

SUMMER 2026

Profiling of CD8⁺ Cytotoxic T Lymphocytes to Identify Optimal Hematopoietic Stem Cell Transplantation Approach in B-cell Acute Lymphoblastic Leukemia

By Yueqiao (Martina) Gao

AUTHOR BIOGRAPHY

Martina Gao is a Grade 11 student at York House School in Vancouver, Canada. She is interested in pediatric cancer diseases that motivated her to pursue research to understand and help children affected by the disease and hopes to pursue future studies in biomedical sciences and public health.

ABSTRACT

B-cell acute lymphoblastic leukemia (B-ALL) is the most common pediatric blood cancer. Despite advances in treatment therapies, the disease remains a clinical challenge due to high relapse rates and poor immune responses. Hematopoietic stem cell transplantation (HSCT) offers a potential cure through graft-versus-leukemia effects, but outcomes vary among different transplantation approaches. In this critical review of existing research and proposed project, I will investigate varied treatment outcomes across three types of HSCT approaches through comprehensively profiling CD8⁺ T-cell phenotype, transcriptional profiles, and functional capacity, to understand the cellular and molecular mechanisms. Using flow cytometry, proliferation assays, single-cell RNA sequencing, T-cell receptor clonotype analysis, and xenograft mouse models engrafted with B-ALL cells *in vivo*, I will characterize CD8⁺ T-cell reconstitution and identify immune features that associate with transplant effectiveness. By comparing the functional characteristics of CD8⁺ T cells across different transplant approaches, this proposal will not only help identify the most effective type of HSCT, but also provides deeper understanding into the factors affecting post-transplant prognosis. In the long term, this approach will provide phenotypic reference to evaluate and optimize current HSCT therapies.

Keywords: *B-cell Acute Lymphoblastic Leukemia (B-ALL), cytotoxic T-lymphocytes (CD8⁺ T-cell), hematopoietic stem cell transplantation (HSCT), haploidentical HSCT (haplo-HSCT), allogeneic HSCT (allo-HSCT), haplo-cord HSCT, graft-versus-host disease (GVHD), graft-versus-leukemia (GVL), relapse, immune reconstitution, T-cell phenotype*

INTRODUCTION AND BACKGROUND

Leukemia is the most prevalent hematological malignancy among children. It is characterized by uncontrolled proliferation of abnormal blood cells in the bone marrow, which then circulates throughout the body (Li, 2022). Among its subtypes, acute lymphoblastic leukemia (ALL) is a common form with occurrences of more than 6,500 cases and a rate of 8 per 100,000 children annually in the United States. ALL is mainly caused by genetically damaged lymphoid progenitors that disrupt the normal growth and differentiation within the bone marrow, bloodstream, and extramedullary areas (Terwilliger & Abdul-Hay, 2017).

B-cell acute lymphoblastic leukemia (B-ALL) represents the predominant subtype of ALL, causing 75% of leukemias among children under the age of 15. Although infants are generally associated with poor prognosis, the long-term rates of remission for childhood B-ALL reach up to 90% (Terwilliger & Abdul-Hay, 2017). B-ALL is characterized by chromosomal abnormalities that substantially influence treatment responsiveness and long-term outcomes. Common chromosomal abnormalities of B-ALL include BCR-ABL1 and KMT24 genes rearrangements, aneuploidy, recurrent translocations, and amplification of chromosome loci such as iAMP21 (Yasuda et al., 2022). These genetic changes disrupt the expression of important transcription factors for normal differentiation and proliferation of early lymphoid cells, resulting in unchecked proliferation and deregulation of key transcription factors contributing to cancerous transformation in B-ALL (Woo et al., 2014).

Due to the high frequency of relapse in B-ALL patients, hematopoietic stem cell transplant (HSCT) treatment has historically been proposed for improved outcomes compared to the traditional treatments of chemotherapy (Narayanan & Shami, 2012). Nonetheless, several new treatment approaches, including immunotherapies, have recently been showing greatly improved survival for B-ALL patients. Chimeric antigen receptor T-cell (CAR-T) therapy has been the most transformative, as the therapy has shown high remission rates among patients, even after the occurrences of relapse after HSCT (Wudhikarn et al., 2021). However, CAR-T therapy include limitations such as the inflammation reaction of cytokine release syndrome, immune effector cell-associated neurotoxicity, and relapse due to CAR-T cell and antigen loss. Additionally, bispecific antibodies binding to CD19 on B-ALL cells and CD3 on T cells are effective in treating minimal residual disease (MRD)-positive patients and leukemia relapse. Antibody-drug conjugates is another treatment that targets surface antigens to deliver cytotoxic drugs directly to leukemia cells, inducing cell death and showing promising results as well (Aureli et al., 2023).

Nevertheless, HSCT remains an essential and leading treatment for stem cell-based therapies. HSCT is a procedure where patients receive healthy hematopoietic stem cells (HSCs) to replace their own damaged or depleted ones. These stem cells are responsible for producing blood cells in the bone marrow, peripheral blood, or umbilical cord blood. Three main types of HSCT exist, which this project will explore. The first is human

SUMMER 2026

leukocyte antigen (HLA)-matched allogeneic HSCT (allo-HSCT), in which destroyed stem cells of patients are replaced with healthy blood-forming stem cells from a donor; allo-HSCT can cure high-risk or relapsed cases. The ideal donor is an HLA-matched sibling or HLA-matched unrelated individual, but over half of the patients lack a fully matched donor. In such cases, the second type of HSCT, haploidentical HSCT (haplo-HSCT), ensures a 50% tissue type match, typically donated from a family member. The third donor source is haplo-cord HSCT, which combines haploidentical stem cells with umbilical cord blood in a 1:1 ratio.

Graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) are important factors that greatly influence the success of leukemia treatment post-hematopoietic stem cell transplant. GVL effects occur after HSCT, when the donor's immune system recognizes and kills residual leukemia, reducing the risk of disease relapse. HSCT is a mainstream therapy for B-ALL, particularly for patients experiencing a high risk of relapse. After HSCT, the reconstitution of cytotoxic and regulatory immune populations, especially T cells and natural killer (NK) cells, is a critical determinant of infection risk, GVHD, and leukemia relapse. Previous studies have characterized the role of cytotoxic T lymphocytes (CD8⁺ T cells) cells in immune reconstitution following HSCT.

Though the CD8⁺ T cells play a pivotal role in the outcome of HSCT therapy, the immunological characteristics of CD8⁺ CTLs in this setting remain poorly characterized. It is still unclear how the phenotype and function of CD8⁺ T cells in haplo-cord HSCT patients compare to those in conventional haplo-HSCT or allo-HSCT patients (Zhou et al., 2022). Clarifying the differences in immune phenotype and function of the T cells correlates with the graft-versus-leukemia effect would reveal further approaches to improve transplant outcomes.

Given this background, this project aims to characterize and compare the phenotype and functions of CD8⁺ T cells following haplo-cord HSCT, haplo-HSCT, and conventional allo-HSCT. The central hypothesis of this proposal is that CD8⁺ CTLs derived from haplo-cord HSCT exhibits a stronger GVL effect, more well-defined CD8⁺ T-cell surface markers, more prominent T-cell activation gene expression profiles, lower exhaustion markers, and potentially even reduced GVHD comparing to those of haplo-HSCT or conventional allo-HSCT (Zhou et al., 2022). This proposal will fill important gaps in our understanding of T-cell immune reconstitution in haplo-cord HSCT and how it differs from other transplantation strategies. This will help establish criteria for evaluating HSCT treatment outcomes and contribute to developing optimized HSCT therapies.

SUMMER 2026

EXPERIMENTAL DESIGN

The investigation will be divided into two aims. The first aim will concentrate on profiling and comparing CD8⁺ T-cell reconstitution across three HSCT approaches (haplo-cord, haploidentical, and allogeneic) through examining their phenotypes, transcriptional profiles, surface markers, functional capacities and responses. The second aim will evaluate how these differences in CD8⁺ T-cell populations affect anti-leukemia responses, both *in vitro* and *in vivo*, and they will correlate with patient clinical outcomes.

Aim 1: To characterize CD8⁺ T-cell reconstitution across three HSCT approaches***Rationale:***

In Aim 1, I will characterize the differentiation states and immunological phenotypes of T-cell reconstitution after haplo-cord HSCT, compared to those after haplo-HSCT and allo-HSCT. This is important because CD8⁺ T cells are a central part of the immune defense, responsible for identifying and destroying abnormal cells through direct interactions with target cells. Specifically, CD8⁺ T cells have a dual function in the post-transplant settings, contributing to both beneficial GVL effects and harmful GVHD. The recovery of T cell immunity after HSCT is key to determine transplant success and the risk of relapse, infections, and GVHD.

Previous studies have shown that different HSCT approaches lead to distinct patterns of T-cell reconstitution. For example, the standard donor allo-HSCT results in reliable GVL with low relapse, but the donor T cells can also cause alloreactive responses against non-hematopoietic normal host tissues, triggering GVHD and higher non-relapse mortality (Falkenburg & Jedema, 2015). Studies have shown that haplo-HSCT displays delayed or altered T-cell reconstitution, which is associated with less effective early GVL and higher risk of relapses (Rambaldi et al., 2021). Haplo-cord HSCT results in better survival and lower relapse compared to other HSCT approaches, suggesting a beneficial GVL with stable GVHD (Zhou et al., 2022). Overall, haplo-cord HSCT appears to obtain the balance of rapid engraftment and strong immune reconstitution with GVL.

To define the T-cell profile of each transplant approach, multicolor flow cytometry, specifically fluorescence-activated cell sorting (FACS) and immunophenotyping, will be employed to identify the key surface and intracellular markers on CD8⁺ T cells. The markers and subsets include differentiation markers like CD45RA/CD45RO to distinguish naïve and memory subsets, activation markers, and inhibitory checkpoint receptors (Rambaldi et al., 2021). I will explore whether haplo-cord HSCT, compared to haplo-HSCT and allo-HSCT, allows CD8⁺ T cells to become more differentiated with functions and memory while exhibiting lower exhaustion markers.

SUMMER 2026

Single-cell RNA sequencing (scRNA-seq) and T cell receptor (TCR) sequencing will characterize transcriptional profiles and identify clonotypes of CD8⁺ T cells from each transplant approach. The results will identify signatures of activation or exhaustion at the single-cell level, gene expression of cytotoxicity and reveal whether effective CD8⁺ T-cell subsets after HSCT, especially following haplo-cord HSCT. I will explore whether haplo-cord HSCT generates healthy CD8⁺ T cells with cytotoxic functions and proliferation of diverse effector cells that support GVL responses.

Evaluating and comparing the functional capacities of CD8⁺ T cells among the three transplant approaches includes assessing the proliferative potential of CD8⁺ T cells in response to stimuli and measuring the proliferation amount. Cell proliferation analysis by EdU assays will be done to achieve the comparison. I will explore whether haplo-cord HSCT generates superior proliferative CD8⁺ T cells that improve functional fitness and responsiveness than haplo-HSCT and allo-HSCT.

Aim 2: To evaluate how these differences in CD8⁺ T-cell populations affect clinical response***Rationale:***

Although profiling the immune cell phenotypes and gene expressions can reveal differences in CD8⁺ T cell reconstitution across HSCT approaches, it is important to determine how these differences translate into functional anti-leukemia clinical responses. During GVL, donor CD8⁺ T cells can recognize leukemia antigens and eliminate malignant cells through antigen-specific cytotoxicity that is mediated by surface markers perforin and granzyme release, as well as cytokine production. However, these interactions of effector mechanisms with healthy host tissues also lead to GVHD initiation, causing proliferation, cytotoxic molecule release, and tissue injury (Rezvani et al., 2007).

To assess the anti-leukemia effect of CD8⁺ T cells from each transplant setting, an *in vivo* model can be adopted. By utilizing immunodeficient mouse models engrafted with human B-ALL cells, comparison of the GVHD and GVL effects, as well as survival outcomes among the models will show which is more effective after transferring CD8⁺ T cells from haplo-cord HSCT, haplo-HSCT, or allo-HSCT into them. I will explore how differences in CD8⁺ T cell phenotype and function influence the effect of GVL, GVHD severity, and survival outcomes.

APPROACH**Blood Sample Collection**

Blood and bone marrow samples will be collected from patients receiving haplo-cord HSCT, haplo-HSCT with the immunosuppressant PTCy, and allo-HSCT. All of the samples will be processed under identical conditions for downstream assays and CD8⁺ T cells will be the primary focus throughout the analysis. All procedures involving human participants and patient sample collection will require prior approval from the Institutional Review Board (IRB), and informed consent will be obtained in accordance with the institutional ethical guidelines before study initiation.

Flow Cytometry Immunophenotyping

Flow cytometry immunophenotyping will be performed to characterize CD8⁺ T cells and their subsets based on surface markers. For example, CD45RA⁺ and CD45RO⁻ will distinguish naïve T cells; CD45RO⁺ and CCR7 will distinguish memory T cells; granzyme B and perforin will distinguish effector T cells; and CD8 will mainly distinguish CTLs (Rezvani et al., 2007). Flow cytometry will determine the presence and abundance of surface markers on the various cells. Blood samples will be processed to isolate peripheral blood mononuclear cells (PBMCs) using Ficoll-Paque density gradient centrifugation. Collected cells will be rinsed with 1% phosphate-buffered saline (PBS) and then surface-stained with fluorochrome-conjugated monoclonal antibodies targeting CD8 (clone OKT8, Pacific Blue, in-house), CD45RA (clone MEM-56, Quantum Dot 655, in-house), CD45RO (UCHL1, PE, ThermoFisher Scientific), CCR7 (clone 3D12, PE-Cyane7, BD Biosciences), granzyme B (clone GB11, Alexa Fluor 647, ThermoFisher Scientific), and perforin (deltaG9, PE-Cyane7, ThermoFisher Scientific) antigens. All antibodies are directly conjugated to their respective fluorochromes. After incubation, the cells will be washed by PBS buffer. A BD FACSCalibur (BD Biosciences) will perform the data acquisition of flow cytometry and FlowJo software will carry out the analysis (McLaughlin et al., 2008). Identification of proportion of T-cell subsets can be based on the expression patterns of surface markers. I anticipate that haplo-cord HSCT will have more expression of activated memory and effector T cells.

RNA Sequencing for CD8⁺ T cells Gene Expression

Cell Culture

Peripheral blood samples from each transplant group will be processed to isolate mononuclear CD8⁺ T cells using density gradient centrifugation with Lymphocyte Separation Medium (Corning). Enrichment of CD8⁺ T cells will be achieved through negative magnetic selection (MojoSort Human CD8⁺ T Cell Isolation Kit; BioLegend), followed by a removal of dead cells (Miltenyi Biotec). Approximately 0.5-1*10⁶ enriched CD8⁺ T cells from each donor will be cultured in a complete medium for 16 hours at 37°C. To initiate T cells in

SUMMER 2026

response to antigens, some cultures will be stimulated by CD3 and co-stimulatory receptor CD28 using Human CD3/CD28 T Cell Activator (STEMCELL Technologies) (Szabo et al., 2019).

Single-Cell RNA Sequencing

Single-cell RNA Sequencing (scRNA-seq) will identify transcriptional signatures associated with T-cell gene expression and exhaustion patterns. Using microfluidic droplet-based scRNA-seq (10x Genomics), single-cell suspensions will be loaded onto a Chromium Single Cell Chip following the manufacturer's instructions for co-encapsulation with barcoded Gel Beads. Each droplet contains a single cell and a Gel Bead that carries thousands of oligonucleotides with a unique cell barcode and unique molecular identifiers (UMIs). During complementary DNA (cDNA) synthesis, the mRNA molecules captured will incorporate both the barcode to label all the transcripts and UMIs to tag individual mRNA molecules. The barcoded cDNA in each droplet will subsequently be pooled into a single tube, where they will be converted into pooled scRNA-seq libraries using the Chromium Single Cell 3' Solution (Szabo et al., 2019). This approach will cluster CD8⁺ T cells into subpopulations based on their gene expression patterns. By comparing the cluster composition between haplo-cord, haploidentical, and allogeneic cohorts, it will be clear if haplo-cord patients have a cluster with significantly more effector T cells or memory T cells, whereas the other patients express more exhausted T cells with genes like PD-1, LAG3, CTLA-4, and TIM-3. This analysis also supports the examination for different gene expressions, such as cytotoxic functions (perforin, granzymes, IFN- γ , TNF) and proliferation in haplo-cord CD8⁺ T cells.

T-Cell Receptor Sequencing

Integrating TCR sequencing with PBMC collected from the three HSCTs can evaluate diversity and expansions within clones of T cells. Nucleic acids will be isolated from PBMCs or sorted T cells, and library preparation will be performed using the approach of multiplex PCR-based assays. Following the sequencing of fragmenting nucleic acids into specific TCR genes, adding barcodes of short DNA sequences onto the fragments for machine recognition, and amplifying fragments for reliable detection, the resulting DNA fragments are prepared for sequencer reading and analysis of TCR diversity. This sequencing approach will identify and quantify clonotypes, listing all unique TCR sequences detected in the sample among the T-cell clonal composition. I anticipate that TCR repertoires from haplo-cord HSCT will display greater diversity and a more balanced clonal distribution, supporting stronger GVL effects.

T Cell Proliferation Assays

SUMMER 2026

Activation and proliferation markers will also be assessed to indicate recent CD8⁺ T cell division and response to antigen. The Click-iT EdU Cell Proliferation Assay Kit (ThermoFisher Scientific) will be used to assay proliferation, following the manufacturer's instructions. The proliferating T cells will incorporate the thymidine analog EdU (5-ethynyl-2'-deoxyuridine) into their DNA when they are stimulated with antigenic stimuli. A copper-catalyzed "click" reaction between EdU and azide-conjugated fluorophore enables detection of EdU incorporated into the DNA of actively dividing cells, without requiring DNA denaturation. The intensity of EdU labeling with fluorescence reflects cell proliferation and replication cycles they undergo. Flow cytometry will analyze the labeled cells with surface and activation marker staining, enabling precise and quantitative measure of CD8⁺ T cell proliferation in response to antigenic stimuli (ThermoFisher Scientific, 2023). I anticipate that CD8⁺ T cells from haplo-cord HSCT will demonstrate significantly higher proliferative capacity in response to antigenic stimuli.

Xenograft Mouse Model *in Vivo*

To test the functional differences of T cells *in vivo*, I will utilize the immunodeficient NOD scid gamma (NSG) mice (Jackson Laboratory) model to engraft with human B-ALL cells due to their capability to support the growth and proliferation of human leukemia cells. All animal experiments will require prior approval from the Institutional Animal Care and Use Committee (IACUC) and will be conducted in accordance with institutional animal care and welfare guidelines. The genetic defects of the NSG mice weaken their immune system and cause them to be highly permissive to human cell engraftment, particularly supporting human HSCs and T cell development (Shultz et al., 2012). Mice will be grouped into cohorts with comparable leukemia burden, and the CD8⁺ T cells from HSCT patients will be injected into them to study the T cell behaviors. The method to establish leukemia in mice utilizes a firefly luciferase-expressing B-ALL model, specifically the B-ALL cell line has been genetically engineered to express luciferase. These modified luciferase leukemia cells will be intravenously injected into NSG mice, allowing non-invasive monitoring of cancer growth through bioluminescence imaging (Mhaidly & Verhoeyen, 2020). After confirmation of successful leukemia engraftment by imaging, mice will be randomly assigned into four groups: CD8⁺ T cells from a haplo-cord HSCT patient, CD8⁺ T cells from a haplo-HSCT patient, CD8⁺ T cells from an allo-HSCT patient, or a control group with no T cells injected. Leukemia progression will be monitored weekly by bioluminescence imaging and tracking the survival of the mice. The measurements of outcome include the leukemia burden over time (luminescence intensity correlates with number of leukemia cells), survival time of the mice, human T-cell persistence *in vivo* (measured by sampling mouse peripheral blood for human CD8⁺ cells by flow cytometry), and any observable signs of GVHD, such as weight loss, deterioration of fur condition, and swelling or reddening at extremities (Nowak et al., 2025). I anticipate that mice injected with CD8⁺ T cells from a haplo-cord HSCT patient will result in outcomes of reduced leukemia burden, prolonged survival, and sustained human T-cell persistence

SUMMER 2026

compared to mice injected with CD8⁺ T cells from a haplo-HSCT patient and CD8⁺ T cells from an allo-HSCT patient.

Quantitative and Statistical Analysis of the Efficacy of Haplo-cord HSCT

Statistical analyses, including ANOVA or Kruskal-Wallis tests, will compare quantitative immune parameters between the three experimental groups, with post-hoc tests for pairing comparisons. Longitudinal data measuring disease progression will be analyzed using mixed-effects models. Pearson or Spearman correlation coefficient will be performed to associate immune profiles, such as phenotype expressions on CD8⁺ T cells (Mishra et al., 2019).

All xenograft experiments will be repeated with T cells from different patients to ensure generalizability. This proposal's integrated approach through combining clinical data with advanced immunological assays will ensure enhanced understanding of the roles of CD8⁺ T cells in haplo-cord versus other approaches of HSCTs.

DISCUSSION

This research proposal aims to determine the most effective HSCT approach through profiling of CD8⁺ T cells. I hypothesized that haplo-cord HSCT will derive CD8⁺ T cells that are more effective against leukemia while reducing risk of GVHD, with superior activation, persistence, and less exhaustion. Flow cytometry, scRNA sequencing, TCR sequencing, and *in vivo* xenograft modeling will be used to characterize the biological markers and functional properties of CD8⁺ T cells under different HSCT approaches, allowing an evaluation of GVL and GVHD across different transplant strategies. I anticipate that mice receiving CD8⁺ T cells from haplo-cord HSCT will show a higher safety profile, demonstrated by reduced GVHD, stable weight and improved survival, while maintaining strong GVL effect. If haplo-cord HSCT creates a more GVL-effective T-cell repertoire, with more effective and less exhausted CD8⁺ T cells, it will provide an immunological explanation for the improved B-ALL outcomes observed with haplo-cord HSCT. Thus, these findings would encourage broader use of haplo-cord HSCT. While the focus of this proposal is CD8⁺ T cells, it is also important to measure helper CD4⁺ T-cell reconstitution and NK cell counts to ensure a more comprehensive understanding since the differences in those compartments could influence the risk of infections and balance between GVL and GVHD.

Beyond providing biological explanations for the efficacy of different HSCT approaches, this proposal also helps identify markers that strongly correlate with GVL and GVHD outcomes. For instance, activation markers and proliferative capacity might predict *in vivo* anti-leukemia activity, while the distribution of T-cell subsets might indicate GVHD risk. Validating the correlations would enable more accurate assessments of

SUMMER 2026

patient prognosis after different HSCT approaches. Furthermore, prognosis indicators identified in the experiments could be applied to optimize existing therapies with higher GVL activity and reduced GVHD, such as through the addition of small molecules or immunomodulatory agents.

Ultimately, this research proposal aims to connect clinical observations with immune mechanisms. A clearer understanding of how haplo-cord HSCT influences CD8⁺ T-cell recovery will provide insights for transplant physicians on adjusting post-transplant care. By determining the immune profiles and functions of CTLs in each transplant approach, I will contribute to the optimization of transplant strategies, which aim to maximize the GVL effect while minimizing relapse and GVHD. This research can thus guide how I can manipulate the immune system to cure leukemia more effectively.

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