core Laboratory for complete blood count analysis.

NK quantification from PBMCs (ALL group): Freshly-isolated PBMCs were resuspended in 3 mL of MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and 100 µL ($<1x10^6$ cells) was used to quantify NK cells. PBMCs were

stained using fluorescently-conjugated antibodies: 2uL anti-human CD3-VioBlue (REA613, Miltenyi Biotec) and 2uL CD56-PE (REA196, Miltenyi Biotec) incubated for 20 min in the dark at 4ºC. Stained cells were fixed (Fixation Buffer, BD biosciences, San Jose, USA) and analyzed by flow cytometry (MACSQuant Analyzer, Miltenyi Biotec) within 1 week of acquisition **(Gating strategy outlined in Supplementary Figure 1A,B)**. $CD3^-/CD56^+$ NK cells were expressed as a percentage of total lymphocytes, and this percentage was then applied to the lymphocyte count from the complete blood count analysis to determine NK concentrations in whole blood. $CD3^-/CD56^{DIM}$ NK cells were also quantified and expressed as a percentage of total NK cells.

NK quantification from whole blood (CNT group): Since CBCs were unavailable for CNT, NK cells were quantified by staining whole blood. For each time point, 100-µL of whole blood was stained with 2 µL of anti-human CD3-VioBlue, CD56-PE, and CD45-APCVio770 (REA747, Miltenyi Biotec) and incubated for 20 min in the dark at 4ºC. Erythrocytes were lysed (BD Pharm Lyse, BD Biosciences, San Jose, USA) as directed by the manufacturer. Samples were fixed (Fixation Buffer, BD Biosciences, San Jose, USA) and assessed using flow cytometry within 1 week of acquisition **(Gating strategy outlined in Supplementary Figure 2)**. NK cells were expressed as proportions (percentage of total lymphocytes), and as a concentration normalized to the volume of the blood sample (100 µL) and reported as NK cells per mL of whole blood. The agreement between PBMC and whole blood NK counts was determined by

comparing a single blood sample (N=4) that was processed using PBMCs and whole blood NK quantification methods, as described above (additional details of this comparison reported in Supplementary Chapter A). A Bland-Altman test suggested comparable NK levels when using PBMCs and whole blood, with limits of agreement from -1.65 to 3.79% NK of total lymphocytes.

NK function: Cryopreserved PBMCs were thawed and cultured overnight (12-18 hours), then split into aliquots for measurement of NK function and receptor expression. The following morning, PBMCs were re-suspended to 5×10^5 c/mL and 100µL of suspension was used for each sample (tube) of the functional assay. The remaining cells were used for receptor analysis (5×10^4 to 1×10^6 cells per tube, including FMO tubes). NK function was measured by assessing NK activation rates using CD107a expression in response to stimulation with target cells and PMA/Ionomycin. To do this, we co-incubated participant PBMCs with K562 cells (HLA-null immortalized myelogenous leukemia cell line; ATCC, Manassas, USA) in an effector to target ratio of 10:1. These samples were run in duplicates and averages were reported. K562 induces NK activation via interaction with its surface receptors and serves to mimic physiological activation. In a second tube, an equal number of participant PBMCs were co-incubated with 100µL of PMA (0.15 µg/mL) and 100 µL of Ionomycin (3 µg/mL), a positive control that induces maximal NK cell activation independent of surface receptor interactions. Due to a limited number of cells available from ALL, a single positive control was performed. For CNT, positive

controls were performed in duplicates and averaged. Finally, spontaneous activation (negative control) was examined by incubating only PBMCs and complete medium. In order to facilitate sample analysis by flow cytometry, an unstained control and CD107a fluorescence minus one (FMO) control were also included. A summary of the samples used in the NK functional assay can be found in **Supplementary Table 1.**

CD107a-APC (REA792, Miltenyi Biotec) was added to all samples except the FMO and unstained controls, and all samples were incubated in the dark at 37ºC in 5% CO_2 for 1 hour. Next, Golgistop (BD Biosciences, New Jersey, USA) was added to all samples, which were then incubated for 3 more hours in the same conditions. When the incubation period was complete, samples were placed on ice to halt the reaction and stained with an antibody cocktail containing 2-µL CD3-VioBlue and 2-µL CD-56PE per sample. Samples were analyzed by flow cytometry immediately after staining **(Gating strategy outlined in supplementary figure 1A,C).** NK function results were reported as net NK activation in K562-induced and maximal activation samples. Net NK activation was calculated by subtracting spontaneous NK activation rates (%NK $CD107a^+$ of total NK) from each of the NK activation rates reported in the K562-induced and maximal activation samples. Some groups have chosen to report NK activation as a percent of maximal activation; however, we find that reporting the two values separately provides more insight into how function changes with exercise. Median fluorescence intensity (MFI) of $CD107a^+$ NK was also reported as an

indicator of protein expression density. MFI values were reported as a net value, calculated as ($MFI_{SAMPLE} - MFI_{FMO\ CONTROL}$).

NK receptors: Receptor analysis was performed in parallel with the functional assay. To assess NK receptor expression, PBMCs were stained with an antibody cocktail containing of each of the anti-human antibodies (2 µL of each antibody per sample) listed in **Table 1** and incubated for 20 min in the dark at 4ºC. All samples were fixed (Fixation Buffer, BD biosciences, San Jose, USA) and assessed using flow cytometry within the same day**.** $CD3^-/CD56^+$ NK cells were identified and further subdivided into $CD56^{DIM}$ or $CD56^{BRIGHT}$ NK cells as outlined in **Supplementary figure A**, **B.** The entire NK cell population was assessed for inhibitory (NKG2A) and/or activating (NKp46,DNAM-1, NKG2D, CD16) receptor expression. Fluorescence minus one (FMO) controls were used for to determine thresholds for positive and negative events for each receptor marker except CD3 and CD56, which could be clearly gated using unstained controls. To assess NK cell receptor expression, $CD3^-/CD56^+$ events expressing the receptor marker of interest at a fluorescence above that of the FMO control were considered positive. Results were reported as a proportion of total NK cells expressing the receptor (% $receptor^+$ of total NK cells). As described with CD107a MFI, background fluorescence was subtracted, and net MFI values were presented as an indicator of receptor density.

Table 1. NK receptor analysis panels

Unstained control	**Panel 1**	**Panel 2**
No markers	CD 3-VB [M] (REA613)	CD 3-VB [M] (REA613)
	CD56-PE [M] (REA196)	CD56-PE [M] (REA196)
	NKG2A-FITC [I] (REA110)	NKp46-APC [A] (REA808)
	CD16-PerCPVio 700 [A] (REA423)	DNAM-1-FITC [A] (REA1040)
		NKG2D-PeVio770 [A] (REA797

Each receptor is displayed the respective fluorochrome used for flow cytometry analysis. [A] Activating receptor, [I] Inhibitory Receptor, [M] NK marker. All antibodies were anti-human and procured from Miltenyi Biotec.

Statistical analysis:

All data are reported using descriptive statistics to avoid misrepresenting our small sample size. Data are presented as means (ranges), and where appropriate standard deviations. For NK quantification and functional data, the area under the curve (AUC) of each group's average response was calculated and used to compare the magnitude of the exercise response between ALL and CNT. Since CNT present higher NK levels and activation at baseline, PRE, POST, and REC values were normalized to each participant's PRE values to allow us to better detect the magnitude of the exercise response for each participant. Differences in the magnitude of the exercise response between groups were reported as fold change. All graphs were created using Prism 5.0a (GraphPad Software).

3.4 RESULTS

A total of 4 ALL participants completed the study; all were males and between 1.3 to 1.6 years into maintenance therapy. CNT were matched by sex and pubertal status (before or after age at peak height velocity, an estimate of pubertal growth spurt), and on average 0.6 (-0.9 to 1.5) years older than ALL. Participant characteristics are provided in **Table 2**. Both ALL and CNT completed the full 30-min of cycling with no breaks. Heart rates during exercise were comparable between groups, with ALL experiencing an average (± standard deviation) heart rate of 150 ± 20 bpm and CNT an average of 140 ± 17 bpm **(Supplementary table 2).**

Table 2. Participant characteristics

Mean (Range)	ALL	CNT
N (% male)	4 (100%)	4 (100%)
Age (years)	13.4 (8.7-18.8)	14.0 (10.2-17.8)
YPHV	-0.59 (-4.14-3.63)	0.40 (-2.93-3.67)
Height (m)	1.53 (1.30-1.82)	1.61 (1.42-1.81)
Height %ile	46.3 (7.6-79.1)	66.4 (38.6-84.3)
Weight (kg)	45.8 (23.9-64.2)	56.2 (34.8-83.0)
Weight %ile	41.8 (11.9-76.1)	70.2 (62.2-89.3)
BF %	16.4 (7.9-29.5)	14.6 (8-20.9)
BF %ile	38.9 (1.9-86.2)	36.1 (1.5-68.8)
Weeks into maintenance	76 (68-82)	N/A

Data are presented as mean (range), unless otherwise indicated. YPHV, years from age at peak height velocity was used as an indicator of pubertal status and calculated using a maturity offset equation by Mirwald et al., where negative values indicate the participant has yet to reach the estimated age at peak height velocity (i.e., pubertal growth spurt), while positive values indicate the participant is past their estimated age at peak height velocity; BF, body fat; %ile, percentile; weight and height percentiles based on normative data from the CDC, body fat percentiles based on normative data from Laurson.

NK cell concentrations:

In ALL, there was a trend towards an increase in NK cell concentrations and NK proportions (% of lymphocytes) from PRE to POST exercise, followed by a decrease from POST to REC (**Figure 1A and C; Supplementary Figure 3 shows enlarged graph**). CNT demonstrated a similar trend but presented with higher NK cell concentrations at each time point (PRE: 4.6x, POST 5.3x, REC 4.9x relative to ALL) as well as greater NK proportions (PRE: 1.9x, POST 2.0x, REC 1.6x relative to ALL) (**Figure 1A-D)**. The overall magnitude of the change in NK concentrations with exercise and recovery was comparable between groups, with CNT demonstrating a 1.1-fold greater change in NK compared with ALL. Similarly, the magnitude of change from PRE to POST alone was 1.2-fold greater in CNT compared with ALL. Finally, both groups showed a slight increase in the proportion of $CD56^{DIM}$ NK cells with exercise that dropped slightly below baseline

by recovery **(Figure1 E,F)**.

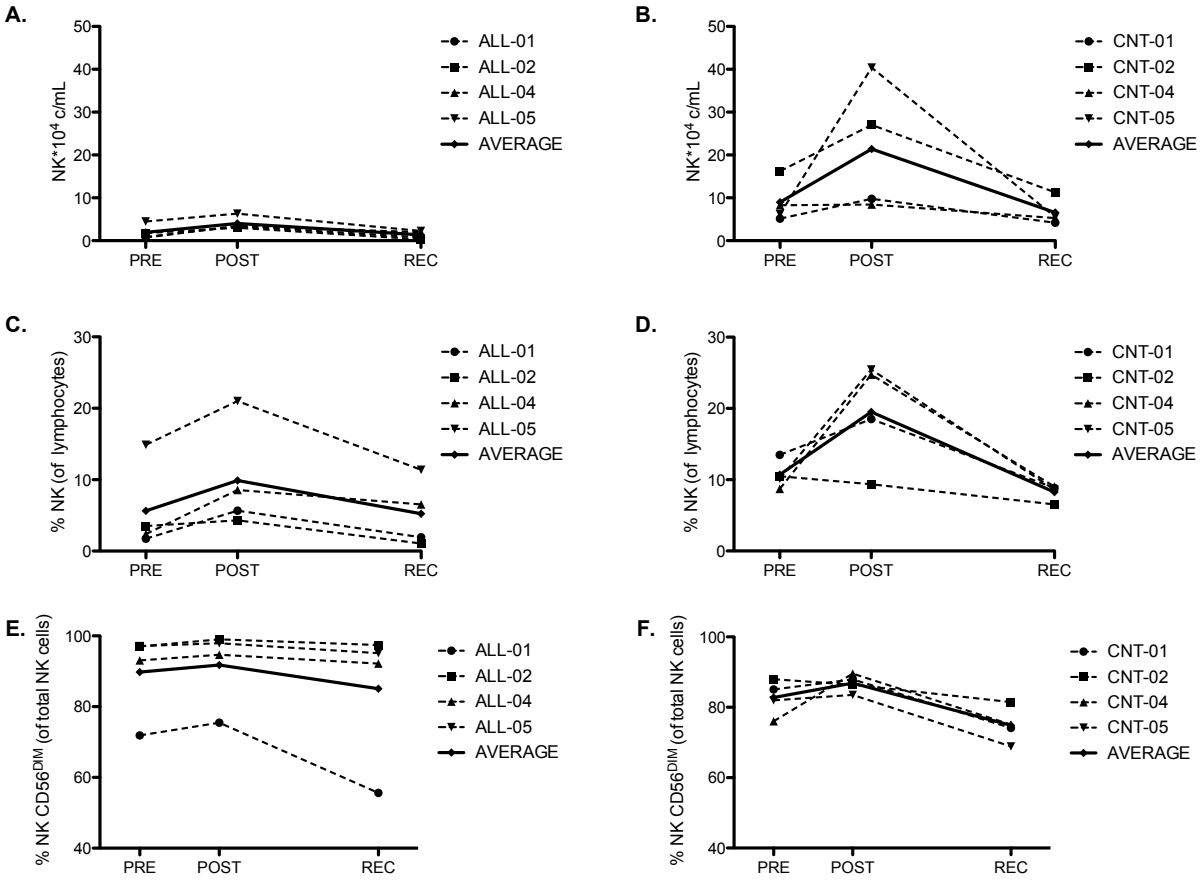

Figure 1. NK cell levels in peripheral blood in response to a bout of exercise. Each participant is depicted by a different symbol and dashed line and the average of all participants in the group is displayed with a thick solid line. NK concentrations **1A.** in ALL (Average AUC=4.29) and **1B.** in CNT (Average AUC=4.82). NK cells as a percent of lymphocytes **1C.** in ALL (Average AUC=4.07), and **1D.** in CNT (Average AUC=4.00). Proportion of total NK cells that were $CD56^{DIM}$ **1E.** in ALL and **1F.** in CNT.

<u>K562-induced NK cell activation:</u>

NK activation with exercise was inconsistent in ALL. Two participants experienced an increase in NK cell activation from PRE to POST and a decrease from POST to REC. Conversely, 2 participants showed decreased NK cell

activation from PRE to POST, one participant decreased POST to REC and one participant remained constant (at 0%) **(Figure 2A)**. Contrarily, CNT demonstrated a clear trend: an increase in NK cell activity from PRE to POST, followed by a decrease from POST to REC **(Figure 2B)**. CNT had greater NK activation at each time point compared with ALL (PRE: 3.5x, POST: 3.9 x, REC: 19.6x). Overall, CNT also had a 1.5-fold more robust NK response to exercise than ALL. There was no clear pattern in the change of CD107a MFI of activated NK cells with exercise in ALL or CNT **(Figure 2C,D)**.

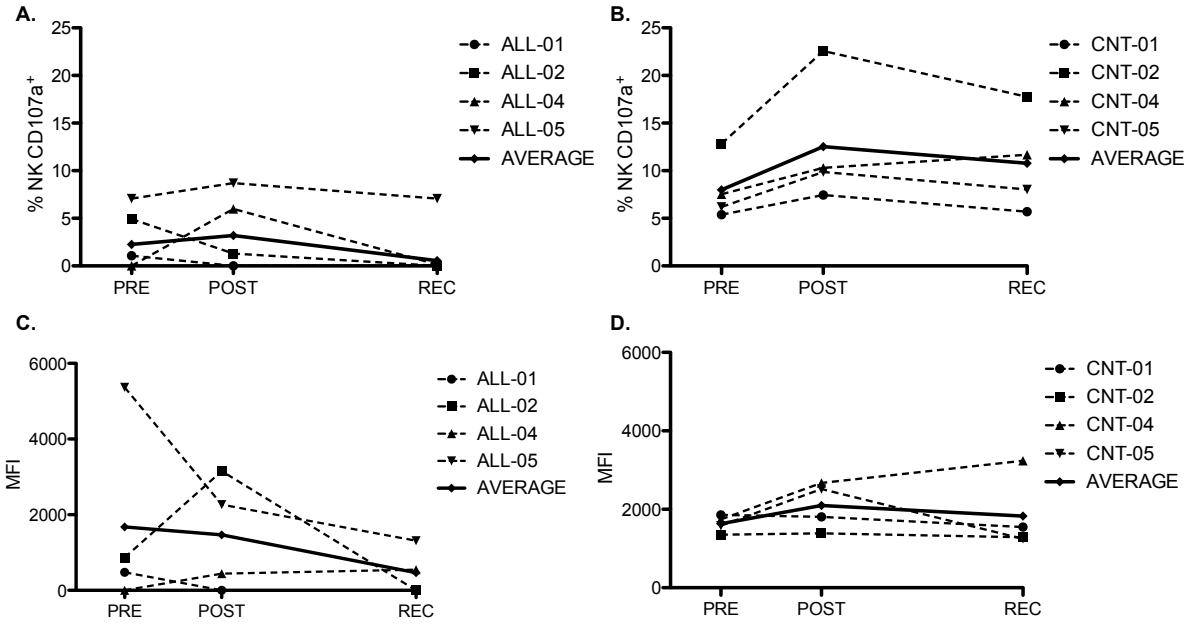

Figure 2. K562-induced NK cell activation in response to a bout of exercise. Intra-assay CV=15.2%. Each participant is represented with a different symbol and dotted line, and the group average is displayed with a thick solid line. The y-axis shows activated NK cells as a percent of total NK cells in the sample (%NK CD107a$^+$). Higher %CD107a$^+$ NK values indicate more activated NK cells (i.e., degranulating and/or secreting cytokines). NK activation **2A.** in ALL (AUC=2.86), and **2B.** in CNT (AUC=4.21). CD107a MFI of activated NK cells **2C.** in ALL, and **2D.** in CNT.

Maximal (PMA/Ionomycin-induced) NK cell activation:

As expected, maximal activation values were higher than those elicited with K562 stimulation. There was a steady trend in maximal activation with exercise in ALL: an increase in activation from PRE to POST and POST to REC in three of four participants **(Figure 3A)**. Meanwhile, CNT presented a trend that was inverse to their K562-induced activation results: there was a decrease in activation from PRE to POST followed by an increase from POST to REC **(Figure 3B).** Here, maximal activation in CNT was only slightly higher than in ALL (PRE: 1.3x, POST: 1.0x, REC: 1.1x). Despite clear trends in NK activation for both groups, there were no consistent, discernable trends in CD107a MFI (protein expression density) with exercise **(Figure 3C,D)**. Average CD107a MFI in CNT was slightly higher than ALL for most timepoints (PRE: 0.9 POST:1.4x REC: 2.7x).

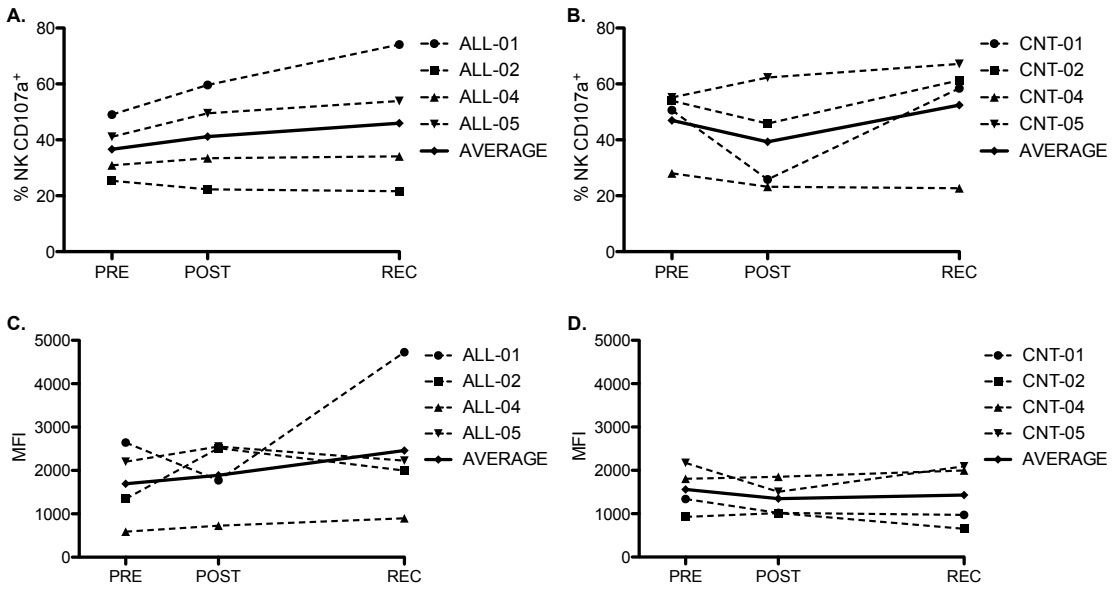

Figure 3. Maximal (PMA/Ionomycin-induced) NK cell activation in response to an acute bout of exercise. Intra-assay CV=7.1%. Each participant is presented with a different symbol and dotted line and the group average is displayed with a thick solid line. The y-axis shows activated NK cells (i.e., $CD107a^+$ events) as a percent of total NK cells in the sample (%$CD107a^+$ NK). Higher %$CD107a^+$ NK values indicate more activated NK cells (i.e., degranulating and/or secreting cytokines). NK activation **3A.** in ALL, and **3B.** in CNT. The y-axis for 3C and 3D shows CD107a MFI. Higher MFI values are related to more granular vesicle release, greater perforin concentrations, and/or more cytokine secretion. CD107 MFI **3C.** in ALL, and **3D.** in CNT.

<u>NK cell receptor expression:</u>

There were no clear patterns in the fluctuation of NK receptor expression with exercise in ALL or CNT. A summary of NK receptor expression in response to acute exercise is displayed in **Figure 4.** Expression of NKG2D, NKG2A and DNAM-1 was different between ALL and CNT. The proportion of NKG2D expression in ALL was consistently lower than in CNT, with 2 of 3 participants lacking NKG2D positive events at multiple timepoints. Meanwhile, ALL consistently exhibited greater NKG2A MFI than CNT. Finally, ALL showed

persistently higher DNAM-1 MFI than CNT. Results of all NK receptors analyzed are summarized in **Supplementary Table 3.**

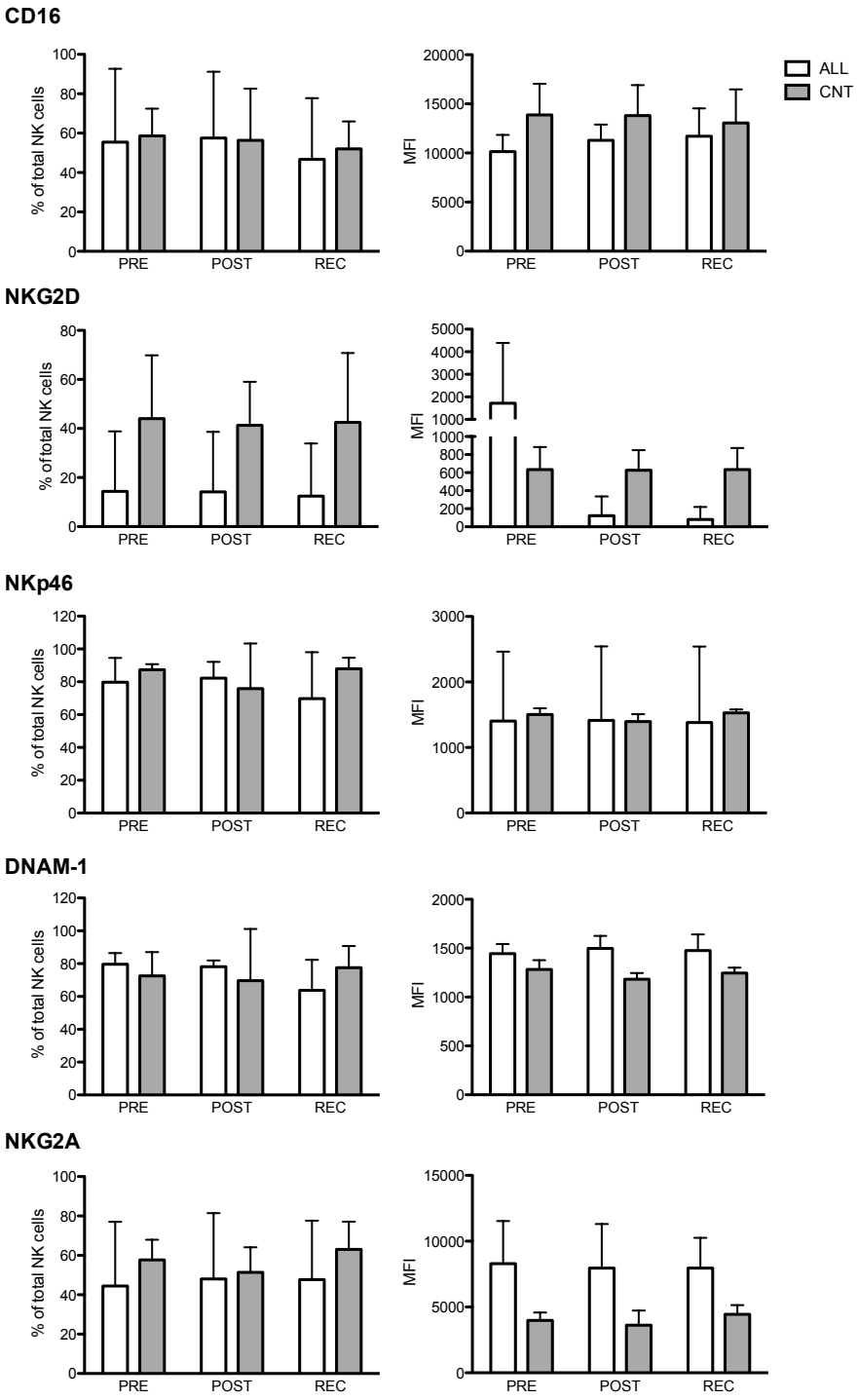

Figure 4. NK cell receptor expression in response to a bout of exercise. Data shown as the average proportion of NK cells expressing the specified receptor (% receptor^{+} of total NK cells) as well as the average net MFI of each receptor. ALL are presented in white, while CNT are shaded. ALL-04 not included due to

insufficient sample to perform analysis, CNT-01 not included in NKG2D, NKp46 and DNAM-1 due to technical error.

Of the receptors assessed, ligands for NKG2D, DNAM-1 an NKG2A are all expressed on K562 cells; however, only fluctuations in NKG2A expression corresponded with changes in NK activation in both CNT and ALL. The increase in the proportion of NK cells expressing NKG2A coincided with an increase in the proportion of NK $CD56^{DIM}$ cells, a decrease in NK function from PRE to POST in ALL participants. Meanwhile in CNT, a dip in NKG2A expression from PRE to POST (both as a proportion of NK cells expressing NKG2A and NKG2A MFI) corresponded with an increase in in the proportion of NK $CD56^{DIM}$ cells and NK cell activation in CNT and vice versa from POST to REC **(Figure 5B)**.

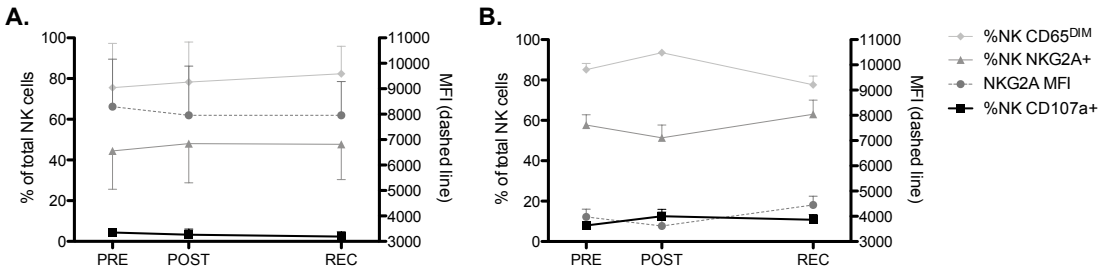

Figure 5. Expression of NKG2A as a percentage of total NK and NKG2A MFI compared to K562-induced activation with acute exercise in ALL and CNT. Average NKG2A expression and NK activation **4A.** in ALL (N=3), and **4B.** in CNT (N=4)

3.5. DISCUSSION

This is the first study to provide preliminary evidence that acute exercise can transiently increase NK cell number and K562-induced activation in pediatric patients treated for ALL, and that this response was comparable to that of healthy counterparts. Exercise-induced changes in NK activation corresponded with

fluctuations in NKG2A expression but not NKG2D or DNAM-1 expression. Moreover, our findings suggest that acute exercise may modulate NK function downstream of receptor expression in both pediatric ALL patients and healthy children.

Both CNT and ALL exhibited a transient increase in circulating NK cell levels in response to a bout of exercise (**Figure 1)**. Exercise-induced NK cell mobilization into the circulation is a well-documented phenomenon in many populations and is thought to occur in response to a combination of increased endothelial shear stress and catecholamine concentrations.[13,21] Similarly, our finding that ALL had fewer NK cells than CNT at every timepoint is consistent with existing literature documenting that children with ALL present with abnormally low NK cell levels during treatment, a side effect that may persist for more than 6 months after the cessation of maintenance therapy.[10,11]

It is interesting to note that despite lower NK cell levels in ALL patients, the *magnitude* of NK cell recruitment with exercise was similar in ALL and CNT. It is commonly thought that acute exercise increases circulating NK cell concentrations by recruiting them from depots rather than by inducing cell proliferation.[21] Given that the cytotoxic therapies used to treat ALL are systemic and not target-specific, it is plausible that NK depots are depleted. Moreover, to our knowledge, there is no evidence to suggest children treated for ALL would have lower catecholamine production in response to exercise, nor that their NK cells would be less susceptible to the adrenergic effects of catecholamines. In

fact, the prednisone administered during maintenance therapy can potentiate the effects of catecholamines, as reported in adults with asthma.[22] Finally, the exercise stimulus was comparable between groups (**Supplementary table 2**), suggesting that shear stress would also be matched between groups. Altogether, it is possible that the NK response to exercise in children with ALL is similar to that of healthy children, and that this response is simply in proportion to their available NK stores.

In this study, ALL demonstrated noticeably lower NK activity compared with CNT at every time point. Currently, literature on the topic is confounding- some suggest NK function is already partially normalized in maintenance therapy while others suggest NK cell function can remain supressed even 12 months post therapy.[11,12] While many studies agree acute exercise can modulate NK function, there is no consensus on the timing or magnitude of the response. For instance, Boas et al. have demonstrated a temporary increase in NK cell cytotoxic function, reported in cytolytic units, against K562 cells immediately post-exercise in children.[14,15] These results align with our findings that CNT consistently demonstrated a transient increase in K562-induced NK cell activation with exercise, while only half of ALL shared that response (**Figure 2)**. It is important to note that in our study, NK function was reported a *percent of total NK cells*, and therefore fluctuations in activation were independent of changes in the concentration of NK cells with exercise. However, multiple studies in adults contest that NK cytotoxicity increased immediately post-exercise as a result of

the increase in NK cell concentration, whereas NK cytotoxic function on a per cell basis only increases by 1-hour post-exercise.[16,23] These results in adults are inconsistent when tested against the same target, and as expected, differ depending on the properties of the target cells (i.e., different ligand expression) used to assess cytotoxicity.[16,23] Current literature suggests that acute exercise primarily modulates NK function by preferentially recruiting more mature, cytotoxic NK cells ($CD56^{DIM}$ cells).[13,21] This preferential recruitment would lead to an increase in the ratio of $CD56^{DIM}$: $CD56^{BRIGHT}$ cells in circulation as well as a shift in NK cell receptor expression.

To complement our functional assay, we assessed NK surface receptor expression with respect to K562-activation. NK cell activation is mediated by an integration of activating and inhibitory signals from cell surface receptors interacting with their cognate ligands on target cells.[8] Therefore, is it important to consider both the proportion of NK cells expressing a receptor, but also the density of that receptor's expression on NK cells. As of yet, there are no methods to reconcile these numbers to provide a clinically relevant value. Of the receptors assessed in this study, only ligands of DNAM-1, NKG2D, and NKG2A are expressed on K562 cells.[24] Yet, only fluctuations in NKG2A expression corresponded to exercise-induced changes in NK activation **(Figure 6)**. Naturally, assessing function against target cells expressing different activating and inhibitory ligands may lead to NK activation via fluctuations in another receptor or receptor combinations.

As $CD56^{BRIGHT}$ cells mature into $CD56^{DIM}$, there is a progressive shift in receptor expression, including a progressive loss of NKG2A (targets non classical HLA-E, expressed on K562)and gain in CD16 is, through subsequent development NK cells acquire inhibitory KIR (targets classical MHC, not expressed on K562) and often lose NKG2A expression altogether.[8,25] With exercise, more mature NK cells are mobilized into circulation in a stepwise fashion, where the most highly differentiated NK cells (defined as $CD56^{DIM}$ /$NKG2A^{-}$/KIR^{+}) are preferentially mobilized with exercise.[23] Therefore, we would expect a dip in NKG2A expression post-exercise, as seen in our healthy cohort. However, the ALL group showed a preferential recruitment of $CD56^{DIM}$ cells, but an increase in NKG2A expression, and decrease in function against an HLA-E bearing target (**Figure 5A)**. This finding may be indicative of the availability of select NK cell phenotypes in depots in children treated for ALL.

Overall, NK cells of ALL patients expressed a higher density of NKG2A receptors than CNT at each study timepoint (Figure 4). The overexpression of NKG2A has already been documented in pediatric ALL and may contribute to the chronically low levels of NK cell activation we observed in response to K562 stimulation.[11] NK cells are poised to remain inactive in the absence of activation signals, and an inhibitory signal can override activation signals.[26] Blocking NKG2A receptors in certain types of cancer can re-establish NK lysis of HLA-E expressing targets.[27,28] Given our observations of elevated NKG2A receptor density together with low NK activation in response to K562 stimulation in ALL,

we propose that future research examine a more detailed inhibitory receptor repertoire with exercise in this population.

Zimmer et al. reported that epigenetic changes mediated an increase in NKG2D expression post-exercise (15 minutes and 24 hours) in adults.[29] However, our results did not indicate an increase in NKG2D expression 1-hour post-exercise in ALL nor CNT. Moreover, the majority of ALL had negligible levels of NKG2D. Interestingly, Rouce et al. noted a potential that pediatric ALL blasts may partake in shedding NKG2D ligands.[11] In studies examining adult malignancies, including ALL, overexposure to NKG2D ligand due to ligand shedding by cancer cells was identified as a potential mechanism that induced NK cell downregulation of NKG2D.[30,31] In our ALL group, chronically low expression of NKG2D may be another factor contributing to the overall low NK activation in response to K562 cells, which bear ULBP and MIC ligands (i.e., NKG2D ligands). This is especially important since NKG2D ligands are only upregulated on stressed cells, therefore NKG2D-mediated signaling can actually overcome inhibitory signals and induce NK activation.[8]Although acute exercise did not increase NKG2D expression in children with ALL, it may be worth exploring an exercise training interventions based on encouraging studies in healthy adults that demonstrate increase NKG2D expression at rest following an exercise training program.[32]

Both high NKG2A expression and low NKG2D expression are consistent with the receptor expression described in exhausted NK cells in people with

various cancers and chronic viral infections.[33,34] Thereby, the overexpression of inhibitory NKG2A (anti HLA-E) combined with the under expression of NKG2D (anti-MICA-B, ULB1-6) seen on the NK cells from children treated for ALL may point to an inhibitory NK phenotype, explaining lower overall activation levels.

Notably, the changes in NK activation were not consistent between the PMA/Ionomycin induced activation and K562-induced assays. Unlike the PMA/Ionomycin NK activation, NK activation in the K562-induced assay depends on 1) the presence of stimulating ligands on the target (K562) cells, and 2) the presence and function of NK surface receptors. Maximal NK activation assessed by incubating PBMCs with PMA/Ionomycin circumvents the need for NK surface receptor stimulation. Instead, the chemicals diffuse through the NK cell membrane and act directly to upregulate downstream signalling pathways (i.e., PKC, NFAT) that lead to NK activation. In CNT, maximal activation transiently decreased, while in the ALL group maximal activation steadily increased with exercise. Few studies have examined the effects of exercise downstream of receptor expression in any population. However, one potential explanation for the decrease observed in CNT might be lactate accumulation. Exercise induces a dose-dependent production of lactate from muscles, skin, adipose tissue and other sources that can decrease in blood and tissue pH.[35] In vitro analyses suggest that lactate may have an inhibitory effect on NK cells, altering expression of certain receptors, inhibiting cytolytic function, and lowering cytotoxic granule concentrations.[36] Other studies have determined that that lactic acid-mediated

intracellular acidification of NK cells can impair NFAT upregulation and energy metabolism needed for NK effector function , even when stimulated with PMA/Ionomycin.[37–39] Interestingly, post-pubertal CNT experienced a more pronounced decrease in NK maximal activation with exercise than pre-pubertal participants. This finding is consistent with the observation that post-pubertal children (and adults) also produce significantly more lactate in response to exercise, which may contribute to acidifying cells in a dose-dependent manner.[40] After 60 minutes of rest, blood lactate levels return to near-resting levels in adults and children alike; this aligns with our recovery sample, when NK cell maximal activation increases again in CNT.[40] The current study did not measure changes in circulating lactate nor intracellular pH levels with exercise. Future studies should strive to assess the effects of exercise on lactate concentrations and intracellular pH of immune cells, which could be a factor contributing to exercise-induced immune modulation.

Unlike CNT, ALL did not experience a decrease in NK maximal activation with exercise, but rather a steady increase. While there are no data on lactate production in response to exercise in pediatric ALL survivors, it is interesting to note that breast cancer survivors produce less lactate compared with healthy counterparts in response to various intensities of submaximal exercise.[41] Therefore, it is plausible that our participants may produce lower levels of lactate with exercise than CNT, and in turn, they may not experience the inhibitory effect of lactate and cellular acidification.

It is plausible that the maximal activation response observed in ALL is related to exercise-induced epigenetic changes. While still a novel field of study, chromatin state dynamics play a central role in NK activation, and histone modification can influence the magnitude of NK degranulation and cytokine expression.[42] Acute exercise can induce epigenetic modifications that may influence function of circulating NK cells.[29,43] Given the effects of exercise on maximal (receptor-independent) NK activation in our ALL group, further efforts should be made to study the effects of exercise on NK function downstream of receptor signaling.

A primary barrier in cancer treatment is overcoming NK inhibition and hyporesponsiveness in patients. Interestingly, the ALL group in our study had comparable NK maximal activation rates and even slightly higher CD107a density (MFI) upon activation than CNT. Higher CD107a density is strongly correlated with higher perforin content in NK cells, which is in turn linked to more efficient killing.[44,45] Our findings suggest that NK cells of children treated for ALL have the *potential* to respond to targets; however, they may lack the ability to initiate that response (i.e., overly inhibitory phenotype/receptor expression).

While the findings of this study provide important insights into the effects of acute exercise on NK cells in children being treated for ALL, our findings must be interpreted in light of several limitations. Among these are a small sample size, a lack of female participants, a large participant age range, and the participants are assessed once over the course of a 2-3 year long therapy). Moreover, while we

collected a reasonable volume of blood, low NK cell numbers in our ALL group made it difficult to perform comprehensive measures of NK cell function. For example, determining NK cytotoxicity by measuring target cell lysis using multiple cancer cell lines (i.e., autologous blasts, and MHC-expressing cancer cell lines, virally infected cells) may provide more clinically relevant data. Furthermore, a more comprehensive analysis of receptor expression is likely required to better understand the effects of exercise on receptor expression, and to contextualize the relationship with NK function. Precautions should be taken to ensure sufficient blood and effective use of sample when working with this population.

3.6. CONCLUSION

This study provided preliminary evidence of a positive effect of acute exercise on NK quantity and function in children being treated for ALL. Chiefly, NK cells of children being treated for ALL may have the potential to respond to exercise robustly but may be hindered by low NK numbers and a predominantly inhibitory NK phenotype. Finally, we detail areas for further research in exercise immunology, particularly with respect to oncology.

3.7. ACKNOWLEDGEMENTS

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3.9. SUPPLEMENTARY INFORMATION

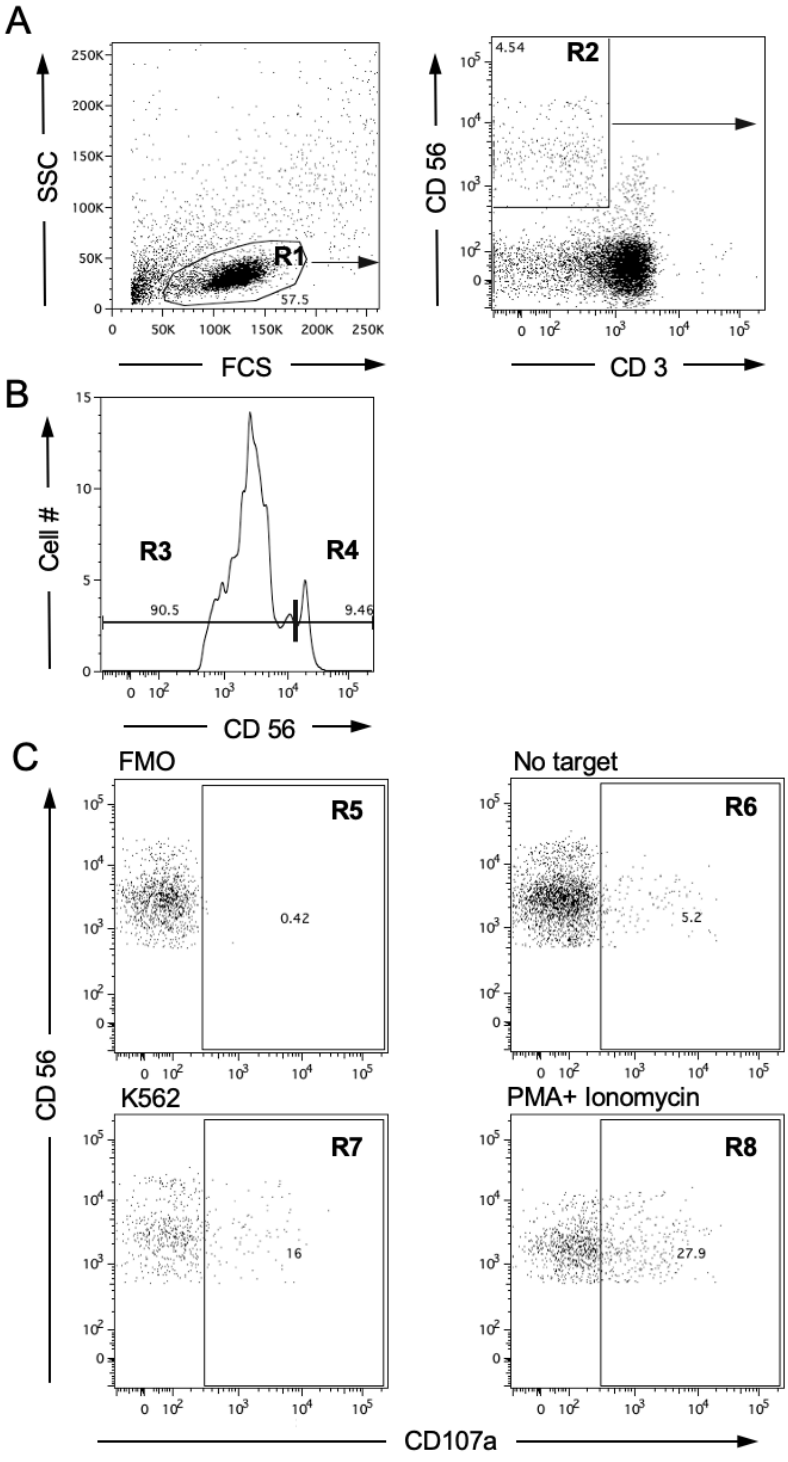
Supplementary table 1. NK Functional assay samples and contents

	Unstained control	**CD107a FMO**	**K562-ind. activation**	**Max. activation**	**Spont. activation**
Medium	Complete RPMI	Complete RPMI	Complete RPMI	Complete RPMI	Complete RPMI
Effector cells	5×10^4 PBMCs	5×10^4 PBMCs	5×10^4 PBMCs	5×10^4 PBMCs	5×10^4 PBMCs
Stimulant	5×10^3 K562s	5×10^3 K562s	5×10^3 K562s	PMA + Ionomycin	None
Protein transport inhibitor	Golgistop	Golgistop	Golgistop	Golgistop	Golgistop
Antibodies	None	CD3 -VB CD56- PE	CD107a – APC CD3 -VB CD56- PE	CD107a – APC CD3 -VB CD56- PE	CD107a – APC CD3 -VB CD56- PE

K562-Induced activation sample run in duplicates, Complete RPMI: RPMI, 10% FBS, 1% Antibiotic/Antimycotic, 5×10^4 PBMC present in each sample, 5×10^3 K562 present in each sample, 1µL Golgistop used in each sample, 100µL of PMA (0.15 µg/mL) and 100 µL of Ionomycin (3 µg/mL).

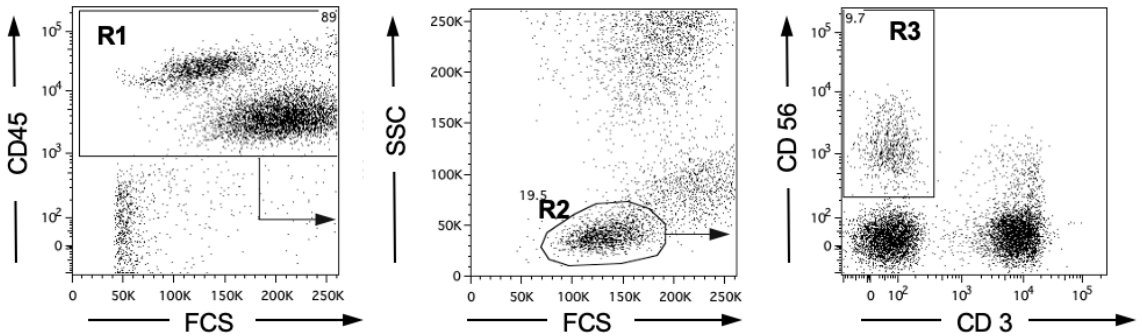
Supplementary table 2. Average heart rates (HR) of study participants during exercise.

ALL Group	**$HR_{Average}$ (range)**	**CNT Group**	**$HR_{Average}$ (range)**
ALL-01	173 (128-191)	**CNT-01**	152 (118-169)
ALL-02	129 (104-153)	**CNT-02**	116 (100-124)
ALL-04	139 (110-154)	**CNT-04**	144 (130-152)
ALL-05	161 (132-180)	**CNT-05**	150 (134-157)

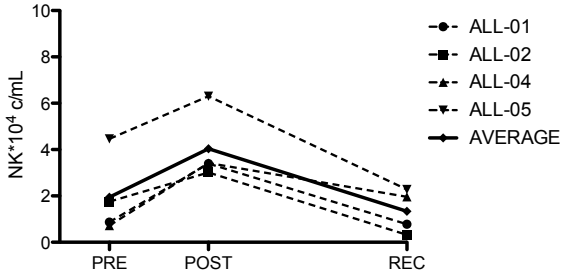


Supplementary figure 1. A representative participant sample depicting the flow cytometry gating strategies used to assess NK concentrations from PBMCs and NK activation from the functional assay. **A.** Identification of $CD3^{-}/CD56^{+}$ NK cells

from fresh PBMCs. Lymphocytes (R1) are gated based on their forward- vs. side-scatter profile and $CD3^-/CD56^+$ NK cells (R2) are identified. **B.** A histogram of CD56 expression is used to identify $CD3^-/CD56^{DIM}$ (R3) and $CD3^-/CD56^{BRIGHT}$ (R4) NK cells for quantification purposes. **C.** Flow cytometric assessment of NK activation. The $CD3^-/CD56^+$ NK cell population identified in **pane A** is further assessed for CD107a expression. The figure depicts examples of fluorescence minus one (FMO) used to set the margin for $CD107a^+$ events (R5), spontaneous activation (R6), K562-induced activation (R7) and maximal activation (R8) samples.



Supplementary figure 2. A representative participant sample depicting the flow cytometry gating strategies used to assess NK concentrations from whole blood. Leukocytes (R1) are identified based on their CD45 expression, lymphocytes (R2) are gated based on their forward- vs. side- scatter profile and $CD45^+/CD3^-/CD56^+$ NK cells (R3) are selected.



Supplementary figure 3. NK cell concentrations in peripheral blood of ALL in response to an acute bout of exercise. Each participant is depicted by a different symbol and dashed line and the average of all participants in that cohort is displayed with a thick solid line. The Y-axis has been adjusted from Figure 1 to clearly view NK cell recruitment in this lymphopenic population.

CHAPTER 4: EVALUATING CHANGES IN THE NK CELL RESPONSE TO ACUTE EXERCISE OVER TIME AND ASSESSING THE LINK BETWEEN HABITUAL PHYSICAL ACTIVITY LEVELS AND NK CELL NUMBER AND FUNCTION IN CHILDREN WITH ALL AT REST

The methods described below supplement the methods sections provided in Chapters 2 and 3. Since the results of Objectives 2b (NK response to exercise over time) and 2c (link between habitual physical activity and NK cell number and function) were not included in the manuscripts intended for publication, the following section will serve to expand on the methods used to address those objectives.

4.1. ASSESSING CHANGES IN THE NK EXERCISE RESPONSE OVER TIME

To address our secondary objective, participants were asked to complete a total of 3 identical exercise study visits separated by 4 weeks each, as described in Chapter 2. We assessed the NK cell response at each exercise visit, per Chapter 3, and compared the magnitude of this response between visits. In order to quantify the magnitude of response for each visit the area under the curve (AUC, described in Chapter 3) was assessed for NK cell number and function. AUC values for exercise visits 1, 2 and 3 were then graphed by participant to examine potential patterns in the magnitude of the NK cell response to exercise as participants progress through their treatment.

There were no clear trends to indicate a difference in NK response as participants progress further into recovery (**Figure 5.1)**. For instance, one participant demonstrated the most robust response in visit 1, while 2 were most responsive at visit 3. This was true for both NK numbers as well as NK function in response to exercise.

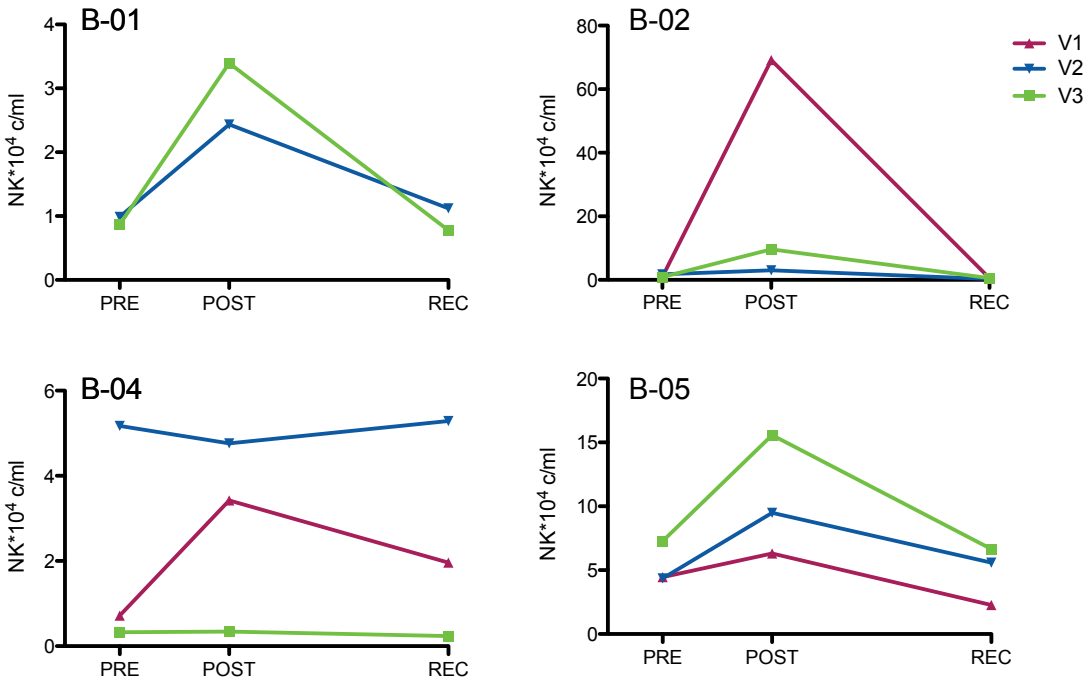


Figure 4.2.1 Fluctuations the NK cell response to acute exercise over the course of the study. Each graph shows data for a single participant and each exercise visit is depicted in a different color and shape. B-01 did not complete the first visit and we were unable to acquire a REC sample.

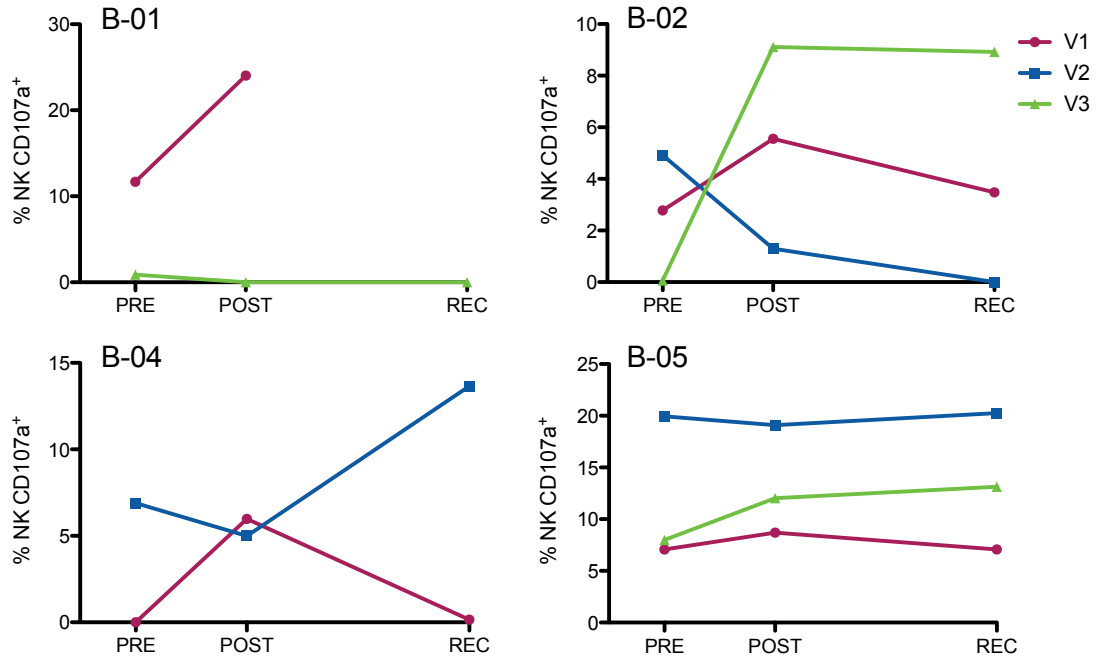


Figure 4.2.2. Fluctuations the NK cell functional response to acute exercise over the course of the study. Each graph shows data for a single participant and each exercise visit is depicted in a different color and shape. Intra-assay CV= 15.2%. B-01 did not complete the first visit, and we were unable to acquire a REC sample. B-01 did not have cryopreserved PBMC samples for visit 2. B-04 did not have sufficient sample to perform functional analysis for visit 3.

4.2. ASSESSING THE LINK BETWEEN HABITUAL PHYSICAL ACTIVITY LEVELS AND NK CELL NUMBER AND FUNCTION AT REST

Participants were asked to wear an ActiGraph accelerometer to monitor habitual physical activity throughout the study (~3 months). Accelerometer files were cleaned in ActiLife software using an automated algorithm designed to flag any 30-min periods of continuous zeros as non-wear time, as described in Chapter 2. Only participants with at least 6 hours of wear time per day, on at least 3 days included in the analysis of this secondary objective. In healthy children, this wear time provides a reliable (84-88%) estimate of habitual physical

activity.[90] Average daily time spent being sedentary, and engaging in total, light, and moderate-to-vigorous physical activity were calculated using activity intensity cut-points.[91]

First, participant sedentary and physical activity levels were compared from month to month to determine if the participant's physical activity habits changed throughout the study. Using a conservative reliability estimate of 84%, we determined that a >16% change in time spent being sedentary or being active represents a meaningful change. [90] Next, we determined the relationship between resting NK cells levels and habitual physical activity. To do this, NK cell concentrations from each resting blood sample (PRE and LP, 3 in total) were plotted against the time spent being sedentary, as well as time in light physical activity, moderate-to-vigorous physical activity (MVPA), and total physical activity for the preceding month.

Overall, sedentary time and physical activity levels of our participants remained fairly consistent throughout the study. Only one visit from each of B-04 and B-05 demonstrated a difference in light and total physical activity levels that exceed what is expected for day-to-day variability (**Figure 5.2**). For context, the Canadian 24-h Movement Behaviour Guidelines recommend children engage in at least 60-min of MVPA per day.[92] None of our participants met these guidelines over the course of the 12-weeks of monitoring.

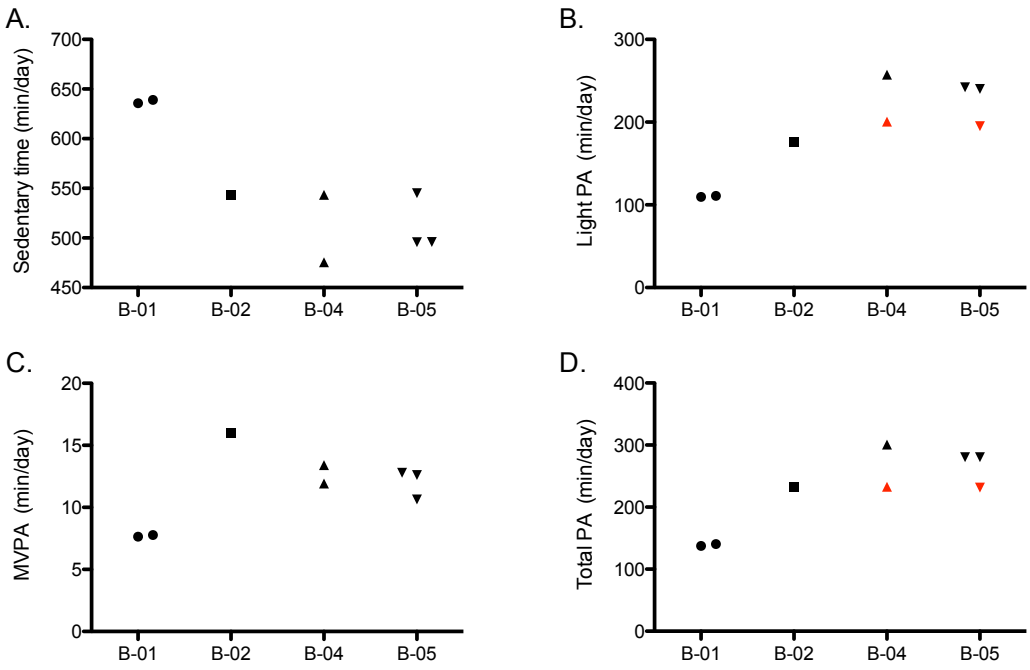


Figure 4.3.1. Habitual physical activity levels in visits 1, 2 and 3 per participant. Each point on the graph represents PA data from a single visit, and each participant is represented with a different shape. Data points highlighted in red differ significantly from the participant's visit 1 data. **A.** Sedentary time (min/day), **B.** Light physical activity (min/day), **C.** MVPA (min/day), and **D.** Total physical activity (sum of light and MVPA, min/day) throughout the study. Missing data points are due to insufficient physical activity data. B-01 was not given an accelerometer at his first visit and the data file at visit 2 for B-04 was corrupted. The accelerometer data from visits 2 and 3 for participant B-02 were excluded on account of having insufficient wear days.

We were unable to assess the relationship between habitual physical activity levels and resting NK cell number and function in children with ALL. **(Figure 5.3, 5.4).** We expect that other factors, including response to chemotherapy, influence resting NK cell levels and function between children to a greater extent than sedentary time or physical activity.

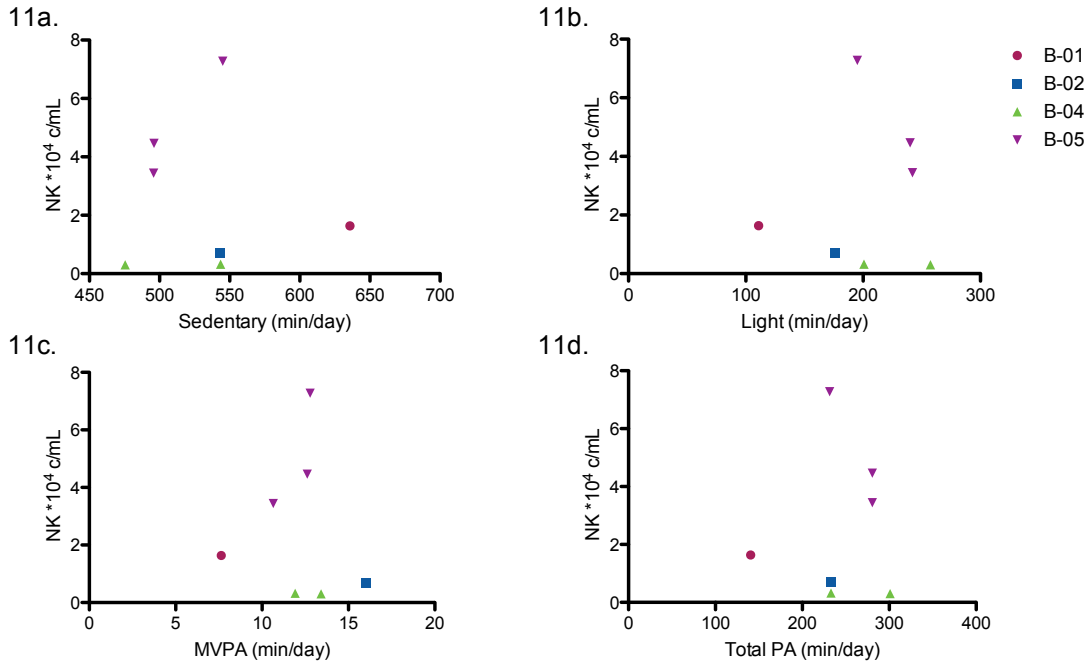


Figure 4.3.2. The relationship between NK concentrations at rest and habitual physical activity levels. Resting NK cell concentrations plotted against **A**. Average daily sedentary time (min/day) **B**. Average daily light physical activity (min/day) **C**. Average daily MVPA (min/day) **D**. Average daily total PA (min/day).

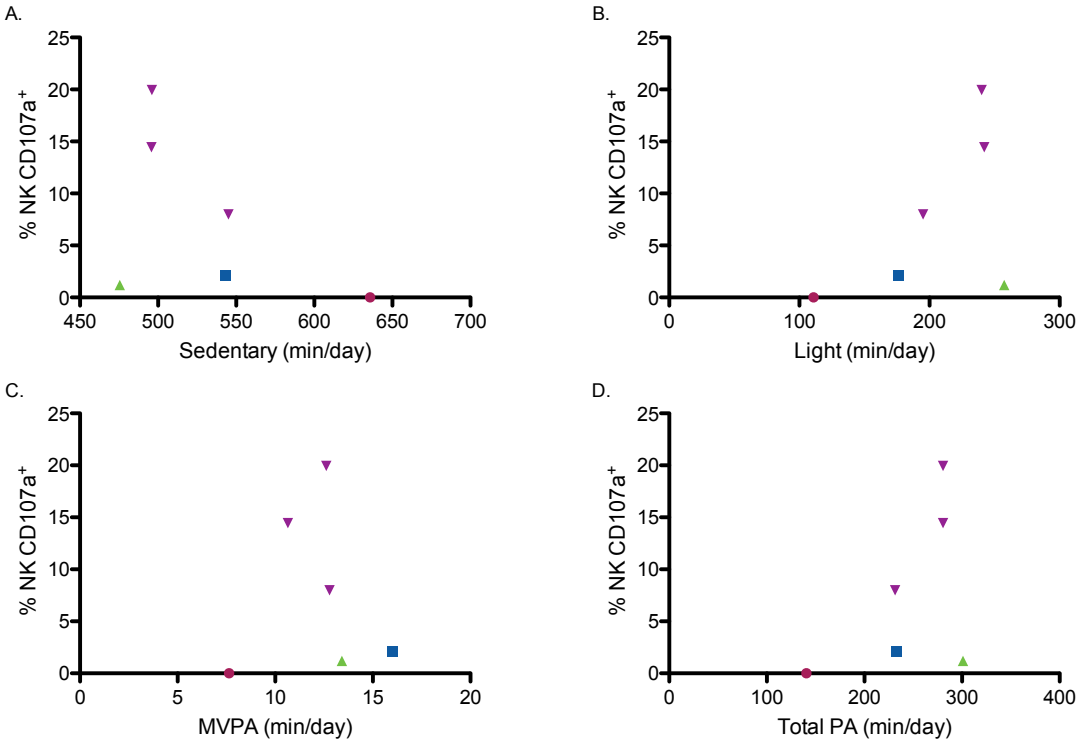


Figure 4.3.3. Assessing the relationship between NK function at rest and habitual physical activity levels. NK cell activation (% of NK cells that were CD107a+) plotted against **A**. Average daily sedentary time (min/day) **B**. Average daily light PA (min/day) **C**. Average daily MVPA (min/day) **D**. average daily total PA (min/day).

CHAPTER 5: SUMMARY OF MAIN FINDINGS AND GENERAL DISCUSSION

5.1. SUMMARY OF MAIN FINDINGS

The overarching aim of this study was to investigate the clinical and experimental considerations for conducting an exercise immunology study in pediatric ALL patients from a pragmatic perspective. As such, the results of this project are intended to support the design and implementation of a larger study examining the effects of exercise on circulating NK cells in children being treated for ALL. This research is crucial for building a solid evidence base to advocate for the implementation of exercise as a standard component of pediatric oncology care.

Altogether, our goal of recruiting 15 participants over a 2-year period was not feasible for our site, the McMaster Children's Hospital. However, the retention of recruited participants and the exercise protocol employed were both feasible for our 4 participants (Chapter 2). Moreover, the exercise protocol and accelerometer wear were deemed acceptable by participants (Chapter 2). The exercise protocol was safe for participants with ALL as there were no adverse events associated with exercise throughout the study (Chapter 2). From a physiological perspective, this thesis is the first to provide preliminary evidence that acute exercise can transiently increase NK cell number *and* function in some children treated for ALL, and to a similar extent to the changes observed in healthy children (Chapter 3). Furthermore, this is the first study to assess the

effects of acute exercise on select NK receptors in a pediatric population. We found that exercise-induced fluctuations in NKG2A expression, but not NKG2D or DNAM-1 expression, corresponded with changes in NK activation (Chapter 3). Moreover, our results suggested exercise may modulate NK function beyond inducing fluctuations in receptor expression (Chapter 3). Finally, there was no clear pattern in the magnitude of the NK cell exercise response over time (4-5 months) as children progressed through maintenance therapy (Chapter 4). We were unable to examine the relationship between physical activity and resting NK cell levels and function due to a small sample size and low ranges of habitual physical activity (Chapter 4). The following section will further discuss findings presented in Chapters 2, 3, and 4 and propose future research directions based on our observations.

5.2. THE FEASIBILITY, ACCEPTABILITY AND SAFETY OF AN EXERCISE STUDY FOR PEDIATRIC ALL PATIENTS

Low participant recruitment numbers are a common drawback in clinical studies. In fact, as many as 80% of clinical studies conducted fail to recruit their goal sample size.[93] Adult patients may hesitate to participate in clinical trials due to safety concerns, study requirements (i.e., additional procedures, appointments, travel), concerns about uncertainty of intervention or trial, and/or concerns surrounding consent. These barriers are amplified in pediatric studies, which require the consent and cooperation of both child and parent.[94] Low pediatric

recruitment rates are especially unfortunate given that children are a historically understudied population, with much clinical practice relying on generalized findings in adults, despite differences in physiology.[95] Therefore, efforts to facilitate recruitment of pediatric participants and conduct pediatric research with adequate sample sizes are necessary.

Previous exercise immunology studies in pediatric oncology patients have had small sample sizes of 3 to 6 participants (including controls), and/or included a heterogeneous diagnosis group.[73–76] However, these studies focus on reporting immune outcomes and often omit data concerning feasibility of recruitment, as well as acceptability feedback from participants. A single study reported recruitment statistics with a brief mention of reasons for refusal.[74] However, it is important to report detailed recruitment feasibility data, and to assess participant acceptability of testing methods in order to provide suggestions to adapt future study approaches and overcome low recruitment rates in this population. For instance, if participants were displeased with cycling as the exercise intervention, future studies may wish to provide more exercise modality options to entice potential participants. If recruitment rates are reported, treatment centres that may have insufficient eligible patients may direct their efforts towards partnering with other study sites or designing simpler studies that require fewer participants.

We were unable to attain recruitment goals. The primary barrier to recruitment was that patients and families had time constraints and were not able to spend more time than necessary at the hospital. Patients that refused faced

long commute times, long hospital wait times and had other commitments to attend to (e.g., other children in the family, work, school). Given these findings, future studies may wish to integrate the acute intervention to align with clinic waiting times. Contrarily, groups looking to conduct exercise training studies may wish to consider incorporating a home-based component to minimize participant travel and allow for flexibility to fit into the patient's schedule. These issues are not limited to the oncology setting. Indeed, studies in patients with other chronic conditions requiring frequent hospital visits, such as chronic kidney disease or cystic fibrosis, may experience similar barriers to study participation. The second most common reason for refusal was anxiety surrounding extended port access, which was most common in younger patients. This barrier is not universally applicable and highlights the importance of identifying and reporting population-specific barriers that might not be widely recognized. Finally, given our 18% study enrollment rate, small and mid-sized hospitals may also consider collaborating with external sites to conduct a multi-site study in order to amass a sufficient sample size.

Additionally, participants provided insight into areas that may improve enjoyment of participation for them and potentially future recruits. Notably, a participant suggested more options for aerobic exercise, such as an elliptical instead of limiting participants to just bike riding. When tracking physical activity levels, nearly all participants agreed a wrist-worn device would be more comfortable and enjoyable to wear. Furthermore, exploring water-proof options

that do not have to be removed for sleep were suggested. Unlike many pharmacological interventions, exercise interventions can provide a certain degree of flexibility without compromising the quality of the intervention. Therefore, it is important to capitalize on that advantage by including participants perspectives when conducting exercise intervention studies. Even when acceptability is not a primary goal, exercise studies may wish to consider incorporating a brief to evaluate participant preference and feedback into the study visit to gain valuable information for further research. Since the ultimate goal of this line of research is to implement exercise as an adjuvant therapy in pediatric cancer, incorporating participant preferences into study designs can support the design of more sustainable interventions.

We would also like to encourage reporting considerations for feasibility of sample collection and processing. Reporting such factors may be especially useful for clinical studies using primary patient samples and time-sensitive assays. For instance, all blood collections were completed within 3 minutes of finishing the exercise. This was made possible by coordinating with clinical staff (i.e., research nurse) to ensure they were present 5-10 minutes before the end of exercise and prepared to draw a sample. For our cell culture experiments, we found that children were lymphopenic, presenting with 0.2-0.7 $x10^9$ c/L throughout their entire visit. Moreover, children with ALL presented lower proportions of NK cells than healthy children (5.9% (0.8-17.2) NK of total lymphocytes at rest across all visits); however, aberrant PBMC distribution for

multiple cell types have also been reported in this population.[52] Therefore, precautions should be taken to acquire adequate blood for the anticipated assays.

Overall, we expect that our findings will encourage future exercise immunology studies in pediatric oncology patients to report limitations in participant recruitment and testing, successful methods and suggestions for improving the likelihood of achieving the target sample size.

5.3. THE EFFECTS OF ACUTE EXERCISE ON NK CELL NUMBER, FUNCTION AND RECEPTOR EXPRESSION IN CHILDREN WITH ALL COMPARED TO HEALTHY CHILDREN

In chapter 3, we reported that NK cell number and function was transiently increased in response to an acute bout of exercise in healthy children and some children treated for ALL. Interestingly, children with ALL and healthy counterparts had a similar magnitude exercise-induced change in NK cell concentrations and proportion. This suggests that children are responding proportionally to their available NK stores. Interestingly, patterns in NK maximal activation, stimulated by PMA/Ionomycin were consistent within populations and did not match the patterns observed with K562-induced NK cell activation. This suggests exercise may regulate NK function downstream of receptor expression; however, the mechanisms governing this modulation are not clear. Nonetheless, NK activation in vivo is limited to receptor-induced activation. Perhaps exploring a combination

of pharmacological therapies in tandem with exercise to remedy overly inhibitory NK phenotypes may provide the most effective increases in exercise.

Aside from NKG2A and NKG2D, which are more extensively discussed in the Chapter 3, the NK activating receptor DNAM-1 should also detect ligands on K562 cells. However, there was no clear association with fluctuations in DNAM-1 expression and NK function in either ALL or CNT (**Chapter 4, Figure 4)**. This is especially interesting considering the relatively high expression of DNAM-1 ligands, CD155 and CD112, on K562 cells.[96]. In CNT, DNAM-1 expression transiently decreased with exercise while activation increased. While the decrease in DNAM-1 we reported is consistent with the decreases in DNAM-1 expression in adults post-exercise, adults also demonstrated a corresponding decrease in NK activation.[59] Interestingly, children treated for ALL had higher densities of DNAM-1 expression on their NK cells, despite globally lower activation rates than healthy children. Evaluating the expression of co-inhibitory receptors CD96 and TIGIT that target ligands of DNAM-1 with a higher affinity than the activating receptor may provide more insight into the activation patterns observed.[97] Moreover, TIGIT may be able to interfere with homo-dimerization of DNAM-1, thereby preventing receptor engagement with ligand and preventing activating signal transduction; this requires further study. [97]

We also examined receptors that were not related to the K562 functional assay in an effort to understand how exercise might mediate the NK response to different cancer types, infected cells, and antibody dependent cell cytotoxicity.

Most notably, we assessed expression of CD16 (FCγRIII), a well-established marker of NK maturation that transmits activating signals upon binding the Fc portion of IgG antibodies. One mechanism of many anti-neoplastic monoclonal antibodies (e.g., rituximab used in adult frontline and refractory ALL treatment) is redirecting NK cell function against malignant cells and inducing NK antibody dependent cytotoxicity.[98,99] The antibody will bind a specific tumor antigen with their FAB portion, and CD16 on NK cells with the Fc portion, thus facilitating NK antibody dependent cytotoxicity and subsequently contributing to the potentiation of the adaptive immune response. [98] Therefore, such antibody therapies may benefit from being administered in tandem with an exercise intervention. As a marker of maturation, CD16 is predominantly expressed on $CD56^{DIM}$ cells, and the density of CD16 expression seems to be correlated with degree of maturation.[27] Given that exercise preferentially mobilizes mature cells, we expected that CD16 expression would also increase after a bout of exercise.[57] However, our data did not show a clear increase in CD16 expression with exercise in ALL or CNT. Moreover, children with ALL experienced lower CD16 MFI than healthy counterparts. None the less, exploring the implication of exercise and antibody therapies in other populations in a tempting explorative line of study.

Finally, NKp46 is an activating receptor expressed on both $CD56^{BRIGHT}$ and $CD56^{DIM}$ NK cells. NKp46 detects ligands associated with a variety of infectious agents, notably disease caused by *S.aureus*, *S. pneumonae,* and a variety of

influenza and parainfluenza strains; incidentally, the same agents that more commonly cause infections in pediatric ALL patients.[100] Therefore, adequate expression of NKp46 on NK cells may be beneficial to prevent these opportunistic infections. [101] However, many children with ALL present with low proportions and density of NKp46, that normalize by maintenance therapy. Indeed, we showed that participants with ALL in expressed comparable levels of NKp46 to healthy counterparts.[23] Little is known about how NKp46 expression responds to acute exercise; however, a single study in healthy adults did not find any changes in NKp46 expression in exercise.[59] This was consistent with our finding that exercise did not affect NKp46 expression in ALL or CNT.

Altogether, we chose to explore a subset of NK receptors that on the basis of their responsiveness to exercise, their interactions with our target cells, or reported alterations in ALL. This was by no means a complete NK receptor profile, but our findings speak to the need for more detailed analysis and understanding of the interactions of NK cell function and receptor expression with exercise, especially in immunodeficient populations.

5.4. CHANGES IN THE NK CELL EXERCISE RESPONSE OVER TIME IN CHILDREN WITH ALL

There were no clear patterns in the magnitude of NK cell response to acute exercise over time, as children progressed through therapy. Existing evidence supports an intensity dependent NK cell response to exercise.[56]

Therefore, in order to account for any minor variation in exercise intensity from visit to visit, we assessed the relationship between average HR, workload and magnitude of the NK cell response (Figure 5.4.1). Overall, there was no clear relationship with exercise intensity and the NK cell response in our population. This is likely due to the narrow range of exercise intensities and heart rates (by design) between visits in our participants. Since neither time between study visits nor exercise intensity accounted for the magnitude of the NK cells response, we can only speculate about external factors that might explain our findings. For instance, acute and chronic psychological stress can modulate NK cell number and function.[102–104] Which may in turn influence the NK response to exercise. Indeed, acute psychological stress may transiently increase NK cell numbers and function to the same extent as acute exercise.[102] However, chronic stress is associated with reduced NK cell number and function, as well as a decrease the proportion of terminally mature $CD57^+$ NK cells.[103,104] Aside from stressors associated with everyday life, children treated for ALL may face additional stressors related to their diagnosis and treatment.[105] Future studies assessing immune status over time may wish to account for participant stress at the time of testing. Furthermore, carbohydrate intake shortly before exercise may attenuate the exercise effect.[106] However, participants were not asked to provide a diet log, nor were they questioned about their adhesion to food intake, or activity rules. Therefore, evaluating participant adherence to prescribed dietary and activity rules may be beneficial for future studies assessing the NK exercise response.

Throughout maintenance therapy children are still receiving regular doses of immunosuppressive drugs, albeit at a lower dose than in previous treatment blocks. Therefore, the immune response to exercise may have similar trends to what is seen with immune reconstitution in these children. Current literature presents conflicting views on the state of NK cell reconstitution in children treated for ALL. Some sources suggest NK cells concentrations and function remain below healthy control values for months after the cessation of maintenance therapy with function recovering slower than number. [22,52] Meanwhile, others have suggested that NK number and function are at least partially restored early in maintenance therapy, when the chemotherapy dose is drastically lower compared to induction and consolidation.[23] Therefore, it is possible that following participants for longer periods of time, and assessing the NK cell response to acute exercise at different stages of therapy (induction, consolidation, start and end of maintenance) and/or post-therapy completion (e.g. 1 and 6 months post-therapy) would provide a more clear insight into trajectories of the immune response to exercise as children progress through therapy.

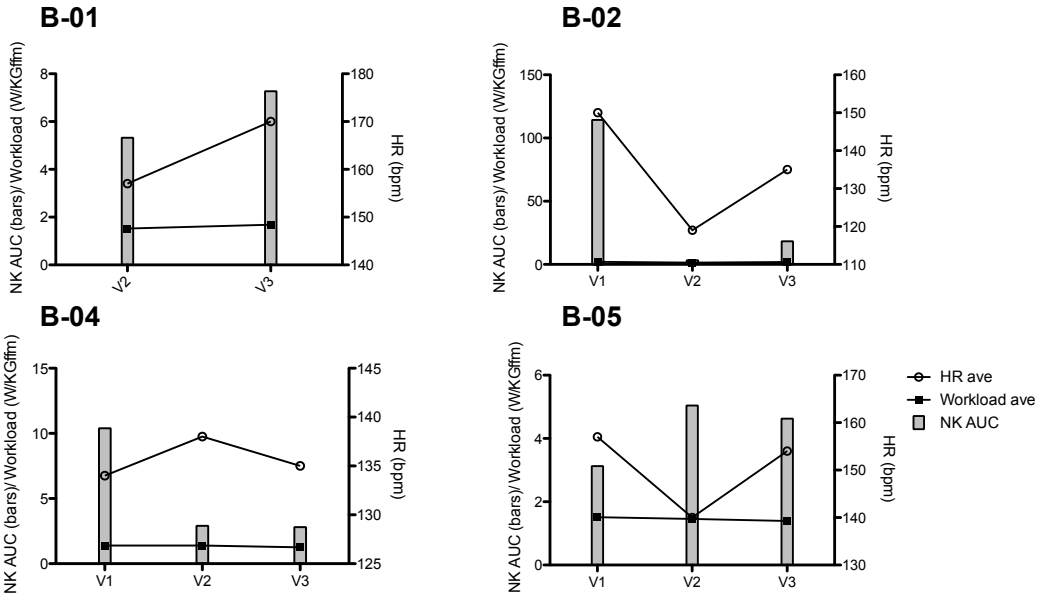


Figure 5.4.1. The magnitude of the NK cell number response compared to the strength of the exercise stimulus at each visit. Average heart rate in beats per minute (bpm) at each exercise session and average workload normalized to fat free mass in Watts/KGffm (W/KGffm) are plotted over the area under the curve (AUC) of the change in NK cell concentrations with acute exercise.

5.5. THE RELATIONSHIP BETWEEN HABITUAL PHYSICAL ACTIVITY LEVELS AND RESTING NK CELL NUMBER AND FUNCTION IN CHILDREN WITH ALL

We were unable to assess the relationship between habitual physical activity levels and resting NK cell number and function in children with ALL. Apart from our small sample size, participants also had a narrow range of physical activity levels, which made it difficult to examine associations across a spectrum of activity levels. Because physical activity levels are typically lower in children with ALL than healthy counterparts, it may be difficult to recruit a cohort of children treated for ALL with a wide spectrum of physical activity levels.[77] Moreover, it may be difficult to detect more subtle effects of physical activity due

to confounding factors may overshadow the exercise effect, such participants regularly taking cytotoxic drugs. Therefore, an exercise training intervention would provide a clearer picture of the effects of physical activity on NK cell concentrations and function at rest.

Perhaps counterintuitively, a study in healthy boys found that trained swimmers who spent significantly more time being active than untrained boys of the same pubertal status had lower resting NK cell levels, as well as a lower baseline NK cytotoxic function.[64] This inverse relationship between physical activity and NK levels/function may not be generalizable to our population. While regular, moderate intensity exercise is beneficial for the immune system, frequent and prolonged high intensity activity (e.g. in trained athletes) may depress the immune system.[107] Clinical interventions with sedentary and exercising (moderate intensity) groups found that the exercising group had a 14% lower incidence of upper respiratory infection, and when illness occurred, the exercising group recovered 23% faster, and experienced 31% less severe symptoms than non-exercising counterparts. [107] However, individuals regularly engaging in vigorous, long lasting exercise may be at an increased risk of infection. [107] Even acute models of exercise in healthy adults have demonstrated the importance of appropriate exercise intensity and duration. For example, when moderate intensity activity for 30-45 minutes (walking) was compared to a far more taxing, high-intensity, and long-lasting activity (42.2 km marathon race), the former induced a transient increase in cellular and humoral immunity while attenuating

inflammation, while the latter suppressed cellular and humoral function and increased inflammation. [107]

Increasing physical activity levels in children with a medical condition who are also sedentary may yield benefits for the immune system; however, chronic overexertion may be harmful. As such, it is important that future work explore interventions to support the development of accurate, disease-appropriate exercise guidelines for children with medical conditions. Studies assessing the effects of different doses of exercise on immune function in children treated for cancer may serve as a stepping-stone for the development of appropriate physical activity guidelines for this population.

5.6. NOVELTY OF FINDINGS

The novelty of the research presented in this thesis are summarized below.

Chapter 3: This is the first study to our knowledge to provide detailed information about recruitment feasibility and overall study acceptability. Major barriers to recruitment were the time commitment, followed anxiety about chemotherapy port access. Overall, participants enjoyed the exercise intervention but suggested future studies should have more exercise options. Concerning activity monitoring, participants suggested using a wrist worn and waterproof device, along with shorter assessment periods.

Chapter 4: This is the first study to demonstrate that acute exercise can induce a transient increase in NK cell number and function in some children with

ALL. Exercise-induced changes in NKG2A receptor expression, but not NKG2D or DNAM-1, corresponded with changes in NK activation in both ALL and CNT. Furthermore, exercise response patterns in receptor-independent NK cell activation were consistent within each group, but different from K562-induced activation, suggesting exercise may regulate NK function downstream of receptor expression.

Chapter 5: Building on the unique goal of assessing NK cell levels and function in response to acute exercise in children being treated for ALL, this study was also the first to attempt to examine the NK cell response to exercise and physical activity levels over 12-weeks of maintenance therapy. There were no clear patterns in the magnitude of the NK cell response over time. Physical activity levels were unchanged over 12-weeks and did not appear to be related to resting NK cell numbers or function.

Altogether, exercise is a simple and accessible intervention that provides many psychological and physiological benefits in children treated for cancer. Unfortunately, the relationship between exercise and the immune system is often overlooked in these studies, although such evidence may help advocate for the incorporation of exercise into pediatric oncology care. Our pilot study took a pragmatic approach to incorporate an acute exercise intervention into the already existing schedule of pediatric ALL patients. By reporting feasibility and acceptability results, we aimed to provide areas for consideration for future study designs. Participant feedback will contribute to developing more practical and

sustainable interventions that are relevant and fun for children. The secondary aims of this pilot study served to compliment the primary goals, by providing preliminary empirical results of the effects of acute exercise on the immune function of children with ALL.

Despite our small sample size, our results have prompted more specific research question, and opened multiple avenues for future research directions. Most notably, encouraging findings that acute exercise can improve NK number and function transiently in children with ALL should be confirmed in a larger cohort. More detailed analysis of the functional response to different targets may provide more precise clinical recommendations about the benefits of exercise for this population. For instance, while exercise may not increase NK function against certain cancer, perhaps it improves targeting of hemagglutinin expressing virally infected cells, which suggests that exercise may be more relevant for prevention of certain infections instead of cancer recurrence. Furthermore, our results cautiously support previous findings suggesting children with ALL (among other cancers and chronic diseases) may have a predominantly inhibitory NK phenotype. Acute exercise leads to a drastic mobilization of NK cells into peripheral circulation and may provide better insight into the overall NK repertoire, particularly in populations where tissue sampling may not be possible. Using an exercise model to assess the inhibitory NK phenotype in these populations might provide more convincing evidence of the extent of the NK cell exhaustion, as well as the potential for exercise to reverse this phenotype. Lastly,

we provide intriguing evidence to support the notion that exercise may regulate NK function downstream of receptor expression. Additional studies assessing exercise-induced systemic changes that can influence NK function, such as changes in lactate and acidity, may provide more insight into these preliminary findings. Finally, our study prudently suggests that exercise may have the capacity to improve NK function, and examining exercise in combination with pharmaceutical interventions to help overcome the inhibitory NK phenotype may be even more beneficial. Overall, the preliminary findings of this pilot study provide a steppingstone to new and exciting research questions to better understand the role of exercise in improving immune health in children with ALL during and beyond treatment.

SUPPLEMENTARY CHAPTER A: ADDITIONAL METHODS

A.1. COMPARISON OF NK QUANTIFICATION METHODS USING WHOLE BLOOD AND PBMCS

In chapter 3, two different methods were used to assess the concentration of NK cells in the blood in healthy children and in children treated for ALL. In children treated for ALL, NK proportions were assessed by combining flow cytometric analysis of isolated peripheral blood mononuclear cells (PBMCs) and complete blood counts (CBC) from the McMaster Core Laboratory. However, since CBCs were not assessed for healthy controls, NK cells were quantified directly from whole blood using flow cytometry. Both methods of NK quantification are described in more detail in the methods section of Chapter 3.

In order to assess the agreement between the two methods of quantifying NK cells, we quantified NK cells using both strategies in a single sample. To do this, 1 × 10-mL EDTA-coated tube of blood was collected from 4 healthy children (2 boys, 2 girls) at rest. From this blood sample, 300-µL whole blood was aliquoted for whole blood staining, which was further divided into 1 × 100 µL for an unstained control, and 2 × 100 µL for NK staining in duplicate. The remainder of the blood sample (~9.7 mL) was used to isolate PBMCs (as described in Chapter 3). Isolated PBMCs were resuspended in 5mL of FACS buffer and 150 µL was taken for analysis. The 150uL of PBMC suspension was diluted 2-fold with MACS buffer and 100 µL was used to prepare an unstained control, with the

remaining 2 × 100µL used for NK staining in duplicate. Both whole blood and PBMC samples were fixed and analyzed consecutively using flow cytometry within 1 week of acquiring the samples. Both whole blood and PBMC were assessed using the gating parameters outlined in Chapter 3, Supplementary Figures 1 and 2. Results were presented as the proportion of NK cells relative to the total lymphocyte population (%NK of total lymphocytes) for both samples, and duplicate stains were averaged. NK proportions derived from whole blood and PBMCs for each participant were compared using a Bland-Altman graph.

The Bland-Altman test suggested PBMC and whole blood methods yielded comparable NK as a proportion of total lymphocytes. The average bias was 1.07%, and 95% limits of agreement were -1.65 to 3.79% NK of total lymphocytes, suggesting that whole blood tended to have slightly higher %NK of total lymphocytes compared with PBMCs **(Figure S1.1.1)**.

To contextualize these findings and determine whether differences between the two methods are clinically meaningful, we assessed the reliability of the duplicates for whole blood and PBMCs separately. For whole blood NK quantification, duplicate samples yielded 95% limits of agreement of -1.08 to 0.59% of total lymphocytes **(Figure S1.1.2 A)**. For PBMC NK quantification, the 95% limits of agreement were -1.9 to 4.54% of total lymphocytes **(Figure S1.1.2 B)**. As expected, the agreement in duplicate samples is slightly lower than that of whole blood and PBMC. Nevertheless, these findings suggest the differences in NK cells measured in duplicate samples were comparable to the differences in

NK cells measured in whole blood and PBMCs. Therefore, we are confident that two methods of quantifying NK cells, from whole blood or PBMC, are equivalent for the purposes of this study.

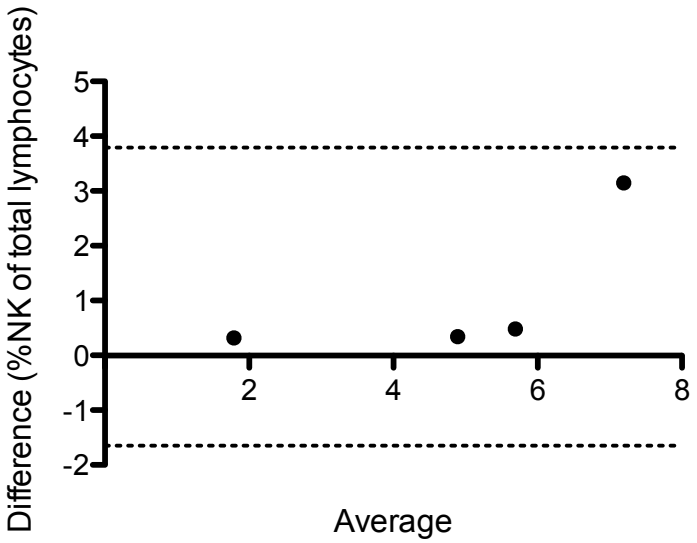


Figure A1.1. Bland Altman plot of %NK of total lymphocytes quantified from PBMC vs Whole Blood. The average is presented as NK % of lymphocytes for whole blood and PBMC. The difference was calculated as whole blood values – PBMC values.

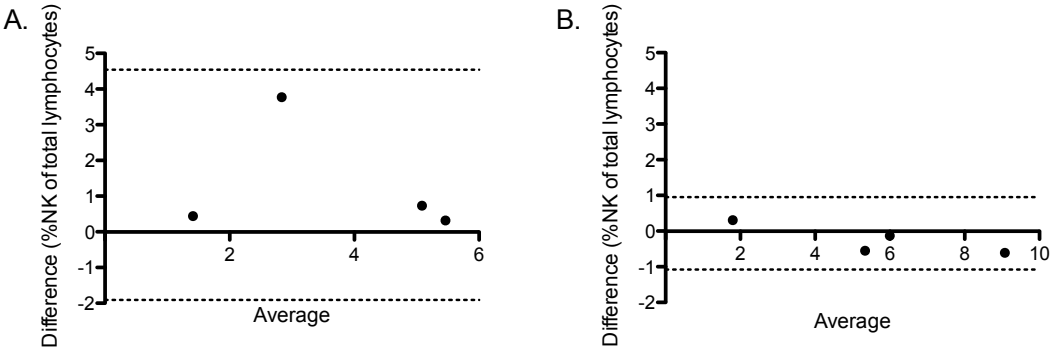


Figure A1.2. Bland Altman plot comparing reliability of NK quantification methods. **A.** Bland Altman plot of %NK of total lymphocytes quantified from duplicate PBMC samples. **B.** Bland Altman plot of %NK of total lymphocytes quantified from duplicate whole blood samples.

CHAPTER 6: REFERENCES

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APPENDIX B: CONSENT AND ASSENT FORMS

PARENT CONSENT FORM

Title of Study:	**Using exercise to BOOST the immune system of children with Acute Lymphoblastic Leukemia**
Principal Investigators:	*Dr. Vicky Breakey (MD), Pediatrics* *Dr. Brian W. Timmons (PhD), Pediatrics*
Co-Investigators:	*Dr. Adam Fleming (MD), Pediatrics* *Dr. Stacey Marjerrison (MD), Pediatrics* *Dr. Joyce Obeid (PhD), Pediatrics* *Dr. Lehana Thabane (PhD), Clinical Epidemiology & Biostatistics*
Funding Source:	Pediatric Oncology Group of Ontario

INTRODUCTION

Your child is being invited to participate in a research study conducted by Dr. Vicky Breaky, Dr. Brian Timmons and colleagues because they are currently receiving maintenance chemotherapy for acute lymphoblastic leukemia. In order to decide whether or not you want to be a part of this research study, you should understand what is involved and the potential risks and benefits. This form gives 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 wish to participate. Your child will be asked to sign another form to confirm that they agree to participate. Take your time to make your decision.

WHY IS THIS RESEARCH BEING DONE?

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer. Treatment for ALL lasts 2-3 years and results in a weakened immune system, putting kids at risk for infection. It's also common for parents to worry that their child is too ill to be physically active and they often limit what they do. The combination of inactivity and the side effects of chemotherapy increase their risk of obesity and other illnesses, which can lead to more health issues in adulthood. To help these kids be healthier during and after cancer treatment, we need to find ways to boost their body's ability to fight sickness. Our research shows that as little as 30 minutes of exercise in healthy kids can boost special cells in the blood called natural killer cells. Natural killer cells help to find and destroy viruses and cancer cells, and can even direct other types of cells to help. Our idea is that exercise can do the same thing for kids with ALL, so we are testing this. Showing an increase

APPENDIX B: CONSENT AND ASSENT FORMS

in natural killer cells after exercise in patients with ALL is the first step in understanding the role that exercise might play in boosting the immune system. By understanding the effects of exercise on the immune systems of children being treated for ALL, we hope to improve their health during therapy and beyond.

APPENDIX B: CONSENT AND ASSENT FORMS

WHAT IS THE PURPOSE OF THIS STUDY?

The purpose of this study is to learn more about the best ways to run an exercise study for children and adolescents who are in maintenance therapy. We also want to learn about the effect that exercise can have on special immune cells called natural killer cells in children and adolescents with acute lymphoblastic leukemia. Our goal is to learn about how these cells change over 3 months of maintenance therapy; and explore if these changes are related to physical activity levels. If we find that exercise and physical activity have a positive effect on these special cells, we can apply this knowledge to improve the health of our patients.

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

If you volunteer to participate in this study, we will ask your child to complete 1 visit each month for a total of 3 months. These visits will take place on the same day as their regularly scheduled chemotherapy sessions. Each visit will be structured in the same manner. During the visit, your child will receive their chemotherapy, followed by a brief period of rest. After this, your child will be asked to perform the following things:

1) **Blood sample:** A small, 40 mL (about 2.5 tablespoons) blood sample will be taken from the central venous catheter that is used for your child's chemotherapy. This will allow us to measure some natural killer cells in the blood. This blood sample will be taken before and immediately after exercise, and again 1-hour after the exercise session.
2) **Exercise:** Your child will be asked to ride a stationary bike at a moderate intensity for 30 minutes. At each clinic visit, their doctor will clear them to perform this exercise.
3) **Physical activity assessment**: After completing the first visit, we will give your child a small pager-like device to wear until their next visit – this device monitors physical activity, kind of like a Fitbit. They can take it off only if they are going to get wet (like in the bathtub or swimming) and at bedtime. We will also ask them to keep track of when they wore the monitor. We will ask you to return the monitor at your final study visit. At that time, we will have a few questions to ask you and your child about any issues that might have arisen while wearing the device between testing visits.
4) **Questionnaires**: We will ask you and your child to fill out some questionnaires that tell us about their physical activity and the issues they see as important for getting or not getting enough exercise. We will also ask you to complete a few questionnaires to better understand your background and your child's disease history. We ask that you complete these to the best of your knowledge. All of your answers will be kept strictly confidential. You should also know that you can choose not to answer any questions that

APPENDIX B: CONSENT AND ASSENT FORMS

make you feel uncomfortable, this will not affect your child's participation in the study.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

The aerobic exercise portion requires your child to pedal on our bike for 30 minutes. This could lead to discomfort from getting tired. However, young people tend to recover very quickly. Wearing the physical activity monitor should not pose any risks or discomforts for your child, nor should completing the questionnaires. A nurse will collect a blood sample using the central venous catheter, where your child routinely gets their medication administered. A small bruise may appear where the needle goes through the skin. While it is very rare, there is also an increased risk of infection from having the central venous catheter beyond the length of your regular appointment; however, a nurse from the oncology clinic will be overseeing each study visit to manage and reduce this risk. There is also chance that your child may feel light-headed after the blood sample. We will have snacks and water on hand to minimize the risk of this happening, but taking this amount of blood will have no major negative effects.

HOW MANY PEOPLE WILL BE IN THIS STUDY?

We are asking a total of 15 children and adolescents to participate in this study. Your participation is voluntary.

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

We cannot promise any personal benefits to you or your child from their participation in this study. We will make each visit fun and enjoyable for your child. Your participation will be very important for us to learn how best to address physical activity, exercise, and their effects on immune cells in other young children with acute lymphoblastic leukemia and to design programs that can help in these areas of health.

WHAT INFORMATION WILL BE KEPT PRIVATE?

All of your child's information will be stored in locked filing cabinets under the supervision of Dr. Brian Timmons for 10 years. **To be able to understand the results of this study, we will need to obtain some of your child's medical information. For example, types of medication they are taking, how long they have had their disease, etc. We can obtain this information from your child's medical chart from the doctor who is responsible for the care of your child's disease. We will not record your child's health card number or any other information that could identify your child.** We will supervise access to your child's information by other people in our group, only if necessary. Your child will be assigned a subject number used to identify them. Records identifying your child

APPENDIX B: CONSENT AND ASSENT FORMS

will be kept confidential. If the results of the study are used in a presentation, your child's identity will remain confidential.

CAN PARTICIPATION IN THE STUDY END EARLY?

If you and your child volunteer to be in this study, you or your child may withdraw at any time with no prejudice. The investigator may withdraw you from this research if circumstances arise which warrant doing so. In no way will withdrawing from this study affect the care you receive from your specialist.

WILL MY CHILD BE PAID TO PARTICIPATE IN THIS STUDY?

We will provide your child $60 as reimbursement for their participation in this study. If you quit the study for personal reasons, we will change the amount for the time completed. If you choose to quit because of a complication from the study, we will give you the full amount. We will pay for your parking expenses at the McMaster Children's Hospital. We will also provide you with a 1-page report of the findings and what they mean.

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 have a research-related injury, you can contact Joyce Obeid at our research office at 905-521-2100 extension 73517 (Daytime) or at 905-928-5538 in the evenings.

If you have any questions regarding your rights as a research participant, you may contact Deborah Mazzetti (Manager) at 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 and to the satisfaction of my son and/or daughter. I agree to allow my child to participate in this study entitled: *"**Using exercise to BOOST the immune system of children with Acute Lymphoblastic Leukemia**".* I understand that I will receive a signed copy of this form.

_____
Name of Participant (child's name)

_____
Name of Legally Authorized Representative

_____ _____
Signature of Legally Authorized Representative Date

APPENDIX B: CONSENT AND ASSENT FORMS

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 for their son and/or daughter to participate in this research study.

_____
Name and title

_____. _____
Signature of Investigator Date

FUTURE RESEARCH
At the end of the study, we may wish to store leftover sample for use in a future study. 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.

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 and to the satisfaction of my son and/or daughter. I agree to have my child's blood stored so it can be used for future research studies approved by the Research Ethics Board other than the one described in this information form.

_____
Name of Participant (child's name)

_____
Name of Legally Authorized Representative

_____ _____

APPENDIX B: CONSENT AND ASSENT FORMS

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 for their son and/or daughter to store blood.

_____
Name and title

_____ _____
Signature of Investigator Date

PHOTO, AUDIO AND VIDEO RELEASE FORM

I, _____, hereby give McMaster University's Faculty of Health Sciences my permission to take and use any photographs, movie films, audio or video tapes made of my child, (name) _____, 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.

I, _____, represent that I am the parent or guardian of the minor named above and that I have the legal authority to execute the foregoing consent and release, and hereby approve the foregoing, and waive any rights in the premises.

Name of Participant	Signature of Participant	Date
Name of Parent/Guardian	Signature of Parent/Guardian	Date

PARTICIPANT CONSENT FORM
(Participant Age: 16+ years)

Title of Study:	**Using exercise to BOOST the immune system of children with Acute Lymphoblastic Leukemia**
Principal Investigators:	*Dr. Vicky Breakey (MD), Pediatrics* *Dr. Brian W. Timmons (PhD), Pediatrics*
Co-Investigators:	*Dr. Adam Fleming (MD), Pediatrics* *Dr. Stacey Marjerrison (MD), Pediatrics* *Dr. Joyce Obeid (PhD), Pediatrics* *Dr. Lehana Thabane (PhD), Clinical Epidemiology & Biostatistics*
Funding Source:	Pediatric Oncology Group of Ontario

INTRODUCTION

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WHY IS THIS RESEARCH BEING DONE?

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer. Treatment for ALL lasts 2-3 years and results in a weakened immune system, putting kids at risk for infection. It's also common for parents to worry that their child is too ill to be physically active and they often limit what they do. The combination of inactivity and the side effects of chemotherapy increase their risk of obesity and other illnesses, which can lead to more health issues in adulthood. To help these kids be healthier during and after cancer treatment, we need to find ways to boost their body's ability to fight sickness. Our research shows that as little as 30 minutes of exercise in healthy kids can boost special cells in the blood called natural killer cells. Natural killer cells help to find and destroy viruses and cancer cells, and can even direct other types of cells to help. Our idea is that exercise can do the same thing for kids with ALL, so we are testing this. Showing an increase in natural killer cells after exercise in patients with ALL is the first step in understanding the role that exercise might play in boosting the immune system.

By understanding the effects of exercise on the immune systems of children being treated for ALL, we hope to improve their health during therapy and beyond.

WHAT IS THE PURPOSE OF THIS STUDY?

The purpose of this study is to learn more about the best ways to run an exercise study for children and adolescents who are in maintenance therapy. We also want to learn about the effect that exercise can have on special immune cells called natural killer cells in children and adolescents with acute lymphoblastic leukemia. Our goal is to learn about how these cells change over 3 months of maintenance therapy; and explore if these changes are related to physical activity levels. If we find that exercise and physical activity have a positive effect on these special cells, we can apply this knowledge to improve the health of our patients.

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CAN PARTICIPATION IN THE STUDY END EARLY?

If you volunteer to be in this study, you can withdraw at any time with no prejudice. The investigator may withdraw you from this research if circumstances arise which warrant doing so. In no way will withdrawing from this study affect the care you receive from your specialist.

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_____
Name of Participant

_____ _____
Signature of Participant 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.

_____
Name and title

_____ _____
Signature of Investigator Date

FUTURE RESEARCH

At the end of the study, we may wish to store leftover sample for use in a future study. We will not store your sample longer than 10 years. All records identifying you will remain confidential. Information about you will not be released. If the results of the study are published, your identity will remain confidential.

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 blood stored so it can be used for future research studies approved by the Research Ethics Board other than the one described in this information form.

_____
Name of Participant

_____ _____
Signature of Participant 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 store blood.

_____

Name and title

_____ _____

Signature of Investigator Date

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