The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

Application of Saccharomyces cerevisiae to improve pediatric acute lymphoblastic leukemia outcomes

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submitted by Matthew Gynn in partial fulfillment of the requirements for
the degree of Master of Science
in Experimental Medicine

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Abstract

Pediatric acute lymphoblastic leukemia (ALL) is the most commonly diagnosed childhood cancer in Canada and continues to need improvements in the care patients receive. Saccharomyces cerevisiae (S. cerevisiae) is a generally nonpathogenic organism that has the potential to affect both stages of leukemia (preleukemia and leukemia) and improve ALL outcomes by acting as a drug delivery vehicle for L-ASNase and/or influence leukemogenesis by inducing early-life, antileukemic immune activity. Mild infectious exposures during infancy and childhood has consistently been shown to influence leukemogenesis. In mice, early-life infections can deplete preleukemic cells and prevent preleukemia developing into leukemia. If preleukemia develops into ALL, children undergo chemotherapy that includes the enzyme L-asparaginase (L-ASNase). ALL cells characteristically stop expressing the enzyme asparagine synthetase (AS) and rely on extracellular sources for L-ASN. L-ASNase depletes extracellular L-ASN, selectively starving ALL cells of L-ASN, and consequently inducing their apoptosis.

To evaluate the efficacy of S. cerevisiae to improve L-ASNase therapy, it was first engineered to constitutively express cell-wall associated L-ASNase-II (AEY – asparaginase expressing yeast). The cell-wall association may improve ALL therapy by shielding L-ASNase from immune detection and consequently reducing the cost, toxicity, and immunogenicity of L-ASNase therapy. In vitro, AEY co-cultured with ALL cell lines can deplete L-ASN levels leading to apoptosis and inhibited cell growth in the asparagine synthetase (AS)-negative cell line RS4;11, but not the AS-positive cell lines BV173 and 697. The AEY biomass required to yield a therapeutic L-ASNase dose exceeds the current ethical limitations for further study in vivo.
To determine if *S. cerevisiae* can activate early-life, antileukemic immune activity, *S. cerevisiae* was injected intraperitoneally into day-6-old Eμ-RET mice and the effect on preleukemic burden was assessed. *S. cerevisiae* is unable to activate the IL-17A and Natural Killer (NK) cell-dependent immune response that has been shown to deplete preleukemic cells. Additionally, both *in vitro* and *in vivo* stimulation of NK cells with IL-17A does not directly lead to their activation. Further investigation into the mechanism leading to the activation of antileukemic NK cells may uncover new immunotherapeutic approaches for ALL.
Lay Summary

Leukemia is a cancer caused by the over proliferation of white blood cells (WBCs). Some children are born with abnormal WBCs that could become cancerous, yet most of these children do not develop leukemia. Common infections at a young age are associated with a decreased risk of leukemia, possibly explaining why most children with abnormal WBCs do not get leukemia. Our lab discovered that infections given to young mice can cause an immune response that kills abnormal WBCs, preventing leukemia. Baker’s yeast is a microorganism that may improve leukemia therapy in two ways. Yeast cells may cause the same immune response as common infections to kill leukemic cells. Yeast cells can also be engineered to deliver the leukemia drug: asparaginase. Unfortunately, yeast cells do not cause immune responses that kill leukemic cells in young mice and too many yeast cells are required to effectively deliver asparaginase.
Preface

All work was completed at either the BC Children’s Hospital Research Institute or the Renaissance BioScience Corporation headquarters, both located in Vancouver, Canada. All animal work was conducted in line with The University of British Columbia ethical guidelines (protocol A15-0187). The work in Chapter 3 was co-conceived and designed with Dr. Gregor Reid (University of British Columbia) and Dr. Matthew Dahabieh (Renaissance BioScience Corporation). The work in Chapter 4 was co-conceived and designed with Dr. Gregor Reid.

Other than the exceptions listed below, I performed the planning and set-up of all the experiments, the data acquisition, the data analysis, the figure presentation, and the interpretation of the results. The work in Chapter 4 contributed to a manuscript which, at the time of writing this thesis, is in preparation for submission to a peer-reviewed journal.

Chapter 3

- The generation of all L-asparaginase yeast strains used in the study were performed by either Ye Wang or Jason Hung at Renaissance BioScience Corporation.
- The yeast cells used for each experiment were inoculated by either Jason Hung or Ye Wang.
- The YEG plates used for experiments were prepared by either Ye Wang or Jason Jung.
- The UPLC analysis of L-asparagine concentrations was performed by Yiqiong Jin at Renaissance BioScience Corporation.
- The transduction of the luciferase gene into the RS4;11 using lentivirus was performed by Reza Rehavi.
• Two of the initial IV injection via tail vein of RS4;11 cells into the NSG mice used for Figure 4.7 for both cohorts were performed by Sumin Jo.

Chapter 4

• The YEG plates used to determine the concentration of S. cerevisiae were prepared by Ye Wang at Renaissance BioScience Corporation.

• In Figure 3.3 A, for 2 of the trials (n=2) for the NK (Lm) and NK (PBS) treatment conditions, the experimental set-up and data acquisition was performed by Dr. Mario Fidanza.

• In Figure 3.4 B, for the PBS treated mice (n=6) and the Lm treated mice (n=4), the data was collected and analyzed by Mario Fidanza.
Table of Contents

Abstract ........................................................................................................................................ iii
Lay Summary ................................................................................................................................. v
Preface ........................................................................................................................................ vi
Table of Contents ......................................................................................................................... viii
List of Tables ................................................................................................................................ xiv
List of Figures ............................................................................................................................. xv
List of Abbreviations ................................................................................................................... xvii
Acknowledgements ................................................................................................................... xxi
Dedication .................................................................................................................................... xxiii

Chapter 1: Introduction ............................................................................................................... 1
  1.1 Characterizing ALL ............................................................................................................. 1
  1.2 Leukemia Statistics .......................................................................................................... 1
  1.3 Two-stage disease model for pediatric leukemia ............................................................. 3
  1.4 Mouse modeling for leukemia ......................................................................................... 5
  1.5 Environmental exposures contribute to leukemogenesis .............................................. 8
  1.6 Pediatric ALL chemotherapy ......................................................................................... 9
  1.7 The role of L-asparaginase in ALL therapy .................................................................. 11
  1.8 Indications for investigating Saccharomyces cerevisiae use in ALL ......................... 12
  1.9 Overall Hypothesis ........................................................................................................ 15

Chapter 2: Methods .................................................................................................................... 16
  2.1 Materials and methods for Chapter 3 ........................................................................... 16
2.1.1 Yeast concentration........................................................................................................16
2.1.2 Determination of heat-inactivation temperature for yeast ........................................16
2.1.3 UV inactivation of yeast ....................................................................................................16
2.1.4 Yeast inoculation and preparation for in vitro experiments ..........................................17
2.1.5 In vitro mammalian cell culture assays ...........................................................................17
2.1.6 L-asparaginase activity assays ........................................................................................18
2.1.7 Western blots ..................................................................................................................19
2.1.8 Transduction of human cell lines with luciferase gene ..................................................20
2.1.9 In vivo analysis of low dose L-asparaginase .................................................................21
2.1.10 Yeast lysates .................................................................................................................21

2.2 Materials and methods for Chapter 4 ..............................................................................22
2.2.1 Infections .......................................................................................................................22
2.2.2 Mouse Genotyping .........................................................................................................23
2.2.3 Processing of mouse spleens ........................................................................................23
2.2.4 Determining preleukemic burden and spleen size of Eμ-RET mice ...............................24
2.2.5 Determining the percentage of activated γδ T cells and NK cells ...............................25
2.2.6 Determining the total number of neutrophils and activated γδ T cells in Eμ-RET mouse spleens ................................................................................................25
2.2.7 In vitro direct killing assays ...........................................................................................26
2.2.8 Phenotyping γδ T cells ....................................................................................................27
2.2.9 Adoptive transfer of γδ T cells .......................................................................................27
2.2.10 NK cell culture .............................................................................................................28
2.2.11 NK cell enriched splenocyte killing assay ....................................................................28
Chapter 3: Investigating the utilization of L-asparaginase expressing *Saccharomyces cerevisiae* to improve L-ASNase therapy for the treatment of ALL

3.1 Aim ................................................................................................................................. 32
3.2 Introduction ...................................................................................................................... 32
  3.2.1 Therapeutic use of L-ASNases .................................................................................. 32
  3.2.2 Current limitations of L-ASNase therapy ................................................................. 33
  3.2.3 Indications for using *S. cerevisiae* as a drug delivery vehicle for L-ASNase ... 34
3.3 Results .............................................................................................................................. 35
  3.3.1 Ultraviolet light vs. heat killing of yeast cells ......................................................... 35
  3.3.2 Asparagine synthetase protein level predicts sensitivity to L-ASNase treatment ... 41
  3.3.3 *S. cerevisiae* expressing *E. coli* l-asparaginase reduces RS4;11 cell viability ....... 54
  3.3.4 Technical and ethical limitations prevent the use of AEY *in vivo* ......................... 59
3.4 Discussion ......................................................................................................................... 65
  3.4.1 Current limitations of using L-asparaginase expressing yeast ................................. 65
  3.4.2 Proposed improvements for AEY therapy ................................................................. 67
  3.4.3 *Saccharomyces cerevisiae* drug delivery for enzyme replacement therapies ........ 71
  3.4.4 Addressing the immunogenicity of L-asparaginase expressing yeast ................. 72
  3.4.5 Concluding Remarks ............................................................................................... 73
Chapter 4: The effects of neonatal *Saccharomyces cerevisiae* exposures on ALL progression

4.1 Aim .............................................................................................................. 74

4.2 Introduction ................................................................................................. 74
  4.2.1 Problems with pediatric ALL chemotherapy ........................................... 74
  4.2.2 The role of infection in influencing pediatric leukemogenesis ............... 74
  4.2.3 Indications for testing the effect of early-life *Saccharomyces Cerevisiae* exposures on leukemogenesis in Eμ-RET mice ................................................................. 75

4.3 Results .......................................................................................................... 79
  4.3.1 Neonatal *Saccharomyces cerevisiae* exposure does not induce depletion of preleukemic cells ........................................................................................................... 79
  4.3.2 *Saccharomyces cerevisiae* does not induce the expansion of activated γδ T cells or activate NK cells ........................................................................................................ 83
  4.3.3 γδ T cells activated in response to infection do not have antileukemic activity *in vitro* ....................................................................................................................... 87
  4.3.4 The effect of IL-17 on NK activation in Balb/c mice .................................. 93

4.4 Discussion ..................................................................................................... 101
  4.4.1 Summary of results ................................................................................ 101
  4.4.2 *S. cerevisiae* may still influence the neonatal immune system and leukemogenesis 102
  4.4.3 The function and contribution of γδT17 cells and IL-17 in generating antileukemic immune activity remains unknown ................................................................. 104
4.4.4 IL-2/IL-15 mediated activation of neonatal NK cell cytotoxicity requires further investigation .................................................................................................................. 111

4.4.5 Concluding Remarks .......................................................................................................................... 112

Chapter 5: Conclusion .............................................................................................................................. 114

5.1 Key Results ........................................................................................................................................... 114

5.2 Future Research ..................................................................................................................................... 114

5.3 Concluding Remarks ............................................................................................................................. 117

References ..................................................................................................................................................... 118

Appendices .................................................................................................................................................. 132

Appendix A - Chapter 3 Flow Cytometry Gating ..................................................................................... 132

A.1 Initial gating for all samples .................................................................................................................. 132

A.2 Asparaginase expressing yeast titrations ........................................................................................... 133

A.3 Overnight, 2-day, and 5-day apoptosis and cell death assays ............................................................... 134

A.4 Proliferation dye assays ....................................................................................................................... 135

Appendix B - Chapter 4 Flow Cytometry Gating ..................................................................................... 136

B.1 Initial gating for all samples .................................................................................................................. 136

B.2 Preleukemic cells in Eμ-RET mice ..................................................................................................... 137

B.3 γδ T cells .............................................................................................................................................. 138

B.4 Natural Killer cells ............................................................................................................................... 139

B.5 Neutrophils .......................................................................................................................................... 140

B.6 289-Luciferase-GFP Cells (NK cell in vitro killing assays) ..................................................................... 141

Appendix C - Formulas .......................................................................................................................... 142

C.1 Formula 1 – L-asparaginase activity calculation .................................................................................. 142
C.2 Formula 2 – Determining the total number of cells of interest in a mouse spleen. 142
List of Tables

Table 3.1 The evaluation of yeast colony formation after 15 mins of incubation at 6 different temperatures .............................................................. 37

Table 3.2 Amount of yeast cells required for 1U of L-asparaginase activity after various treatment protocols .......................................................... 39

Table 3.3 The number of colonies formed after 3 x 5min UV exposure for multiple different starting concentrations of yeast .............................................. 39

Table 3.4 ED<sub>50</sub> values for E. coli L-asparaginase vs. AEY treatments for 6 different cells ........ 49
List of Figures

Figure 1.1 The two-stage disease progression of pediatric acute lymphoblastic leukemia .......... 4

Figure 3.2 The effect of heat vs. UV-light killing of yeast cells on subsequent L-asparaginase activity ........................................................................................................................................................................... 38

Figure 3.3 ALL cells cannot be cultured in a highly concentrated yeast environment ............ 40

Figure 3.4 The sensitivity of 6 different ALL cell lines to L-asparaginase and AEY ............... 47

Figure 3.5 Comparison of ED50 for 6 ALL cell lines. ................................................................. 48

Figure 3.6 The effect of E. coli L-asparaginase vs. AEY on the growth kinetics of 3 ALL cell lines ........................................................................................................................................................................... 51

Figure 3.7 The effect of E. coli L-Asparaginase vs. AEY on apoptosis and cell death for 3 different ALL cell lines ........................................................................................................................................................................... 53

Figure 3.8 The efficacy of S. cerevisiae constitutively expressing E. coli L-ASNase in depleting l-asparagine and inducing cell death in an asparagine synthetase negative cell line ............ 58

Figure 3.9 Overview of the GFP-sorting strategy used to generate RS4;11-luciferase-GFP cells62

Figure 3.10 The effect of low dose E. coli L-asparaginase on ALL tumour growth in vivo ....... 64

Figure 4.11 The current understanding of the mechanism underlying the depletion of preleukemic cells in Eμ-RET mice ................................................................................................................................. 78

Figure 4.12 The effect of neonatal Saccharomyces cerevisiae exposures on splenic preleukemic burden and spleen size ................................................................................................................................. 82

Figure 4.13 The effect of Listeria monocytogenes and Saccharomyces cerevisiae on NK cells, γδ T cells, and neutrophils in the spleen of Eμ-RET mice ........................................................................................................ 86

Figure 4.14 The contribution of IFNγ-producing γδ T cells in neonatal, antileukemic immune responses ........................................................................................................................................................................... 91
Figure 4.15 The effect of culturing sorted, activated γδ T cells in the presence of a leukemia cell line........................................................................................................................................... 92

Figure 4.16 The effect of IL-17 on NK activation and NK-mediated depletion of leukemic in vitro ........................................................................................................................................... 98

Figure 4.17 The effect of IL-17 on NK cell activation in vivo ................................................................. 100

Figure 4.18 Two possible mechanisms explaining the role of IL-17 in the murine antileukemic, early-life immune responses ........................................................................................................... 110
List of Abbreviations

289-Luc  289 cell line that constitutively expresses a luciferase enzyme conjugated green fluorescent protein

ASP3  *Saccharomyces cerevisiae* L-asparaginase Gene

ASP3-AEY  Asparaginase Expressing Yeast Constitutively Expressing the ASP3 Gene

AEY  Asparaginase Expressing Yeast

ALL  Acute Lymphoblastic Leukemia

AML  Acute Myeloid Leukemia

ANOVA  Analysis of Variance

AnsB  *Escherichia coli* L-asparaginase Gene

AnsB-AEY  Asparaginase Expressing Yeast Constitutively Expressing the AnsB Gene

AS  Asparagine Synthetase

ASP3  *Saccharomyces cerevisiae* L-Asparaginase-II Gene

B-ALL  B cell Acute Lymphoblastic Leukemia

BD  Becton Dickinson

BHI  Brain Heart Infusion

BSA  Bovine Serum Albumin

BV  BV 173

cfu  Colony Forming Units

CLL  Chronic Lymphoblastic Leukemia

CML  Chronic Myeloid Leukemia

CNS  Central Nervous System

d  Day(s)
DC  Dendritic Cell

_E. coli_  Escherichia coli

ECL  Enhanced Chemiluminescence

Erwinase  _Erwina carotovora_ L-Asparaginase

FACS  Fluorescent-activated Cell Sorted

FBS  Fetal Bovine Serum

FSC-A  Forward Scatter-area

GAPDH  Glyceraldehyde 3-phosphate Dehydrogenase

GFP  Green Florescent Protein

h  Hour(s)

IFN\(_\gamma\)  Interferon-gamma

Ifng  Mouse interferon-gamma gene

IgH  Immunoglobulin Heavy Chain

IL-17  Interleukin-17A; IL-17A

IP  Intraperitoneal(ly)

IV  Intravenous(ly)

JKT  T-ALL cell line, Jurkat

KO  Knockout of a specified gene

L-ASN  L-asparagine

L-ASNase  L-asparaginase

Lm  _Listeria monocytogenes_

min  minute

MRD  Minimal Residual Disease
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>N6</td>
<td>B-ALL cell line, Nalm6</td>
</tr>
<tr>
<td>NK Cell</td>
<td>Natural Killer Cell</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Non-obese diabetic/severe combined immunodeficient</td>
</tr>
<tr>
<td>NS Mice</td>
<td>NOD-SCID KO Mice</td>
</tr>
<tr>
<td>NSG Mice</td>
<td>NOD-SCID-gamma Mice</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDVF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>PEG-L-ASNase</td>
<td>Monoethoxypolyethylene Glycol Succinimidyl Conjugate of <em>E. coli</em> L-Asparaginase</td>
</tr>
<tr>
<td>RC</td>
<td>B-ALL cell line, RCH-ACV</td>
</tr>
<tr>
<td>RFP</td>
<td>Receptor tyrosine-protein kinase (RET) Fusion Protein</td>
</tr>
<tr>
<td>RS</td>
<td>B-ALL cell line, RS4;11</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SB</td>
<td>Sleeping Beauty</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SSC-A</td>
<td>Side Scatter-Area</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T cell Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline with 0.1% Tween</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype <em>S. cerevisiae</em></td>
</tr>
<tr>
<td>YEG</td>
<td>Yeast Extract Glucose</td>
</tr>
<tr>
<td>γδT17</td>
<td>IL-17 Producing γδ T Cells</td>
</tr>
</tbody>
</table>
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Thank you to the studentships and operating grants from both MITACS and CIHR that made both projects possible.

I would like to thank my friends for their on-going emotional support throughout this degree. Moving to Vancouver, BC was a tough choice, their support has enabled me to stay focused and motivated to complete this degree.

Lastly, I would also like to thank my parents David and Sonja and my sister Stephanie. Their on-going support continues to amaze me. I am thankful to having a supportive family.
Dedication

I would like to dedicate this work to my late grandfather, Frederick Macarthur Gynn. His battle with leukemia inspired me to join the fight against cancer.
Chapter 1: Introduction

1.1 Characterizing ALL

ALL can be further divided into two subtypes B cell ALL (B-ALL) and T cell ALL (T-ALL), representing 80% and 20% of ALL diagnoses respectively\(^1\)\(^-\)\(^4\). B-ALL can be further classified based on the presence of common gene translocations, such as the t(9;22)(q34.1;q11.2);*BCR-ABL1* translocation, the t(12;21)(p13.2;q22.1);*ETV6-RUNX1* translocation, and the t(1;19)(q23;p13.3);*TCF3-PBX1* translocation, hyperdiploidy or hypodiploidy\(^5\)\(^,\)\(^6\). T-ALL is further classified into early T cell precursor lymphoblastic leukemia or Natural Killer (NK) cell lymphoblastic leukemia/lymphoma\(^5\). As B-ALL represents the majority of pediatric ALL cases, this type of leukemia will be the primary focus of this study.

1.2 Leukemia Statistics

Leukemia, the cancer of circulating white blood cells, can be divided into four categories based on the proliferation of immature blast cells vs. mature cells (acute vs. chronic) and whether the cancer cells are of lymphocyte or myeloid origin (lymphoblastic or lymphocytic vs. myeloid): acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML)\(^1\)\(^,\)\(^7\). Overall, in Canada, leukemia is the 7\(^{th}\) deadliest cancer for men and 6\(^{th}\) deadliest cancer for women, representing 3.9% and 3.3% of total cancer-related deaths respectively\(^8\). In 2018, around 6,200 Canadians will be diagnosed with leukemia and approximately 3,000 cancer-related deaths will be due to leukemia\(^8\).

Forty-two percent of leukemic cases are acute leukemias, with ALL representing 10% of cases and AML representing 32% of cases, whereas the chronic leukemias CLL and CML represent 35% and 15% of leukemia cases\(^9\)\(^-\)\(^{12}\). Acute leukemias typically progress faster and have worse
prognoses compared to chronic leukemias\textsuperscript{13-21}. Risk of developing leukemia typically increases with age, except for ALL\textsuperscript{13,15,16,21}. ALL is the only type of leukemia that is more commonly diagnosed in children than adults\textsuperscript{7}. The risk of developing ALL is highest in childhood, drops in early adulthood, and then slowly increases again with age\textsuperscript{7}.

The global incidence of ALL is 3 per 100 000 with the highest incidence occurring in children between 1-9 years old\textsuperscript{7,22}. Although the long-term survival for children (age 1-10) with ALL is approaching 90\%, the prognosis for infants (age 0-1), adolescents (age 10-19), and adults (age 20+) still needs improvement with survival rates in these groups estimated to be 60\%, 75-80\%, and <40\% respectively\textsuperscript{13,23-29}. Additionally, 15-20\% of children experience relapse\textsuperscript{30-33}. If ALL relapse was considered its own distinct cancer diagnosis it would represent the fifth most common childhood malignancy\textsuperscript{32}. The long-term survival for children (age 1-10) experiencing ALL relapse drops to 40-50\%\textsuperscript{32,33}. For infants (age <1) and adolescents (age ≥ 10), the 5-year survival rate for relapsed patients drops to approximately 20\% for both cohorts\textsuperscript{33}. Other risk factors that predict inferior chances of post-relapse survival include male gender, central nervous system (CNS) infiltration, and T cell ALL\textsuperscript{33}. Despite favourable outcomes for most pediatric ALL patients, the high frequency of the disease relative to other childhood cancers still makes it the second leading cause of childhood cancer death in Canada\textsuperscript{2}. In the United States, ALL is the most common death due to cancer before age 20\textsuperscript{31}. These grim statistics highlight that despite high ALL cure rates, there is still an urgent need to continue to develop new approaches for ALL therapy, in particular for relapsed ALL.
1.3 Two-stage disease model for pediatric leukemia

Greaves’s hypothesis predicts childhood ALL to be a two-stage disease progression. The first stage of the disease occurs in utero. A percentage of children are born with genetic mutations in some of their lymphocytes that predispose them to later develop childhood leukemia. However, these in utero mutations alone are not sufficient to develop leukemia. For example, the ETV6-RUNX1 (or TEL-AML1) fusion gene is the most prevalent translocation in pediatric ALL, yet, the ETV6-RUNX1 gene fusion has been reported to be present in up to 5% of healthy newborns. In contrast, only 0.0016% of children develop ALL and only 25% of those children have the ETV6-RUNX1 gene fusion (approximately 0.0004% of total children). Hematopoietic cells containing the ETV6-RUNX1 fusion gene, or other genetic abnormalities, such as mutations in the ARID5B, IKZF1, CEBPR or CDKN2A genes are thus classified as preleukemic or predisposed to become leukemic. Currently, preleukemia can be “defined as a condition that has modifying mutations in the bone marrow that either cause myelodysplastic syndromes or cause clonal hematopoietic expansion initially without disease but associated with progression to leukemia.” In the second stage of leukemia, exogenous or endogenous exposures result in additional mutations that result in a child developing leukemia (see Figure 1.1).
Figure 1.1 The two-stage disease progression of pediatric acute lymphoblastic leukemia

Figure depicting the two-stage disease progression of pediatric acute lymphoblastic leukemia. A small percentage of children incur initiating events in utero leads to a detectable population of pre-leukemic cells detectable from birth. A small percentage of children will incur additional mutations that leads to pediatric ALL. Approximately 20% of children diagnosed with ALL will subsequently experience relapse.
1.4 Mouse modeling for leukemia

Effective disease modeling is essential for developing new therapeutic approaches for ALL. Several mouse models currently exist to help researchers to both understand the leukemogenesis of ALL and to test new therapeutics in preclinical models. Xenograft-derived ALL mouse models are an effective tool to complement *in vitro* studies, determine the rate of growth of a human leukemic sample, analyze biologic markers, and to test drug efficacy\(^4^4\). One of three types of mice are usually chosen as the hosts in a xenograft mouse model: nude mice, NOD-SCID (NS) mice, and NOD-SCID-gamma (NSG) mice\(^4^4\). Each strain of mice is immunocompromised to varying degrees to prevent immune rejection and encourage engraftment of leukemia. Nude mice have a mutation in *Foxn1* that results in lack of T cells, but these mice still have B cells, NK cells, monocytes and granulocytes. NS mice have a deletion of *Prkdc* that results in no B or T cells, but functional NK cells. NSG have an additional knockout of the *Il2rg* gene to render an immune system deficient in all lymphocytes. When choosing which mice strain to utilize as the host in a xenograft model, the more immunocompromised a mouse the greater the chance of engraftment; however, there is reduced ability to study the effects of the immune system on tumour growth\(^4^4\). Either an ALL cell line or a patient-derived tumour sample can be injected into the host mouse. Cell lines are useful for preliminary studies as they are more readily accessible and reproducible; however, cell lines lack the tumour heterogeneity present in patient-derived tumour samples, and the latter are thus better representations of disease complexity.

Additionally, selective pressures of *in vitro* culture can change the expression profile of genes in cell lines which do not revert during engraftment, further reducing their ability to effectively model disease\(^4^5\). Overall, xenograft-derived mouse models are useful to complement *in vitro* findings and to test drug efficacy, but the lack of immune system does prevent a more complete
representation of disease. Moreover, currently, there is no way to study the two-step disease progression of acute lymphoblastic leukemia using a xenograft model.

Transgenic mice are better models for the two-step progression of leukemia. Transgenic models can be used to test drug efficacy; however, the delayed, variable onset of disease makes using these models time consuming and expensive for drug discovery. Currently, there are three transgenic models primarily used to study ALL: the ETV6-RUNX1 model, the E2A-PBX1 model, and the Eμ-RET model. Although the ETV6-RUNX1 translocation is the most common translocation in pediatric ALL, constitutive expression of an ETV6-RUNX1 transgene under the immunoglobulin heavy chain (IgH) enhancer, does not lead to leukemia in mice. To overcome this obstacle, the Sleeping Beauty (SB) transposase is co-expressed with the ETV6-RUNX1 transgene and these mice are crossed with mice carrying a SB transposon array, leading to additional genetic abnormalities in addition to the ETV6-RUNX1 translocation. In this model, 20% of mice go on to develop B-ALL and tracking the insertional sites of SB has identified both known and novel genes that contribute to leukemogenesis in this model. Further building on this model, co-expressing the ETV6-RUNX1 with SB on a Pax5+/− background has provided further insight into genetic events that contribute to leukemogenesis: STAT pathway activation and Trp53 mutations. Pax5 mutations occur in 31.7% of childhood B-ALL cases. This example highlights how manipulation of the genetic background surrounding the ETV6-RUNX1 and SB construct can be used as a model to provide further insight into the role of the transgene and genetic events that lead to leukemia.
The *E2A-PBX1* mouse model utilizes the t(1;19) translocation (*E2A-PBX1*) which occurs in \( \approx 5\% \) of ALL cases\(^{50-54} \). When the *E2A-PBX1* transgene is constitutively driven by the IgH enhancer, mice go onto to develop aggressive T cell leukemia within 5 months; however, when crossed with a CD3\( \varepsilon \)-negative background the mice develop B-ALL\(^{50,55} \). When the *E2A-PBX1* transgene is conditionally expressed during B cell development the incidence of leukemia varies from 5-50\% depending on the promoter used to drive *E2A-PBX1* expression\(^56\). In this same model, cells expressing *E2A-PBX1* are GFP-positive, creating a model with a detectable preleukemic population that precedes leukemia\(^56\). Additionally, the loss of the *Pax5* gene cooperates with the *E2A-PBX1* transgene to increase the penetrance and shorten the latency until developing leukemia, highlighting how the *Pax5* gene plays a key role in leukemogenesis in more than one mouse model\(^{48,56} \). The *E2A-PBX1* model has been used to identify key secondary mutations in JAK and STAT signaling pathways that leukemic cells require for proliferation\(^56\).

Both the *ETV6-RUNX1* with SB and *E2A-PBX1* mouse models can generate B-ALL; however, the penetrance of ALL is less than 100\% in immune competent mice\(^{50,56} \). The E\( \mu \)-RET mouse model of B-ALL provides an advantage over these models by achieving 100\% penetrance for mice developing B cell precursor leukemia\(^57\). In the E\( \mu \)-RET model, RET tyrosine kinase expression is constitutively driven by the SV40 promoter and the IgH enhancer\(^57\). The RET fusion protein (RFP) generates a population of nonfunctional, pro-B cells that are BP\(-1^{hi}/B220^{int}/CD43^{int} \) and can be detected from birth in the spleen\(^57 \). These nonfunctional, pro-B cells can be considered preleukemic since they are IL-7 dependent and by themselves are not leukemic\(^58 \). The constitutive activity of the RFP allows for the ongoing survival of the nonfunctional pro-B cell population, a population of cells that would normally undergo apoptosis.
in a wild-type genetic background. Overtime, the nonfunctional pro-B cell population incurs additional mutations that eventually yield an IL-7-independent BP-1<sub>hi</sub>/B220<sup>int</sup>/CD43<sup>int</sup> population of leukemic cells. The Eµ-RET mouse model thus effectively models the two-step progression of leukemia; mice are born with preleukemic cells (stage one) that must sustain additional mutations to overcome their IL-7 dependency and become leukemic (stage two)<sup>58</sup>. Each mouse model presents different advantages depending on the nature of the study. The ETV6-RUNXI with SB and E2A-PBX1 mouse models may be more relevant for understanding the genetic events that lead to B-ALL, whereas, the Eµ-RET model has a higher penetrance of leukemia and an easily detectable preleukemic population that can be used to study the effects of environmental exposures on preleukemia.

1.5 Environmental exposures contribute to leukemogenesis

Exposures that have been linked to leukemia include exogenous exposures such as infection, ionizing radiation, and pesticides, and endogenous exposures such as inflammation and oxidative stress<sup>13,34,35,59-63</sup>. Of interest, infection has long been hypothesized as a leading factor in pediatric leukemia progression<sup>34,35</sup>. However, the epidemiological findings have reported contradictory results: infection can both increase and decrease the risk of developing leukemia, with the timing of the infection playing a critical role<sup>59,64-67</sup>. Recently, our lab uncovered the first mechanistic explanation for the protective role of infection and observed a role for timing of that infection in leukemogenesis<sup>68,69</sup>. In Eµ-RET mice, mild infections delivered during the neonatal period of life yield an NK-cell dependent immune response that results in the depletion of preleukemia, delaying the onset of leukemia<sup>69</sup>. 
Once diagnosed with leukemia, children are risk stratified and put on the corresponding remission induction therapy regimen. Patients are considered high risk based on several features such as age (less than 1 year or greater than 10 years), CNS involvement, testicular involvement, unfavourable cytogenetics (ex. hypodiploidy or the t(9;22) translocation). High-risk patients undergo an induction therapy regimen that includes a combination of glucocorticoid (prednisone or dexamethasone), vincristine, and L-asparaginase (L-ASNase), and an anthracycline (doxorubicin or daunorubicin). Low-risk patients only receive a glucocorticoid, vincristine, and L-ASNase to reduce toxicity. Remission induction therapy lasts 4-6 weeks with the goal of complete remission, defined as “less than 5% detectable blasts on microscopic morphology at the end of induction.” The detection of blasts occurs using flow cytometry or polymerase-chain reaction (PCR) which can detect 1 blast in 10,000-100,000 cells. Patients with between 0.01%-5% blasts after induction therapy will be classified as minimal residual disease (MRD) positive, and subsequently stratified as high-risk and placed on the appropriate treatment plan.

Recently, the flow cytometry and PCR-based assays used to determine the percentage of detectable blasts have been questioned for their sensitivity and speed respectively. New high-throughput sequencing and multidimensional flowcytometry technologies are being explored as more effective techniques for determining if a patient should be classified as MRD-positive.

Additionally, during remission induction therapy patients receive intrathecal chemotherapy, which has been shown to almost eliminate CNS relapse in pediatric ALL. After remission induction therapy, children undergo consolidation therapy to eradicate the submicroscopic residual disease. The duration and intensity of the consolidation protocol depend on the
Consolidation therapy utilizes a wider variety of drugs to prevent the build-up of drug resistance: mercaptopurine, methotrexate, thioguanine, cyclophosphamide, etoposide, and cytarabine. The last stage of chemotherapy, maintenance therapy, lasts 2-3 years as children undergo oral administration of antimetabolites (methotrexate or mercaptopurine).

Relapse occurs in 15-20% of patients when they fail to maintain complete remission of their ALL. Reinduction therapy after the first relapse includes the same four drugs as the original induction therapy: glucocorticoid, vincristine, and L-ASNase, and an anthracycline. Relapse ALL patients are also risk stratified, primarily based on the length of the first complete remission and the site of the relapse. Early relapses (relapse <18 months after diagnosis) are typically associated with a poor prognosis (0-48% survival) whereas prognosis improves with intermediate (relapse between 18 and 36 months after diagnosis; 10-75% survival) and late relapses (relapse ≥ 36 months after diagnosis; 33-87% survival). Relapses occurring in the bone marrow are associated with a worse prognosis than those occurring in the testes or CNS.

Patients experiencing B-ALL early relapse or T-ALL (early or late relapse) usually undergo hematopoietic stem cell transplants whereas late relapse B-ALL can usually be cured with just chemotherapy. Adults and children undergoing allogenic bone marrow transplants have overall survival rates between 40-60% and 60-80% respectively; but patients that are disease-free 2 years post-transplant have an 84% probability of surviving at least 10 years. New therapeutic approaches are needed for these high-risk patients as bone marrow transplants have, at best, modest survival rates due to common, life-threatening complications such as graft-versus-host-disease. Additionally, relapsed ALL can often be traced back to ancestral clones.
The presence of ancestral clones supports the two-step model for ALL. In this model, the relapsed leukemia develops from an ancestral population of cells that could be classified as preleukemic. After these persistent preleukemic cells survive chemotherapy, they can gain additional mutations and cause relapse. Developing new techniques to target the eradication of both leukemia and preleukemic cells may further reduce the risk of relapse and improve the rate of survival. Despite the overall survival rate of pediatric ALL approaching 90%, the 5-year event-free survival for children who experience ALL relapse is still between 40-50%, highlighting the need for new approaches to specifically prevent ALL relapse.

1.7 The role of L-asparaginase in ALL therapy

Both ALL standard induction therapy and relapse therapy include a combination of a glucocorticoid, vincristine, and L-ASNase. Of interest, L-asparaginase has a long history with ALL therapy. L-asparaginase catalyzes the hydrolysis L-asparagine (L-ASN) into L-aspartate and ammonia. The reciprocal enzyme to L-ASNase, asparagine synthetase (AS), catalyzes the conversion of aspartate and glutamine into asparagine and glutamate. ALL cells characteristically stop expressing asparagine synthetase (AS) and rely on extracellular sources for the amino acid L-ASN. L-ASNase therapy works by depleting serum levels of L-asparagine to starve the leukemic cells of an essential nutrient to inhibit tumor growth and induce apoptosis. Since healthy cells retain AS activity, and thus the ability to synthesize L-ASN, L-ASNase is a therapeutic that selectively targets ALL cells for death. The reason ALL cells silence AS remains unknown, but, a CpG island has been identified in the gene’s promoter and the methylation of this promoter is correlated with loss of AS activity in leukemic patients. Although many species of bacteria and fungi produce L-ASNase, currently, only two...
preparations are used clinically: *Escherichia coli* (*E. coli*) and *Erwina carotovora*\textsuperscript{13,87,93}. The monoethoxypolyethylene glycol succinimidy conjugate of *E. coli* L-ASNase (PEG-L-ASNase) has predominantly replaced the native *E. coli* product because of its superior half-life and decreased frequency of immune silencing and hypersensitivity reactions\textsuperscript{13,94-96}. *Erwina carotovora* L-ASNase (Erwinase) is typically reserved for patients who experience immune complications with PEG-L-ASNase such as allergic reactions and/or high anti-L-ASNase antibody titers\textsuperscript{13,87,97,98}. Despite the proven efficacy of L-ASNase as an ALL therapeutic, it is not devoid of problems. High costs, immune complications, and toxicity are all limitations for current L-ASNase therapies.

1.8 *Indications for investigating Saccharomyces cerevisiae use in ALL*

To this end, *Saccharomyces cerevisiae* (*S. cerevisiae*), commonly known as baker’s yeast, presents a novel approach as a therapeutic for both stages of childhood leukemia. *S. cerevisiae* is also a model organism for genetic manipulation and an established host to produce a wide range of chemicals\textsuperscript{99-102}. Moreover, *S. cerevisiae* utilizes two L-ASNases to metabolize asparagine: ASP1p and ASP3p, an intracellular L-ASNase I and an extracellular, cell wall-associated L-ASNase II, respectively\textsuperscript{103-105}. Both enzymes have been explored as potential L-ASNase therapeutics\textsuperscript{106,107}. Importantly, *S. cerevisiae* is also generally a non-pathogenic, commensal microorganism. In immuno-competent patients, yeast cells act as an adjuvant to promote robust CD8 T cell responses\textsuperscript{108,109}. This effect is mediated by yeast cell-wall components that activate toll-like-receptor (TLR) 2 and mannan receptors on dendritic cells (at a minimum). *S. cerevisiae*’s status as a non-pathogenic organism has already led to the development of *S. cerevisiae*-based vaccine vectors for cancer and viral diseases\textsuperscript{108-111}. 
Since L-ASNase II is native to *S. cerevisiae* and *S. cerevisiae* is a generally non-pathogenic organism in immuno-competent individuals, *S. cerevisiae* genetically manipulated to constitutively express L-ASNase II (ASP3) presents a novel approach to L-ASNase therapy. Under normal conditions, L-ASNase II is only secreted during conditions of nitrogen deprivation, and thus *S. cerevisiae* requires genetic manipulation to ensure its constitutive activity as a L-ASNase therapeutic\(^{112}\). Like *E. coli* L-ASNase, ASP3 is active at human body temperature and pH\(^93\). However, the cell-wall association of *S. cerevisiae* L-ASNase II may both shield the L-ASNase from the immune system and increase its half-life (t\(_{1/2}\)), therefore lowering the frequency with which L-ASNase may need to be given to the patient\(^{93,106}\). Moreover, identifying a new approach for L-ASNase therapy will greatly benefit relapsed patients who are more likely to have already built up immunity to PEG-L-ASNase and/or Erwinase\(^{87,113}\). Thus, using *S. cerevisiae* as a vector for L-ASNase may reduce the risk for both immune complications and toxicity currently associated L-ASNase therapy, yielding major benefits to the patient. Any reduction in immune complications will also decrease the need to transfer patients to the more expensive L-ASNase options. Alternatively, *S. cerevisiae* may mildly activate the immune system and yield antileukemic activity that eliminates leukemic cells and ancestral ALL clones, subsequently lowering the risk of relapse. Thus, *S. cerevisiae*-based drug delivery may double as an effective immunotherapeutic. Additionally, using a yeast-based vector will make L-ASNase therapy cheaper by removing the costs of enzyme purification. The benefits to both the patient and the payer of using *S. cerevisiae* as a vector for L-ASNase therapy are the focus of further study in Chapter 3.
Building on Chapter 3, I examine the immunogenicity *S. cerevisiae* by determining if early life exposures to *S. cerevisiae* induce the same IL-17 producing-γδ T cell-dependent, NK cell-mediated immune response as mild infections to deplete preleukemic cells, as seen with infection agents such as Lm⁶⁹. The role of the microbiome and nonpathogenic organisms in the development of leukemia is currently poorly understood. Recent research has begun to examine the effects of chemotherapy on the composition of the microbiome, but at this time, no research has investigated nonpathogenic, microbiota exposures and the subsequent effects on leukemia progression¹¹⁴-¹¹⁶. The fungal microbiome, or mycobiome, is emerging as an important component of the microbiome¹¹⁷-¹¹⁹. Of interest, *Saccharomyces cerevisiae* is a generally considered a nonpathogenic, commensal organism that is present in food and drink¹⁰²,¹²⁰. Although, *S. cerevisiae* cannot colonize the human gastrointestinal tract, exposure in early life is imminent though dietary and environmental exposures¹⁰²,¹¹⁸. In Chapter 4, I will evaluate the effects of early-life *S. cerevisiae* exposure on leukemogenesis in Eμ-RET mice. Additionally, I will further explore the role of IL-17 producing γδ T cells and IL-17 in the neonatal immune response that depletes preleukemic cells. The results shown in this Chapter will provide insight into the immunogenicity of *S. cerevisiae*-based drug delivery and whether this drug delivery vehicle may double as an immunotherapeutic that can kill preleukemic cells/ancestral ALL clones.

*S. cerevisiae* presents as a novel organism that may be able to affect both leukemogenesis and act as a drug delivery vehicle for the ALL therapeutic. Evaluation of the effect early-life exposures to *S. cerevisiae* on preleukemic burden will provide insight into the role of the *S. cerevisiae* and the microbiome in regulating preleukemia. Assessing the efficacy of using *S. cerevisiae* as a drug
delivery vehicle for the ALL therapeutic L-ASNase may reduce costs, toxicity, and immune complications currently associated with L-ASNase therapy. Additionally, any immune responses driven by S. cerevisiae may target ancestral preleukemic clones and reduce the risk of relapse when used as a therapeutic. In Chapter 3, I will analyze the feasibility of using S. cerevisiae cells as a drug delivery vehicle for L-ASNase. In Chapter 4, I will determine the potential of S. cerevisiae as an immunotherapeutic. Overall, the potential of S. cerevisiae to affect both stages of leukemia and consequently improve pediatric ALL outcomes warrants further investigation.

1.9 Overall Hypothesis

I anticipate that the cell wall-association of S. cerevisiae L-ASNase II will protect the protein from dilution and degradation in vivo, and may prevent adaptive immunological detection, while maintaining the ability to break down soluble L-asparagine. Additionally, the innate immune activity elicited by S. cerevisiae will yield antileukemic immune activity. This novel formulation will, therefore, provide L-ASNase therapeutic activity and double as an immunotherapeutic, while reducing the side-effects of current L-ASNase standard-of care formulations.
Chapter 2: Methods

2.1 Materials and methods for Chapter 3

2.1.1 Yeast concentration

*S. cerevisiae* constitutively expressing ASP3 (*S. cerevisiae* L-ASNase II), AnsB (*E. coli* L-ASNase) or the wild-type/parental control strain was supplied by Renaissance BioScience Corporation. *S. cerevisiae* constitutively expressing ASP3 will herein be referred to as L-asparaginase expressing yeast (AEY). The yeast concentration was measured by diluting the yeast solution 100x in distilled water, 1mL final volume, and measuring the absorbance at 600nm (OD\textsubscript{600}). This step was always performed in triplicate and the average OD\textsubscript{600} was used as the basis for dosing in subsequent experiments. By diluting 1mL of 1OD\textsubscript{600} 1 in 10\textsuperscript{6}, it was determined that 1OD\textsubscript{600} was equivalent to 2.12 x 10\textsuperscript{7} +/- 0.8 yeast cells/mL (n=6).

2.1.2 Determination of heat-inactivation temperature for yeast

2 x 10\textsuperscript{7} yeast cells were suspended in 1mL of distilled water and heated at either 30, 40, 50, 60, 70, 80 degrees Celsius for 15 mins. Samples were centrifuged at 13,100 rpm for 1 min and the supernatant was discarded. An inoculation loop was dipped in the yeast pellet and spread out on a YEG-agar plate using a cell spreader. YEG plates were supplied by Renaissance BioScience Corporation (Vancouver, BC). Plates were incubated overnight at 30\textdegree C and the presence of colonies was determined the next day. Each condition was performed in triplicate.

2.1.3 UV inactivation of yeast

Yeast cells were diluted in 15mL PBS and exposed to UV light for 5 mins in a 15mm x 100mm petri dish. The cell suspension was pipetted up and down for 30s to resuspend any yeast cells
stuck to the bottom of the petri dish after 5 mins. These two steps were repeated two additional times for a total of 15 mins of UV exposure. The cell suspension was collected and centrifuged at 5000rpm for 5 mins at 4°C in a 50mL centrifugal tube. The supernatant was discarded and the cell pellet was resuspended to yield a final concentration of 1.1 OD$_{600nm}$. 100μL of the final cell suspension was plated on YEG-agar plates and incubated overnight at 30°C. To determine the efficiency of UV inactivation, the total number of colonies present after overnight incubation was counted and compared to diluted, untreated yeast control plates.

2.1.4 Yeast inoculation and preparation for in vitro experiments.
Yeast strains were inoculated in 5mL of YEG media and incubated overnight in a rotating incubator at 30°C. 1mL aliquots of the inoculated yeast solution was heat-inactivated in a 60°C water bath for 15 mins. Herein, “heat-inactivated” yeast refers to yeast cells that have been heat-inactivated in a 60°C water bath for 15 mins. The yeast concentration was determined as previously described. Yeast cells were washed in 1mL PBS three times before being used for subsequent in vitro experiments.

2.1.5 In vitro mammalian cell culture assays
Six human ALL cell lines 380, 697, BV 173, Nalm6, RCH-ACV, and RS4;11 were obtained from the American Type Culture Collection (Manassas, VA) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, GER). Cells were cultured in RPMI media containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin, 2mM L-glutamine, 0.01M HEPES, and 5μM 2-mercaptoethanol. All media reagents were purchased from Thermo Fisher Scientific (Waltham, MA). For overnight and 2-day assays, 50,000 cells were incubated in
100μL of RPMI in a 96-well plate. For 5-day assays, 10,000 cells were plated for the cell lines 380, 697, BV 173, Nalm6, and RCH-ACV. For the slower growing cell line RS4;11, 25,000 cells were plated for 5-day assays. All 5-day assays were also done in a 96-well plate. Cells were incubated in either the presence of asparaginase-expressing yeast (AEY), wild-type *S. cerevisiae* (WT), purified *E. coli* L-ASN or untreated. Cells were washed with FACS buffer (PBS with 2% FBS, 0.01M sodium azide) followed by staining with flow cytometry antibodies. For apoptotic assays, cells were stained with Annexin V-FITC, 7-AAD and anti-human HLA-A/B/C-PE (clone W6/32). For cell proliferation assays, cells were stained with fixable viability dye-eFlour 520, and intracellular staining proliferation dye, eFlour 670. Unless otherwise stated, all flow cytometry antibodies herein were purchased from BioLegend (San Diego, CA) and counting beads were purchased from Thermo Fisher Scientific. After staining, cells were washed and resuspended in 100μL FACS buffer and counting beads (1.5μL or 3μL beads (concentration 49,000-52,000 beads/50μL) in 100μL FACS buffer). Samples were run on the BD Accuri flow cytometer. 500-1000 bead events were collected. Each experiment was analyzed in triplicate yielding an average measurement for each trial. Flow cytometry samples were run on a Becton Dickinson (BD) Accuri or BD LSRII cytometer (Franklin Lakes, NJ) and analyzed on FlowJo Version 10.0.

### 2.1.6 L-asparaginase activity assays

*E. coli* L-ASNase (0.1U/mL) or yeast cells (1 OD₆₀₀) were incubated in 0.5g/mL L-ASN solution for 15 mins in a 37°C water bath. Purified *E. coli* L-ASNase was purchased from Sigma-Aldrich (St. Louis, MO). Prior to suspension in the L-ASN solution, yeast cells were washed 3 times in 1 mL PBS. After each wash step, a cell pellet was obtained by centrifuging at 13,100rpm for 1 min
and the supernatant was discarded. After incubation, L-ASNase was inactivated in an 80°C water bath for 15 mins. At 80°C, both the viability of yeast cells and the activity of L-ASNase is eliminated. At 60°C, only the viability of the yeast cells is eliminated and some L-ASNase is retained (more in the results section). The starting and final L-ASNase concentrations were determined either by using a UV spectrophotometer-based L-ASN Assay Kit (Megazyme) or a UPLC-based AccQ-Tag UPLC kit. Ultraviolet-based L-ASN concentration kits were purchased from Megazyme (Bray, IRE). The AccQ-Tag UPLC kit for amino acid analysis was purchased from Waters Corp (Milford, MA). The L-ASNase activity was determined by subtracting the final from the initial L-ASN concentration, divided by time (see Formula 1 in Appendix C.1).

2.1.7 Western blots
Cell lysates were prepared for each cell line (380, 697, BV 173, Nalm6, RCH-ACV, RS4;11) by collecting 3x10⁷ cells, washing once with PBS to remove media, and lysing the cells using 300μL Cell Lysis Buffer with a mammalian cell protease inhibitor cocktail. The Cell Lysis Buffer was purchased from Cell Signaling Technology Inc. (product number: 9803; Danvers, MA) and the protease inhibitor cocktail for mammalian cell lysates was purchased from Sigma-Aldrich (product number: P8340). The cell suspensions were incubated on ice for 15 mins, mixing each suspension every 5 mins. The cell suspension was then centrifuged for 10 mins at 15,000g and the supernatant was collected and stored at -80°C until use. Protein concentrations were determined using the Pierce™ BCA protein assay kit purchased from Sigma-Aldrich. 20ug of protein was diluted to a final volume of 40μL using sodium dodecyl sulfate (SDS) loading buffer. Samples were then denatured at 95°C for 5 mins before loading onto gel. Samples were loaded into the wells of SDS-polyacrylamide gel (10% resolving gel, 6% stacking gel using
0.25M Tris-HCl buffer, pH 6.8 and 8.8 respectively). Samples were run at 80V while in the stacking gel and 105V while in the resolving gel. Gels were run in Towbin’s buffer (10% SDS (v/v), 25mM Tris, 192mM glycine, pH 8.3) and then the blot was transferred onto PDVF membrane overnight at 4°C at 40V in Towbin’s buffer (10% SDS (v/v), 20% methanol (v/v), 25mM Tris, 192mM glycine, pH 8.3). The membrane was blocked for 1h at room temperature using 5% BSA in tris-buffered saline with 0.1% Tween 20 (TBST). The membrane was then incubated in Asparagine Synthetase (AS) primary antibody (1:2000 dilution in 5% BSA in TBST), shaken overnight at 4°C. The membrane was then washed 3 times for 5 mins with TBST and incubated with anti-rabbit-HRP secondary antibody (1:2000 dilution) for 70 mins at room temperature in TBST (5% skim milk). The AS primary antibody was purchased from Novus Biologicals (product number: NBP2-14879; Littleton, CO) and the anti-rabbit-HRP secondary antibody was purchased from Thermo Fisher Scientific (product number 31460). All other Western blot reagents were also purchased from Thermo Fisher Scientific. After two 5-min washes in TBST followed by one 5-min wash in TBS, the membrane was developed using enhanced chemiluminescence (ECL) kit in dark room with exposures of 10s, 30s, 90s, and 5 mins onto X-ray film.

2.1.8 Transduction of human cell lines with luciferase gene

Cells were suspended in 24ug/mL polybrene in RPMI media. Lentivirus carrying the luciferase-GFP construct was incubated with cells overnight. The multiplicity of infection for the overnight incubation was 0.05 and the overall percentage of viable cells was maintained ≥ 90%. Cells went through 2 rounds of fluorescence-activated cell sorting (FACS) based on GFP expression to purify a 100% luciferase-positive population.
2.1.9 In vivo analysis of low dose L-asparaginase

NOD-SCID-Gamma (NSG) mouse/mice were originally purchased from Jackson Laboratories (strain number: 005557; Bar Harbor, ME) and a breeding colony is maintained in the BC Children’s Hospital Research Institute animal facility. $1 \times 10^6$ RS4;11-luciferase-tagged cells were intravenously (IV) injected into NSG mice via tail vein. 100U/kg L-ASNase was injected intraperitoneally (IP) in 200μL 10 days and 14 days post cell engraftment. 200U/kg L-ASNase was injected IP in 200μL 18 days post cell engraftment. Mice were imaged using a bioluminescent imager (Ami-x, Spectral Instruments Imaging, Phoenix, AZ) on day 0, 4, 7, 10, 14, 17, and 21. Serum was collected on day 21 and mice were sacrificed on day 21-22. Bone marrow cells were harvested and were cultured with/without L-ASNase (0.05U/mL). Serum was analyzed for its L-ASN concentration using AccQ-Tag UPLC kit.

2.1.10 Yeast lysates

$1.06 \times 10^7$ yeast cells were washed with 1mL water three times. Cell pellet incubated in 40μL zymolase solution (5mg/mL, dissolved in 1M sorbitol) for two hours at 30°C to degrade the cell wall. Cell lysates were then either subjected to mechanical lysis as previously described\textsuperscript{121} or osmotic lysis using double distilled water with yeast protease inhibitor cocktail purchased from Sigma-Aldrich (product number P8215).
2.2 Materials and methods for Chapter 4

2.2.1 Infections

Eμ-RET mice were generously donated from Dr. Stephan Grupp at The Children’s Hospital of Philadelphia. Interferon-gamma (Ifng) KO (strain number: 002286) and Balb/c (strain number: 000651) mice were originally obtained from Jackson Laboratories. For all mice, a breeding colony is maintained in the BC Children’s Hospital Research Institute animal facility. Eμ-RET/Ifng KO mice were generated after 10 generations of backcrossing Eμ-RET onto Ifng-KO. Eμ-RET, Eμ-RET/Ifng KO or wild-type Balb/c mice were infected with either attenuated *Listeria monocytogenes* (Lm) or *S. cerevisiae* between days 5-8 or 4-6 weeks of life. Lm was generously provided by Dr. Tobias Kollman (BC Children’s Hospital Research Institute, Vancouver, BC) and *S. cerevisiae* was obtained from Renaissance BioScience Corporation. Both microorganisms were injected intraperitoneally (IP) suspended in sterile PBS in a total volume of 50μl. For Lm, 10⁴ colony-forming units (cfu) were infected per mouse. For *S. cerevisiae*, either 10⁴, 10⁵, 10⁶ or 10⁷ cfu were injected. Each infection was performed in a volume of 50μL for neonates and 200μL for adults. IP injections were chosen because of the small tail vein vasculature of 6-day-old mice and the subsequent high degree of skill required to inject intravenously (IV). Additionally, IP injections were deemed acceptable because one of the organs listeriosis manifests in is the spleen. Cfu was determined using brain heart infusion (BHI)-agar plates for Lm, and yeast extract glucose (YEG)-agar plates for *S. cerevisiae*. Mice were either sacrificed 72h or 8d after the initial IP injection, depending on the experiment.
2.2.2 Mouse Genotyping

Ear snips were taken from each mouse in Eμ-RET litters where one parent (the sire in our breeding colony) is hemizygotic for the RFP transgene. The ear snips were digested for a minimum of 2h at 56°C in DirectPCR (Ear) Lysis reagent with 0.9mg/mL Proteinase K to obtain DNA in a total volume of 150µL. Both the DirectPCR Lysis reagent and Proteinase K were purchased from Viagen Biotech (Los Angeles, CA). PCR analysis was performed to detect the presence of RFP as a 350bp amplicon using the primers sense-CATTGGGGACACATTGAGCAG and antisense-TAGCAGTGGATGCAGAAGGCAGAC. Amplicons were generated under the following PCR conditions: 1) 10-mins initial incubation time at 94°C 2) the PCR conditions were then 94°C for 60s followed by 57°C for 30s followed by 72°C for 30s for 30 cycles and 3) after 30 cycles, the samples were incubated for an additional 7-mins at 72°C. Only mice identified as transgene positive (PCR band at 350bp) were included in any figures depicting preleukemic burden. Transgene-negative mice were treated as wild-type Balb/c mice.

2.2.3 Processing of mouse spleens

Mice were sacrificed either 72h or 8d after the initial injection of either Lm, S. cerevisiae or PBS. The spleen was harvested from each mouse and mushed through a 70µm nylon filter using the back of a 0.5mL insulin syringe. The splenocytes were washed though the filter using 10mL of phosphate buffered saline (PBS). The splenocytes were centrifuged at 500g for 5min at 4°C and the supernatant was discarded. The cell pellet was resuspended in 2mL 0.16M Tris-Ammonium Chloride pH 7.2 buffer for 5 mins to lyse red blood cells. After 5 mins, 8-10mL of PBS was added to terminate cell lysis. Cells were then centrifuged at 500g for 5min at 4°C and
the supernatant was discarded again. The cell pellet was then resuspended in 2mL FACS buffer (PBS with 2% FBS, 0.01M sodium azide) for subsequent experiments.

2.2.4 Determining preleukemic burden and spleen size of Eμ-RET mice

Eμ-RET mice were identified as previously described. 2.5-5% of processed splenocytes were stained with anti-mouse BP-1 (clone 6C3), B220 (clone RA3-6B2), and 7-AAD. 6μL of counting beads (52,000 beads/μL) were added to each sample after staining for a final volume of 200μL. 1000 counting bead events per sample were collected on the BD LSRII flow cytometer. Preleukemic cells were identified by the presence of a BP-1+/B220int/7-AAD− population of cells that are only present in RFP-positive mice. An RFP-negative littermate (i.e. a wild-type Balb/c mouse) was analyzed in parallel to act as a negative control to properly gate the population of preleukemic cells (see Appendix B.2 below). Using the total number of BP-1+/B220int/7-AAD− and bead events, and “spleen multiplier” the total number of preleukemic cells was calculated using Formula 2 (see Appendix C.2). The spleen multiplier is one over the proportion of total splenocytes stained for flow cytometry. For example, if 5% (0.05) percent of the total number of splenocytes were stained, the spleen multiplier would be 20. The spleen size was determined using the same approach, except using the 7-AAD− population of cells. Each sample was measured in duplicate, yielding an average measurement. Samples were analyzed using FlowJo Version 10.0 (Ashland, OR) and subsequent calculations were performed using Microsoft Excel (version: 1708; Redmon, WA).
2.2.5 Determining the percentage of activated γδ T cells and NK cells

Either Balb/c or Eμ-RET mice were sacrificed 72h post-infection. Processed splenocytes were stained with two antibody panels. Panel 1: anti-mouse γδ TCR (clone eBioGL3), CD3 (clone 17A2), CD69 (clone H1.2F3), 7-AAD. Panel 2: anti-mouse CD49b (clone DX5), CD335 (clone 29A1.4), CD3, CD69, 7-AAD. The γδ T cell receptor (TCR) antibody which was purchased from Thermo Fisher Scientific. γδ T cells were identified as a CD3+/γδ TCR+/7-AAD− population. 6μL of counting beads were added per sample and 1500-2000 counting bead events were recorded using the BD LSRII flow cytometer. NK cells were identified as a CD49b+/CD335+/7-AAD− population. To determine the percentage of activated γδ T cells or NK cells, a gate was drawn on the CD69+ γδ T or NK cells respectively. CD69 is an early activation marker for both γδ T and NK cells123,124. To determine the placement of the gate, the CD69− cell population was first identified in the PBS treated spleens.

2.2.6 Determining the total number of neutrophils and activated γδ T cells in Eμ-RET mouse spleens

For neutrophil counts, either Balb/c or Eμ-RET mice were sacrificed day 8 post-infection. 2.5-5% of processed splenocytes were stained with anti-mouse Ly-6G (clone 1A8), CD11b (clone N418), and 7-AAD. Neutrophils were identified as a Ly-6Ghi/CD11b+/7-AAD− population. For γδ T cell counts, mice were sacrificed 72h post infection. 2.5-5% of processed splenocytes were stained with γδ T cell TCR, CD3, CD69, 7-AAD. Activated γδ T cells were identified as γδ-TCR+/CD3+/CD69+/7-AAD−. 6μL of counting beads was added per sample and 1000-1500 bead events were recorded using the BD LSRII flow cytometer. The total number of both neutrophils and activated γδ T cells in each spleen was determined using Formula 2 (see Appendix C.2).
2.2.7 *In vitro* direct killing assays

Between 6-9 Balb/c or Eμ-RET mice were injected IP with $10^4$ cfu Lm or PBS and 72h post-infection were sacrificed for fluorescent-activated cell sorting (FACS). Mice were between age 6-8d when injected. Processed splenocytes were stained with the following antibody panel: anti-mouse CD49b, CD335, CD3, γδ TCR, CD69, and 7-AAD. For Lm treated mice, two populations were sorted: NK cells and activated γδ T cells. For PBS treated mice, only NK cells were sorted. NK cells were identified as a CD49b+/CD335+/7-AAD population. Activated γδ T cells were identified as a γδ TCR+/CD3+/CD69+/7-AAD population. NK cells and γδ T cells were sorted using either the BD Aria or Beckman Coulter Astrios (Brea, CA) cell sorters at 4°C. After sorting, cells were cultured in DMEM media containing 20% fetal bovine serum, 100pg/mL IL-7, 100U/mL penicillin-streptomycin, 2mM L-glutamine, 0.01M HEPES, 1x nonessential amino acids and 5μM 2-mercaptoethanol overnight (16h) at 37°C in the presence of luciferase-GFP positive murine leukemic cell line 289 (289-Luc) at a ratio of 50:1 (sorted cells:289) in a 96-well plate. The next day, the cells were stained for BP-1, B220, and 7-AAD. 3μL of counting beads was added per sample and 1,000-2,000 bead events were collected on the BD LSRII. The total number of viable 289 cells, identified as a GFP+/BP-1+/B220+/7-AAD population, was determined for each condition. The proportion of viable cells relative to the untreated 289 control was determined for each cell condition. Each population of sorted cells was plated in triplicate and an average proportion of 289 cells relative to the control was calculated. For two trials (N=2) both untreated and Lm treated mice were sorted in parallel and plated overnight in the same culture conditions, yielding a direct head to head comparison for the three different populations sorted.
2.2.8 Phenotyping γδ T cells

Splenocytes were processed and stained for activated γδ T cells as previously described. In addition, splenocytes were also stained with anti-mouse CD44 (clone IM7) and CD27 (clone LG.3A10). Within the activated γδ T cell population, CD44+ and CD27+ are respectively, indicative of IL-17 and IFNγ producing cells\textsuperscript{125}.

2.2.9 Adoptive transfer of γδ T cells

Nine Balb/c mice were infected with 10\textsuperscript{4} cfu Lm at day 6 of life. 72h post infection, the mice were sacrificed, and the processed splenocytes were pooled and stained using anti-mouse CD3, γδ TCR, CD69, and 7-AAD. The activated γδ T cells (γδ TCR+/CD3+/CD69+/7-AAD\textsuperscript{-}) were sorted on the Beckman Coulter Astrios. After sorting, the viable cells were counted using a hematocytometer and trypan blue. Either 100,000 or 300,000 activated γδ T cells suspended in PBS were injected IP into mice age 8 or 9 days. Additionally, control mice were injected with only PBS. The recipient mice were sacrificed 40h after the adoptive transfer and the spleens were harvested and processed. 5% of the processed splenocytes were stained with anti-mouse CD49b, CD3, CD69, and 7-AAD. 6μL of counting beads were added per sample and 1500 bead events were collected on the BD LSRII flow cytometer. The percentage of activated NK cells and the mean CD69 fluorescent intensity was determined for both adoptive transfer recipients and control mice.
2.2.10 NK cell culture

Mice were infected with $10^4$ cfu Lm or PBS at age day 6-8 of life. 72 post-infection, mice were sacrificed and spleens were harvested. 2 x $10^6$ processed splenocytes were cultured per well in a 24-well plate. Splenocytes were cultured in RPMI media containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin, 2mM L-glutamine, 0.01M HEPES, 1x nonessential amino acids, and 5µM 2-mercaptoethanol with the addition of either IL-2 (25ng/mL), IL-15 (25ng/mL), IL-2 and IL-15 (both 25ng/mL) or PBS control. Additionally, for each culture condition, cells were cultured with and without the presence of IL-17A (25ng/mL). Cell culture media reagents were purchased from Gibco (Thermo Fisher Scientific) and all recombinant cytokines were purchased from BioLegend. Cells were cultured overnight (16h) at 37°C and stained the next day with a panel of anti-mouse CD3, CD49b, CD335, CD69, and 7-AAD. Both the number and percentage of CD69 activated NK cells was determined for each culture condition (8 in total). The number and percentage of CD69$^+$ NK cells was determined as previously described.

2.2.11 NK cell enriched splenocyte killing assay

Three Balb/c mice were sacrificed at day 8-9 of life. The spleens were processed and pooled for each group of three mice. NK cells were selected for using an enrichment kit purchased from Stemcell Technologies (Vancouver, BC). NK cells were cultured overnight (16h) at 37°C in a 96-well plate in RPMI media containing 10% fetal bovine serum, 250pg/mL IL-7, 100U/mL penicillin-streptomycin, 2mM L-glutamine, 0.01M HEPES, 1x nonessential amino acids, and 5µM 2-mercaptoethanol with addition of IL-2, IL-15, and IL-15, IL-2 and IL-17A, only IL-17A or PBS control. The concentration of each cytokine was (25ng/mL). Additionally, for each culture condition, cells were cultured in the presence of the murine leukemic cell line 289 at a
ratio of 50:1 NK cell-enriched splenocytes to 289-Luc cells. After overnight incubation, each well was stained with anti-mouse B220, BP-1, 7-AAD. 3μL of counting beads was added per sample. 1000 bead events were collected on the BD LSRII and the total number of viable 289-Luc cells was calculated per well (GFP+/BP-1+/7-AAD+ population). The proportion of viable 289-Luc cells relative to the PBS control was determined for cytokine treatment. Additionally, the same experiment was performed with non-enriched-splenocytes as a control for the direct effects of IL-2 and IL-15 on 289-Luc viability and NK cell enrichment. Each condition was analyzed in duplicate or triplicate, yielding an average measurement for a given trial.

2.2.12 IL-17 Injections

Adult or 9-day-old pup Balb/c mice were injected IP with either 0.5μg IL-17A (BioLegend) or PBS. IL-17A recruits neutrophils to the peritoneal cavity to the site of injection. The IL-17A or PBS was injected in a 250μL or 50μL total volume, for adults and pups respectively. The adult and pup mice were sacrificed 4hs and 16hs after the injection respectively. After being sacrificed, the skin was removed from the abdomen of the mice. For adults 2.5mL of PBS containing 3mM EDTA was injected IP. For pups, 700μL was injected into the peritoneal cavity. A small incision was made in the peritoneal lining and the lavage fluid was collected. For adults 1mL, and pups 200μL, of additional PBS containing 3mM EDTA was added to the peritoneal cavity, pipetted up and down, and removed and additional 2-3 times. For the pups, the spleen was also harvested and processed as previously described to determine the activation of NK cells. The total lavage fluid was centrifuged at 300g for 5 mins at 4°C and the supernatant was discarded. The cells were resuspended in 1mL FACS buffer. Either 50% or 5% of total cells were stained with a panel of anti-mouse CD11b, Ly-6G, TER-119 (clone TER-119), and 7-AAD.
antibodies. The total number of neutrophils in the peritoneal cavity was determined for each mouse. Neutrophils were identified as previously described.

2.3 Microorganism Strains

<table>
<thead>
<tr>
<th>Title</th>
<th>Abbreviation</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental Control (Saccharomyces cerevisiae)</td>
<td>N/A</td>
<td>NDSIV: Industrial bakery yeast strain.</td>
</tr>
<tr>
<td>Asparaginase expressing yeast</td>
<td>AEY</td>
<td>NDSIV with 3 copies of the S. cerevisiae ASP3 gene (cell wall-associated L-ASNase) under the constitutive action of the PGK1 promoter. The gene constructs were inserted into the URA3 or LEU2 genomic locations by CRISPR/CAS9.</td>
</tr>
<tr>
<td>E. coli-asparaginase expressing yeast</td>
<td>AnsB-AEY</td>
<td>NDSIV with 1 copy of the E. coli AnsB gene under the constitutive action of the PGK1 promoter. The AnsB gene construct also utilized the same leader sequence (first 20 amino acids) as the ASP3 gene, RSLNTLLLSLFVAMSSGAP. The gene construct was inserted into the LEU2 genomic location by CRISPR/CAS9.</td>
</tr>
<tr>
<td>Attenuated Listeria</td>
<td>Lm</td>
<td>ΔTrpS;ActA attenuated strain of Listeria monocytogenes.</td>
</tr>
</tbody>
</table>

2.4 Statistics

All statistical tests were performed using GraphPad Prism version 7.04 (San Diego, CA). For all statistical comparisons, samples were first analyzed for normality using the Shapiro-Wilk test. If the assumptions of normality were met, the appropriate parametric statistical test was used.
(Student t-test, Two-way ANOVA, etc.). If one sample did not pass the normality test, the appropriate nonparametric statistical test was performed (Mann-Whitney U test, Kruskall-Wallis test, etc.). P-values are labelled as follows $P > 0.05$ (ns), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***)$ $P \leq 0.0001$ (****). Throughout this dissertation, a “statistical difference” (or lack thereof) refers to a statistically significant difference ($P \leq 0.05$) between the means of two (or more) sets of data analyzed using the above-mentioned methods.

### 2.5 Figures

All figures were prepared using GraphPad Prism version 7.04. For every figure, the mean value for each measurement is plotted, and where the software would allow standard error bars are shown.
Chapter 3: Investigating the utilization of L-asparaginase expressing
Saccharomyces cerevisiae to improve L-ASNase therapy for the treatment of
ALL

3.1 Aim
Analyze the efficacy of using S. cerevisiae cells engineered to constitutively express L-ASNase-II as a drug delivery vehicle for L-ASNase therapy in the treatment of ALL.

3.2 Introduction
3.2.1 Therapeutic use of L-ASNases
L-asparaginase (L-ASNase) is an integral component of ALL induction and relapse therapy\textsuperscript{13,32}. ALL cells characteristically stop expressing asparagine synthetase (AS) and rely on extracellular sources for the amino acid asparagine (L-ASN)\textsuperscript{87}. L-ASNase therapy thus selectively induces apoptosis in asparagine synthetase-negative ALL cells by depleting serum levels and starving leukemic cells of an essential nutrient\textsuperscript{87-90}. Healthy cells maintain AS activity and can thus synthesize L-ASN in the absence of extracellular sources\textsuperscript{89,90}. Two preparations of L-ASNase are currently used clinically: monoethoxypolyethylene glycol succinimidyl conjugate of \textit{E. coli} L-ASNase (PEG-L-ASNase) and \textit{Erwina carotovora} L-ASNase (Erwinase). PEG-L-ASNase is the therapeutic of choice and Erwinase is typically reserved for patients who experience immune complications to PEG-L-ASNase, such as allergic reactions and/or high anti-L-ASNase antibody titers \textsuperscript{13,87,94-98}. 
3.2.2 Current limitations of L-ASNase therapy

Despite the proven efficacy of L-ASNase as an ALL therapeutic, it is limited by high cost, immune complications and toxicity. The cost of 30 weeks of *E. coli* L-ASNase (native or PEG-L-ASNase) treatment for the payer is approximately $60 000 USD\textsuperscript{127}. Key factors driving the cost are the need for enzyme purification and the conjugation of polyethylene glycol (PEG). Although PEG-L-ASNase is 9-10x more expensive to manufacture, its increased half-life and subsequent decreased dosing frequency make the price difference between native *E. coli* and PEG-L-ASNase negligible\textsuperscript{128-130}. Both native and PEG-L-ASNase are prone to either immune silencing (detected by high L-ASNase antibody titre in the patient’s serum), occurring in 26-42% and 2-11% of patients respectively, and/or allergenic hypersensitivity reactions, occurring in 24-29% and 3-14% of patients respectively\textsuperscript{97,131-133}. Hypersensitivity reactions can range from mild erythema near the site of injection to near-fatal anaphylaxis. Perpetual dosing of these formulations increases the risk of developing these immune complications, ultimately limiting the use of L-ASNase, especially in the case of relapse\textsuperscript{87}. Both immune complications necessitate the transfer to Erwinase which doubles the cost of 30 weeks of treatment to $120 000 USD\textsuperscript{127}.

The decreased frequency of both, immune silencing and hypersensitivity reactions associated with PEG-L-ASNase, shows how preventing these immune complications ultimately saves the payer substantial costs.

Although L-ASNase is a relatively safe therapeutic, some patients still experience toxicity, especially in adults and adolescents\textsuperscript{93}. The common forms of serious toxicity are pancreatitis and thrombosis. These side-effects require secondary treatment which can impact the long-term health and survival of the patient\textsuperscript{93}. The rates of these complications vary based on the treatment
regimen, but are estimated to range from 5-10% for pancreatitis and 10-20% for thrombosis. Additionally, rare cases in adults have shown L-ASNase therapy can lead to fatal toxic encephalopathy. The less frequent dosing schedule required for PEG-L-ASNase is believed to contribute to lower rates of toxicity, highlighting how L-ASNase formulations with longer half-lives and lower immunogenicity yield benefits to the patient.

3.2.3 Indications for using S. cerevisiae as a drug delivery vehicle for L-ASNase

S. cerevisiae genetically manipulated to constitutively express L-ASNase II (ASP3) presents as a novel drug delivery vehicle approach L-ASNase therapy. S. cerevisiae L-ASNase II is an extracellular, cell-wall associated L-ASNase which is activated at human body temperature and pH. Importantly, the cell-wall association of S. cerevisiae L-ASNase II may shield the L-ASNase-II from the immune system. Evading the immune system may increase the L-ASNase half-life (t½) and lower the risk of immune complications. By increasing the half-life and lowering the risk of immune complications, S. cerevisiae-delivered L-ASNase would require a less frequent dosing schedule compared to the standard L-ASNase therapeutics. Less frequent dosing schedules are valuable as they have been correlated with decreased costs and reduced toxicity. Moreover, relapsed ALL patients may especially benefit from a novel approach to L-ASNase drug delivery as these patients are more likely to have already built up immunity to PEG-L-ASNase and/or Erwinase. Lastly, using a S. cerevisiae-based vector may further reduce the costs of L-ASNase therapy by removing the costs of enzyme purification.

Thus, using S. cerevisiae as a vector for L-ASNase drug delivery may reduce or eliminate many of the current problems associated with L-ASNase therapy: cost, toxicity, and adverse immune
complications. Overcoming these current limitations will yield benefits to both the patient and the payer and thus warrants further investigation in this chapter.

3.3 Results

3.3.1 Ultraviolet light vs. heat killing of yeast cells

Killing the yeast cells prior to their administration as a therapeutic will be necessary to minimize toxicity since the chemotherapy regimen for ALL patients compromises the immune system. Although *S. cerevisiae* has been shown to be nonpathogenic, live yeast cells present a greater risk of side-effects in immune-compromised patients. Thus, I investigated two techniques to kill the yeast cells while attempting to maintain L-ASNase activity: heat vs. UV-radiation killing.

The temperature required to kill yeast cells is lower than the *S. cerevisiae* L-ASNase-II denaturation temperature (80°C)\(^{138}\). To determine the optimal temperature to kill the yeast cells, I incubated \(2.12 \times 10^6\) yeast cells (1mL of 1OD\(\text{600}\) *S. cerevisiae* was determined to equal \(2.12 \times 10^7\) yeast cells; results in Chapter 2.1.1) at 6 different temperatures for 15 mins. The yeast cells were then plated on YEG-agar plates and the plates were evaluated for the presence of colonies after overnight incubation at 30°C; colony formation indicates < 100% killing of yeast cells. Table 3.1 shows that 60°C was the threshold for the complete killing of yeast cells.

UV-light is capable of inducing DNA damage in yeast cells and preventing their replication\(^{139,140}\). Furthermore, damaging the DNA of the yeast cells may prevent cell replication while maintaining L-ASNase activity. To determine if UV-based inactivation is feasible, a protocol treating yeast cells with 3 x 5 mins of UV-light exposures was tested and achieved \(\geq 99.99\%\) killing (see Figure 3.2 A). Next, both heat and UV treatments were applied to L-
asparaginase expressing S. cerevisiae (AEY) and the ratio of yeast cells to L-ASNase units was calculated for both treatments (see Figure 3.2 B). Table 3.2 summarizes the L-ASNase activity for each condition shown in Figure 3.2 B. Although the UV treatment is significantly better at retaining the L-ASNase activity of the AEY (P < 0.05), < 100% killing will cause unwanted artifacts in comparing AEY to purified E. coli L-ASNase in subsequent *in vitro* and *in vivo* assays. The short doubling time of yeast compared to human cells (90 mins vs. 24h) raises concerns that even one live yeast cell can grow in culture and make interpreting assays longer than 1 day very difficult (Note: The L-ASNase activity and recovery assays occurred immediately after the UV treatment protocol, so there is negligible time for yeast cell growth to impact the results). The direct comparison of purified *E. coli* L-ASNase units to the yeast cell count becomes challenging as the continual growth of yeast cells constantly changes the number of yeast cells and subsequent L-ASNase activity. Additionally, live yeast cells can grow to concentrations greater than or equal to 72M yeast cells/mL (3.4OD<sub>600</sub>) which create unfavorable culture conditions as even control yeast with no constitutive L-ASNase activity can inhibit cell viability at this concentration (see Figure 3.3). To further illustrate the technical limitations associated with UV killing, smaller starting amounts of yeast (one half and one quarter the initial 2.3 x 10<sup>7</sup> yeast cells) were subject to the same UV treatment protocol. Table 3.3 illustrates that decreasing the starting amount of *S. cerevisiae* does not result in 100% efficient killing of yeast cells. Additionally, the total yeast cell recovery percentage wanes as the starting yeast concentration decreases (see Table 3.3), creating further technical problems with utilizing the UV killing approach. The inability of the UV treatment protocol to kill the yeast with 100% efficiency while maintaining efficient (defined as ≥ 80%) cell recovery warrants the use of heat-killed yeast in subsequent assays.
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Presence of Colonies?</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Yes</td>
</tr>
<tr>
<td>40</td>
<td>Yes</td>
</tr>
<tr>
<td>50</td>
<td>Yes</td>
</tr>
<tr>
<td>60</td>
<td>No</td>
</tr>
<tr>
<td>70</td>
<td>No</td>
</tr>
<tr>
<td>80</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 3.1 The evaluation of yeast colony formation after 15 mins of incubation at 6 different temperatures
Figure 3.2 The effect of heat vs. UV-light killing of yeast cells on subsequent L-asparaginase activity

A) Comparison of the number of yeast colony forming units after overnight incubation at 30°C. Yeast cells were either subject to 3 x 5 min of UV radiation (n = 12) or untreated (n=4) and diluted to obtain a countable number of colonies (Welch’s t-test). B) L-Asparaginase activity (U/10^6 yeast cells) for L-asparaginase expressing yeast (AEY) after heat and UV treatment protocols (n=4) or no treatment (n=6) (One-way ANOVA).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>1U L-Asparaginase (10^6 yeast cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live (Untreated)</td>
<td>261.0 +/- 30.6</td>
</tr>
<tr>
<td>UV</td>
<td>233.8 +/- 0.9</td>
</tr>
<tr>
<td>Heat</td>
<td>433.6 +/- 1.15</td>
</tr>
</tbody>
</table>

Table 3.2 Amount of yeast cells required for 1U of L-asparaginase activity after various treatment protocols.

<table>
<thead>
<tr>
<th>Starting amount of yeast cells (10^6 yeast cells)</th>
<th>Treatment (UV exposure)</th>
<th>Average cfu/10^6 yeast cells</th>
<th># plates with colonies</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.2</td>
<td>3 x 5min</td>
<td>823.8 +/- 123.6</td>
<td>3/4</td>
<td>-</td>
</tr>
<tr>
<td>11.7</td>
<td>3 x 5 min</td>
<td>106 +/- 29.9</td>
<td>3/4</td>
<td>64 +/- 1.6</td>
</tr>
<tr>
<td>5.8</td>
<td>3 x 5min</td>
<td>115.5 +/- 50.0</td>
<td>1/4</td>
<td>20 +/- 0.9</td>
</tr>
<tr>
<td>23.2</td>
<td>0min</td>
<td>Confluent</td>
<td>2/2</td>
<td>-</td>
</tr>
<tr>
<td>2.3 x 10^{-4}</td>
<td>0min</td>
<td>2.0 x 10^7 +/- 5.7 x 10^6</td>
<td>2/2</td>
<td>-</td>
</tr>
<tr>
<td>2.3 x 10^{-5}</td>
<td>0min</td>
<td>2.1 x 10^5 +/- 2.3 x 10^6</td>
<td>2/2</td>
<td>-</td>
</tr>
<tr>
<td>23.2</td>
<td>Heat Treatment (15min, 60°C)</td>
<td>0</td>
<td>0/2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.3 The number of colonies formed after 3 x 5min UV exposure for multiple different starting concentrations of yeast.
Figure 3.3 ALL cells cannot be cultured in a highly concentrated yeast environment

Forward scatter-area (FSC-A) vs. side scatter-area (SSC-A) flow cytometry plots that show that culturing an ALL cell line in highly concentrated yeast environment can eliminate viable conditions. A) RS4;11 cells cultured in 3.4 OD<sub>600</sub> WT. B) RS4;11 cells cultured in 3.4 OD<sub>600</sub> WT. C) Merge of A (Red) and B (blue). Note: Loss of viable cell cluster in A.
3.3.2 Asparagine synthetase protein level predicts sensitivity to L-ASNase treatment

Recent reports have identified that the low protein levels of asparagine synthetase (AS) are the most important determinant of high sensitivity of ALL cells to L-ASNase therapy. To determine if AS expression predicts sensitivity to L-ASNase-expressing yeast (AEY), six human ALL cell lines (380, 697, BV173 (BV), Nalm6(N6), RCH-ACV (RC), and RS4;11(RS)) were evaluated for AS protein levels by Western blot analysis. Jurkat (Jkt) cells (a T-ALL cell line) were included as a positive control for AS expression. RS was the only cell line that displayed no AS band at 64 kDa (see Figure 3.4 A).

To determine if AS protein expression predicts sensitivity to AEY treatment, all 6 cell lines were titrated against heat-inactivated AEY. Additionally, each cell line was titrated against the equivalent number of units of purified E. coli L-ASNase (based on the 1U L-ASNase:4.3 x 10⁸ yeast cell ratio presented in Table 3.2) to compare differences in efficacy between the two L-ASNase treatments. The proportion of viable ALL cells (fixable viability dye-eFluor 520⁻ (FVD⁻ eF520), HLA-A/B/C-PE⁺) relative to the untreated control was determined for each cell line at each of the 6 different L-ASNase concentrations for both AEY and purified E. coli L-ASNase (see Figure 3.4 B). Additionally, each cell line was plated with the parental control strain lacking constitutive L-ASNase expression at the highest yeast concentration (0.34OD₆₀₀/7.2 x 10⁶ cells per well) to control for any cell death due to the presence of high concentrations of yeast (see Figure 3.4 C). Except for RCH-ACV, the AEY can significantly reduce cell viability compared to the parental yeast control in all the cell lines, showing that the constitutive expression of L-ASNase-II is primarily responsible for the reduction in cell viability. Using non-linear regression, the ED₅₀ (L-ASNase concentration in which there is 50% cell viability compared to
parental control) was analyzed for each cell line for the *E. coli* and AEY titrations (see Table 3.4). A comparison of the ED$_{50}$ for each cell line for both L-ASNase treatments was plotted and analyzed for statistical significance (see Figure 3.5). Every cell line that yielded an AS band via Western Blot analysis had an ED$_{50}$ calculated outside the concentration range used in the titrations for this experiment. As shown in Figure 3.5, the only cell line with a statistical difference in ED$_{50}$ between the two L-ASNase treatments was RS4;11, with the ED$_{50}$ for the AEY treatment being significantly higher than purified *E. coli* L-ASNase (P < 0.05). More accurate ED$_{50}$ calculations could not be made since increasing the yeast concentration began to inhibit cell growth for some cell lines (see Figure 3.3). Moreover, the calculated ED$_{50}$ for RS4;11 is, at a minimum, one order of magnitude lower than every other cell line for both L-ASNase treatments. RS4;11’s low ED$_{50}$ and the lack of an AS band identified RS as the only ALL cell line tested that is highly sensitive to L-ASNase.

Current L-ASNase therapeutics can either induce apoptosis in AS-negative cells or retard growth in AS-positive cells due to the depletion extracellular L-ASN. Three cell lines assessed for proliferation, apoptosis, and cell death to determine the cause of AEY-induced reductions in cell viability. The three cell lines were selected based on their differing sensitivity to L-ASNase based on the calculations in Table 3.1: RS4;11 (low ED$_{50}$), 697 (moderate ED$_{50}$), and BV173 (high ED$_{50}$).

To analyze the effect of AEY on cell proliferation, two approaches were used. First, the fold increase in the total number of viable cell was analyzed for the three cell lines (697, BV, RS) after 2-day or 5-day incubations. Second, the cells were stained with proliferation dye-eFlour
670 prior to culture and the average e670 intensity of viable cells relative to day 0 was measured at each time point. The lower the mean fluorescent intensity, the greater the rate of proliferation since dividing cells distribute the dye evenly among the two daughter cells. It is important to note that after 5-days of either the AEY or purified *E. coli* L-ASNase treatments, the average eF670 intensity of dead RS cells had to be used since there was either zero or a very limited number of viable RS cells at this time point. Cells were also cultured with equivalent concentrations of purified *E. coli* L-ASNase to further evaluate for differences in efficacy between the two treatments. Lastly, cells were also cultured in equivalent concentrations of the parental control yeast lacking L-ASNase expression to control for any changes in cell viability due to the concentrated yeast growth environment. For both assays, cells were treated with either AEY or purified *E. coli* L-ASNase and compared to both the yeast parental control lacking constitutive L-ASNase expression and an untreated control. The results for these two assays are shown in Figure 3.6. None of the cell lines displayed any statistical differences between treatment groups after overnight incubation. In terms of the relative fold increase in the number of viable cells after 2-days, RS was the only cell line to have statistical differences between treatment groups, with both AEY and the purified *E. coli* L-ASNase being able to significantly reduce the number of viable cells compared to the untreated, but not the parental, control. However, in the proliferation dye assay at 2-days, *E. coli* L-ASNase, but not AEY, significantly increased the mean log eF670 intensity of 697 cells after 2-days of incubation compared to the untreated and parental controls. Both AEY and *E. coli* L-ASNase significantly increased the mean eF670 intensity of viable RS cells after 2-days compared to both controls. After 5-days of incubation, AEY and the purified *E. coli* L-ASNase treatments yielded statistically significant differences (P ≤ 0.05) compared to both controls for both 697 and RS cell lines in both assays.
Additionally, in the RS proliferation dye assay, an additional statistical difference (P < 0.05) was observed between AEY and purified *E. coli* L-ASNase, with the average eF670 intensity being higher in the *E. coli* treated group than the AEY treated group (i.e. less proliferation). At 5-days, for 697, no difference in log proliferation dye intensity was observed between AEY and purified *E. coli* L-ASNase. In both assays at every time point, no statistical differences were observed between treatment groups for BV. Moreover, although both 697 and RS display statistical differences compared to the parental and untreated controls, the L-ASNase treatments only reduced the total number of viable cells relative to the day 0 time point for RS. For 697, the number of viable cells increased throughout each assay, just at a slower rate compared to the L-ASNase lacking controls.

To explain the growth kinetics for each cell line, the cell death and apoptosis were assessed by first culturing the cells at the same conditions as Figure 3.6 and then staining them with 7-AAD and AnnexinV to determine the percentage of apoptotic and dead cells via flow cytometry after overnight, 2-day, and 5-day incubations. AnnexinV+/7-AAD− cells were classified as apoptotic and AnnexinV+/7-AAD+ cells were classified as dead. The percentage of both apoptotic and dead cells was plotted for each condition and compared to the untreated control (Figure 3.7). For both 697 and RS, the AEY and purified *E. coli* L-ASNase treatments significantly increased the percentage of apoptotic and dead cells compared to both the parental and untreated controls at 2 days and 5 days, but not after overnight incubation (P < 0.001). There were no statistical differences (P > 0.05) when comparing the AEY to purified *E. coli* L-ASNase treatment and the parental control to the untreated control, showing that L-ASNase activity is necessary to induce both apoptosis and subsequent cell death for the RS and 697 cell lines. The magnitude of the
effect on increasing the percentage of dead cells was significantly higher in RS compared to 697 at the 2 and 5-day timepoints (P <0.05). For the BV cell line, there were no statistical differences (P > 0.05) between any of the four conditions after overnight and 2-day incubations. After 5-days, for BV, the only two comparisons that yielded statistical differences (P ≤ 0.05) were the difference in the percentage of dead cells for the AEY vs. untreated control and parental control vs. untreated control comparisons (see Figure 3.7). However, the magnitude of these differences is small, -1.99% and -2.12% respectively. One possible explanation for the lower percentage of dead cells compared to the untreated control is that the highly concentrated yeast provides a survival advantage for the BV cell line, albeit the magnitude of this advantage is small.

The results shown in Figure 3.6, in combination with amount of induced apoptosis and cell death for each cell line (Figure 3.7), show that only the AS-lacking cell line (RS) is susceptible to complete elimination by AEY. Other ALL cell lines that maintain AS expression can have differential responses to AEY that range from no effect on cell death and proliferation (BV) to small increases in apoptosis and reductions in the overall rate of proliferation (697).
**A**

Cell Line | Proportion of viable cells relative to untreated control
--- | ---
JKT | 64 kDa
380 | 64 kDa
697 | 64 kDa
BV | 64 kDa
N6 | 64 kDa
RC | 64 kDa
RS | 64 kDa

**B**

*E. coli* L-ASNase

![Graph of relative viability vs. L-Asparaginase Concentration](image)

AEY

![Graph of relative viability vs. L-Asparaginase Concentration](image)

**C**

Proportion of viable cells relative to untreated control

![Bar chart of cell line viability](image)
Figure 3.4 The sensitivity of 6 different ALL cell lines to L-asparaginase and AEY

A) Western blot bands for asparagine synthetase (AS) (64kDa) and the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 37 kDa) for 6 ALL cell lines and one positive control (Jurkat (JKT) cell line). B) Left Panel: 48h purified *E. coli* L-ASNase titration using 5 ten-fold serial dilutions for 6 ALL cell lines. Starting concentration: 0.017U/mL. Right Panel: 48h AEY titration using 5 ten-fold serial dilutions for 6 ALL cell lines. Starting concentration: 0.017U/mL or 0.34OD_{600}. C) Comparison of cell viability after treatment culturing in either 0.34OD_{600} L-Asparaginase expressing yeast (AEY) (n=5) or the control parental yeast strain lacking constitutive L-ASNase expression (n=4) (Multiple Student’s t-tests).
Comparison of ED$_{50}$ for 6 ALL cell lines. Comparison of the purified E. coli L-ASNase and AEY ED$_{50}$ for six cell lines (380, 697 BV, N6, RC, and RS) calculated using non-linear regression from Figure 3.2 C (n=18; Welch’s t-test).
<table>
<thead>
<tr>
<th>ALL Cell Line</th>
<th>Treatment</th>
<th>E. coli L-ASNase</th>
<th>L-ASNase Expressing Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED₅₀ (U/mL)</td>
<td>R²</td>
<td>ED₅₀ (U/mL)</td>
</tr>
<tr>
<td>380</td>
<td>0.21 +/- 0.37</td>
<td>0.494</td>
<td>0.029 +/- 0.01</td>
</tr>
<tr>
<td>697</td>
<td>0.11 +/- 0.09</td>
<td>0.7798</td>
<td>0.032 +/- 0.02</td>
</tr>
<tr>
<td>BV173</td>
<td>3.8 x 10¹ +/- 2.0 x 10²</td>
<td>0.3673</td>
<td>0.080 +/- 0.06</td>
</tr>
<tr>
<td>Nalm6</td>
<td>0.19 +/- 0.17</td>
<td>0.7651</td>
<td>0.051 +/- 0.02</td>
</tr>
<tr>
<td>RCH-ACV</td>
<td>1.1 x 10⁴ +/- 1.2 x 10⁵</td>
<td>0.1736</td>
<td>0.049 +/- 0.11</td>
</tr>
<tr>
<td>RS4;11</td>
<td>8.1 x 10⁻⁴ +/- 1.7 x 10⁻⁴</td>
<td>0.9178</td>
<td>1.5 x 10⁻³ +/- 2.6 x 10⁻⁴</td>
</tr>
</tbody>
</table>

Table 3.4 ED₅₀ values for E. coli L-asparaginase vs. AEY treatments for 6 different cells

ED₅₀ values calculated using nonlinear regression for both, Purified E. coli L-ASNase and AEY treatments (n=3), for 6 ALL cell lines. Corresponding R² values for the equations used to calculate the ED₅₀ values are also included.
**Figure 3.6 The effect of *E. coli* L-asparaginase vs. AEY on the growth kinetics of 3 ALL cell lines.**

A) The relative fold increase in the number of viable cells and the B) change in proliferation dye-eFluor 670 intensity after overnight, 2-days or 120h treatment in either i) 0.34OD$_{600}$ AEY, ii) 0.34OD$_{600}$ parental yeast control, iii) 0.17U/mL purified *E. coli* L-Asparaginase or iv) untreated control for the 697 (left panel), BV173 (middle panel), and RS4;11(right panel) cell lines (n=3; Two-way ANOVA).
Figure 3.7 The effect of *E. coli* L-Asparaginase vs. AEY on apoptosis and cell death for 3 different ALL cell lines.

The percentage of apoptotic cells ( AnnexinV*, 7-AAD– ) and dead cells ( AnnexinV*, 7-AAD+ ) after overnight, 48h or 120h treatment in either i) 0.34OD_{600} AEY, ii) 0.34OD_{600} parental yeast control, iii) 0.17U/mL purified *E. coli* L-Asparaginase or iv) untreated control for the 697 (left panel), BV173 (middle panel), and RS4;11 (right panel) cell lines (n=3; Two-way ANOVA).


3.3.3 *S. cerevisiae* expressing *E. coli* l-asparaginase reduces RS4;11 cell viability

Since purified *E. coli* L-ASNase displayed a significantly lower ED_{50} (see Table 3.4) compared to AEY, in partnership with Renaissance BioScience Corporation, a new yeast strain was developed using CRISPR-Cas9 technology to constitutively express the *E. coli* L-ASNase gene (AnsB-AEY). The AnsB gene construct also utilized the same leader sequence (first 20 amino acids) as the ASP3 gene, RSLNTLILLSLFVAMSSGAP, in an attempt to target *E. coli* L-ASNase to the *S. cerevisiae* cell wall. To determine the L-ASNase activity for the new AnsB-AEY, the same approach to evaluate L-ASNase activity as seen in Figure 3.2 B was used. The activity of AnsB-AEY was compared to the previously evaluated AEY, the parental control yeast strain lacking constitutive L-ASNase expression, and a CRISPR-Cas9 control yeast that had the extracellular yeast-L-ASN inserted instead of the *E. coli* gene (ASP3-AEY). The results are shown in Figure 3.8 A. Notably, the AnsB-AEY yielded no L-ASNase activity and its activity was not significantly different than the parental control yeast (P > 0.05). Additionally, it is important to note that AEY has three copies of the ASP3 gene under the PGK1 promoter whereas the control ASP3-AEY generated using CRISPR-Cas9 has only one copy, accounting for the major differences observed in activity between the two yeast strains. As determined by Renaissance BioScience Corporation, above three copies of the ASP3 gene there is no further increase in L-ASNase activity is observed.

To further evaluate L-ASNase activity in the AnsB-AEY, RS4;11 was titrated against AEY, AnsB-AEY, and the parental control. RS4;11 was the only ALL cell line used as it was previously identified to be the only cell line highly sensitive to L-ASNase treatment. The results in Figure 3.8 B show that despite no L-ASNase activity in the 15-min L-ASNase assay, the
AnsB-AEY was still able to significantly reduce RS4;11 viability compared to the parental control (P < 0.0001). Moreover, the ED$_{50}$ for the AEY and AnsB-AEY RS4;11 viability titrations displayed no statistical difference (P > 0.05; see Figure 3.8 C). These results suggest that the AnsB-AEY does have L-ASNase activity. Indeed, after incubating AEY, AnsB-AEY, and the parental control in L-ASNase solution for 48h at 37°C, only AEY and AnsB-AEY significantly deplete the L-ASN (see Figure 3.8 D) (P < 0.001). The assay used to determine L-ASNase activity in Figure 3.8 A was designed to detect extracellular, cell wall-associated L-ASNase activity. Due to the discrepancy between extracellular L-ASNase activity and the reduced RS4;11 cell viability, two additional experiments were performed to determine the source of L-ASNase activity for AnsB-AEY. Since it has been shown that AnsB-AEY has L-ASNase activity, but the enzyme is unlikely to be attached to the extracellular surface of the cell wall, two experiments were designed to evaluate if _E. coli_ L-ASNase was trapped intracellularly. First, to determine if the heat-inactivation procedure increased the fluidity of the yeast cell wall, both untreated (live) and heat-inactivated AnsB-AEY and the parental control strain were incubated in 0.5g/mL L-ASN solution for 48h at 37°C. As shown in Figure 3.8 E, there is no statistical difference (P > 0.05) in the final L-ASN concentration after 48hours between untreated and heat-inactivated yeast for both strains. These results indicate that both untreated and heat-inactivated yeast can deplete L-ASN. Notably, even the parental control appears to have some L-ASNase activity after a 2-day incubation. One possible explanation for this observation is the parental control strain may still express low levels of either ASP3 (extracellular L-ASNase) or ASP1 (intracellular L-ASNase). Another possible explanation is that the intracellular fluid comes to an equilibrium with the L-ASN solution, effectively diluting the starting concentration. To further evaluate if L-ASNase was trapped intracellularly, cells were treated with zymolase to break
down the cell wall and then lysed using both mechanical and osmotic techniques\textsuperscript{121}. Despite using established techniques to lyse yeast cells, no L-ASNase activity was detected in the supernatants of any yeast strain, including AEY (see Figure 3.8 F). The lysis procedure may inactivate L-ASNase, so no conclusions were drawn from this experiment. Although these experiments were not able to definitively show the cellular location and mechanism of L-ASNase depletion of AnsB-AEY, this information would not change the overall outcome of the project, and thus, no further investigation was undertaken. However, overall, the results show that indeed yeast cells can be used as a drug delivery vehicle for trans-species L-ASNases, and potentially other enzymes.
**Figure 3.8 The efficacy of *S. cerevisiae* constitutively expressing *E. coli* L-ASNase in depleting l-asparagine and inducing cell death in an asparagine synthetase negative cell line**

A) The proportional amount of L-ASNase activity for the *S. cerevisiae* constitutively expressing either ASP3 (*S. cerevisiae* L-ASNase; n=3) or AnsB (*E. coli* L-ASNase; n=3) inserted using CRISPR-Cas9 (n=6) relative to AEY (n=3). L-ASNase activity was determined by measuring the amount of L-ASN depleted from a L-ASN solution (0.5g/ml) by 2.12 x 10^7 yeast cells in 15 minutes. (One-way ANOVA) B) 48h AEY (n=3), AnsB-AEY (n=6), and WT parental control (n=3) titration using 4 two-fold serial dilutions for RS4;11. Starting concentration: 0.017U/mL or 0.34OD_600_. (Multiple Student’s t-tests) C) ED50 for both AEY and AnsB-AEY RS4;11 titrations from plot B (Welch’s T-test). D) Final L-asparagine concentration after 48h incubation at 37°C for live AEY, AnsB-AEY, and parental control yeast. Yeast cells were incubated at a concentration of 1 OD_600_ in 0.5g/ml L-ASN dissolved in PBS. (n=3; One-way ANOVA) E) Comparison of final L-asparagine concentration after 48h incubation at 37°C for live vs. heat inactivated yeast for the AnsB-AEY and parental control yeast strains. Yeast cells were incubated at a concentration of 1 OD_600_ in 0.5g/ml L-ASN dissolved in PBS. (n=3; Two-way ANOVA) F) L-asparaginase activity for the yeast cell lysates for 3 strains: AEY, parental control, AnsB-AEY. 21.2M cells for each strain was lysed (n=2).
3.3.4 Technical and ethical limitations prevent the use of AEY \textit{in vivo}

With sensitivity to L-ASNase established in the RS4;11 cell line, it was the cell line of choice to test the efficacy of AEY against an L-ASNase-sensitive ALL cell line \textit{in vivo}. To track the RS4;11 tumor growth \textit{in vivo}, an established bioluminescent imaging approach utilizing luciferase positive cells was used\textsuperscript{142}. To generate the luciferase positive cell line, the RS4;11 cells were transduced with a luciferase-GFP construct using lentivirus. The GFP positive cells were then sorted to establish a homogenous population of RS4;11-luciferase-GFP cells (RS-Luc). The cells were then sorted a second time for GFP positivity to ensure a homogenous luciferase-positive population and to increase the intensity of the luciferase signal for the cell population. This increase in luciferase intensity was accomplished by selecting the top tenth percentile of cells in terms of GFP signal intensity during the second sort. The greater the GFP intensity, the more copies of luciferase being produced by the cell and thus the luciferase signal should also increase in magnitude. The sorting process for generating the RS-Luc cell line used in subsequent experiments is shown in Figure 3.9. Once the RS-Luc cell line was established, it was titrated against AEY and the parental RS (RS-WT) as a control to confirm no change in L-ASNase sensitivity occurred during the transfection and sorting process. The resulting titration shown in Figure 3.10 A reveals no statistical differences $(P > 0.05)$ at any AEY concentration.

Next, the amount of AEY required to give a therapeutic dose was determined using the previously determined ratio of $4.336 \times 10^8$ heat-inactivated AEY cells/U L-ASNase and a literature search. Previous L-ASNase murine studies have shown eradication of leukemia with a single IP dose of 2000U/kg\textsuperscript{143-145}. To monitor development of a human leukemic cell line \textit{in vivo}, a xenograft model using NSG mice was used. The average adult NSG mouse weighs between 25-30g. At this weight, the dosage required for AEY treatment would be between be between
21.6-26.0 x 10^9 yeast cells (calculated using the ratio shown in Table 3.2). Practically, a 110-150mL overnight culture of AEY would be required for a single therapeutic dose of AEY. A visual for how many yeast particles are in a 55mL overnight culture is shown in Figure 3.10 B. Giving a dose of billions of yeast cells presented two major obstacles. First, from an ethical standpoint, it would be inhumane to inject such a large number of cells into the mice. The increased risk for adverse outcomes such as embolisms, pain, suffering, and ultimately death compared to currently available L-ASNase therapies results in an ethical restriction in testing the efficacy of AEY in vivo. Second, from a technical standpoint, the volume of the yeast culture alone exceeds the maximum volume recommended by the Canadian Council on Animal Care (CCAC) guidelines for intraperitoneal injections of 10mL/kg (i.e. 250-300μL in 25-30g mice). Thus, it is not possible to administer the AEY in the preferred route of administration for this study. Due to the limitations of injecting 2000U/kg of AEY in NSG mice, we wanted to determine if lower doses of L-ASNase could still be efficacious. To do so, 1x10^6 RS-Luc cells were injected intravenously into adult NSG mice 10 days prior to the beginning of L-ASNase therapy. On days 10 (day 0 treatment) and 14 (day 4 treatment) post RS-Luc injection, a cohort of mice was treated with a dose of 100U/kg purified E. coli L-ASNase and on day 18 treated with a dose of 200U/kg. Mice were imaged after injection of a luciferin substrate on days -10, -6, -3, 0, 4, 7, and day 11 to monitor disease progression. The days are numbered relative to the first day of L-ASNase treatment (day 10 post RS-Luc injection). The greater the number of photons emitted after a luciferin injection by a given mouse, the greater the number of RS4;11 cells. The log ratio of the number of photons emitted for each day relative to Day 0 of therapy was plotted for L-ASNase and PBS treated mice. The results shown in Figure 3.10 C show no statistical differences (P > 0.05) in tumor growth between the L-ASNase and PBS treated mice. To confirm
drug action in the L-ASNase treated cohort, serum samples were collected on Day 21 and analyzed for the L-ASN concentration. The results in Figure 3.10 D show the L-ASNase treated group had significantly lower L-ASN levels in their serum compared to the untreated controls (P < 0.01). To determine if the RS4;11-Luc cells had become resistant to L-ASNase in vivo, bone marrow was harvested from each mouse and cultured with or without 0.05U/mL L-ASNase for 48h in vitro. At 48h, the cells were stained with 7-AAD and anti-human HLA-A/B/C (hHLA) antibody and analyzed using flow cytometry. The total number of viable L-ASNase treated hHLA+/GFP+ cells were compared to the untreated control. The RS-Luc cells used for injections at the beginning of the study were maintained in culture and used as a control. As shown in Figure 3.10 E, the RS-Luc cells in the bone marrow of PBS and L-ASNase treated mice are still sensitive to L-ASNase, but no differences (P > 0.05) were observed between the two groups. This result shows that no L-ASNase resistance developed in the L-ASNase treated cohort. The bone marrow derived RS-Luc cells from L-ASNase and PBS treated mice showed significantly lower viability after L-ASNase treatment compared to the cell line control at 48h (P < 0.05). One possible explanation is that the sensitivity to L-ASNase increases when cultured in the presence of bone marrow cells. The serum and bone marrow culture data shows, that using a dosing schedule of 100U/kg (day 0 and day 4) and 200U/kg (day 8) is not sufficient to effectively treat RS4;11-Luc xenografts in NSG mice. The inability of lower doses of L-ASNase to effectively treat RS-Luc in vivo, in combination with the large number of AEY cells required to obtain a therapeutic L-ASNase dose, prevented the completion of the in vivo evaluation of AEY treatment of ALL.
Figure 3.9 Overview of the GFP-sorting strategy used to generate RS4;11-luciferase-GFP cells

Proportion of GFP positive (GFP+) RS4;11 cells A) after transduction/before sorting B) after one sort for GFP+ cells C) after two rounds of sorting for GFP+ cells. D) Merge of before sorting (red), one GFP+ sort (blue), two GFP+ sorts (orange).
**Figure A**

Proportion of viable cells relative to untreated control vs. AEY concentration (OD<sub>600</sub>).

**Figure B**

Days after 1<sup>st</sup> L-ASNase treatment.

**Figure C**

Log photons/s relative to day 0 treatment.

**Figure D**

Serum L-asparaginase concentration (mg/L).

**Figure E**

Percentage of viable cell count compared to untreated control.
Figure 3.10 The effect of low dose E. coli L-asparaginase on ALL tumour growth in vivo

A) Four two-fold titration of AEY starting at 0.34 OD₆₀₀. 50 000 RS4;11 wild-type and RS4;11-luciferase were cultured in the presence of AEY for 48h. The proportion of viable cells relative to the untreated control is plotted for each concentration (n=3; multiple Student’s t-tests). B) A picture of a S. cerevisiae pellet obtained from a 55mL overnight culture. C) The tumor size of RS4;11 xenografts in NSG mice relative to day 0 (start of L-ASNase treatment) over time (n=4; multiple student t-tests). D) Serum L-ASN concentrations at Day 11 after the start of L-ASNase treatment for RS4;11-Luc xenograft mice (n=4; Welch’s t-test). E) The proportion of viable bone marrow derived GFP⁺/hHLA⁺ RS4;11 cells cultured in the presence of 0.05U/mL E. coli L-ASNase for 2-days relative to the untreated control. 4 x 10⁵ bone marrow cells were cultured per well. 50 000 RS-Luc cells were cultured per well as a control (n=4; One-way ANOVA).
3.4 Discussion

3.4.1 Current limitations of using L-asparaginase expressing yeast

*S. cerevisiae* can act as a drug delivery vehicle for L-ASNase; however, the current biomass required to yield therapeutic doses has suspended further investigation. In *in vitro* culture settings, AEY is capable of depleting L-ASN and inducing apoptosis in the AS-negative ALL cell line, RS4;11, and inhibiting proliferation in the AS expressing ALL cell line, 697. When working with a mouse xenograft model for ALL, the number of yeast cells required to reach a therapeutic dose exceeded both ethical and technical limits. Ethically, it would be inhumane to subject the mice to potential suffering without any *in vitro* evidence that AEY is a superior L-ASNase therapeutic. Additionally, the animal care guidelines do not allow injecting IP the volume that would be required for an injection on the order of $10^{10}$ yeast cells\[^{146}\].

Calculations showed that 21.6-26.0 billion yeast cells were required to reach a therapeutic dose of L-ASNase in a 25-30g mouse. However, this calculation may be an underrepresentation of the number of yeast cells needed. The ratio of units of L-ASNase to AEY OD\(_{600}\) was calculated in saturated L-ASN conditions, but *E. coli* L-ASNase and *S. cerevisiae* L-ASNase have different K\(_m\) properties. *E. coli* L-ASNase is $1.25 \times 10^{-5}$ M whereas *S. cerevisiae* L-ASNase is $2.5-3.5 \times 10^{-4}$ M\[^{147,148}\]. The smaller K\(_m\) value for *E. coli* L-ASNase indicates that it can maintain maximal L-ASNase activity at lower concentrations than *S. cerevisiae* L-ASNase. These differences in activity at low L-ASN concentrations may require more AEY to maintain L-ASN levels at the therapeutic threshold.
The ED$_{50}$ for the AEY titration was also significantly higher than the ED$_{50}$ for the purified *E. coli* L-ASNase titration for RS4;11 (see Figure 3.5). Although no statistical differences ($P > 0.05$) in ED$_{50}$ were observed for the other 5 cell lines: 380, 697, BV, N6, and RC, the $R^2$ values were low ($< 0.9$) making it difficult to interpret the results. Although more robust ED$_{50}$ calculations ($R^2$ closer to 1.0) would be obtained by increasing the starting concentration for the titrations, technically, it was not possible to perform the assay with higher AEY concentrations (0.17U/mL(0.34OD$_{600}$)). At concentrations higher than 7M cells/mL, the yeast cell density is too high to culture viable leukemia cells and properly analyze using flow cytometry, which is a limiting factor when culturing in the presence of AEY (see Figure 3.3). The higher ED$_{50}$ for AEY titration for RS4;11 further supports that notion that AEY may require more copies of enzyme than purified *E. coli* L-ASNase to obtain a therapeutic dose.

With the calculated ratio of units of L-ASNase to yeast cells, over 20 billion yeast cells are required for a 25g mouse for a therapeutic dose. In a 10-20kg child, the number of yeast cells required for a single therapeutic dose would be in the trillions. Giving such a large dose of AEY in a single injection would likely be a severe risk for embolisms that could result in death. The use of an intravenous line could potentially overcome this obstacle, however, the extra time required for a safe infusion may drain valuable medical resources and increase the costs of L-ASNase therapy. Moreover, any additional time required for AEY therapy may inconvenience the patient and thus may not be their therapy of choice. On top of the risks of embolisms, the risk of severe immune reactions by giving patients large doses of AEY would discourage the use of AEY. Even though *S. cerevisiae* is a nonpathogenic organism, the large dose of particles with numerous TLR ligands may trigger a cytokine storm or other life threatening immune reactions.
Comprehensive research into the immunology behind both large and repeated AEY dosing must be completed before this therapy could be considered viable for the patient.

Although it was not possible to inject AEY intraperitoneally (IP), a subcutaneous (SC) injection of AEY could have been feasible. The CCAC guidelines allow up to a 3mL SC injection into a 25g mouse before additional ethical approval and monitoring is required\textsuperscript{146}. However, the bioavailability of SC injections is less than IP injections, thus more AEY would likely be required to reach a therapeutic dose. Gaining ethical approval for this technique would also be difficult, as the large mass of cells being injected subcutaneously would likely be very painful for the mouse and consequently it would unlikely to be permitted by any animal care committee. Also, the reduced bioavailability and increased discomfort of SC injections from having a large mass underneath the skin, make them an unrepresentative model of how AEY would likely be administered clinically. So, no investigation into the efficacy of SC injected AEY therapy took place.

3.4.2 Proposed improvements for AEY therapy

Currently, the biggest hurdle to making AEY a viable drug delivery vehicle for L-ASNase therapy is the incredibly large biomass required for a therapeutic dose. To make AEY a feasible drug delivery approach, the number of yeast cells required for an IP injection in mice should be reduced from the order of $10^{10}$ to $10^8$-$10^7$. For reference, a study utilizing recombinant \textit{S. cerevisiae} as a vaccine vector utilized $10^7$ cells per injection\textsuperscript{111}. A start to reducing the biomass would be to improve the inactivation procedure. In this study, heat-inactivation was utilized because it was the only way to ensure 100\% inactivation of the AEY. The problem with the heat-
inactivation of yeast cells is that the activity recovery percentage is 60-70%. In this study, it was shown that UV inactivation can inactivate >99% of cells while maintaining >90% of the L-ASNase activity. Part of the problem with the UV inactivation was the simplicity in the resources available. To UV inactivate the AEY, cells were simply diluted and plated in an empty petri dish and exposed to a single UV light source for 5-min intervals. As the cell density increased, yeast cells suspended at the top of the solution blocked out the UV exposure to the bottom of the dish. Even after resuspended the cells between exposures, a very small percentage of cells remained viable. Multiple UV light sources and/or an apparatus that effectively mixes the yeast cells while they are being exposed to UV light may allow 100% inactivation. Alternatively, higher intensity, DNA damaging light rays such as x-ray or gamma irradiation could be used to inactivate the yeast cells. One reason that further investigation into improving the efficiency of the UV-inactivation protocol was not performed was because even with 100% efficiency and 100% activity recovery, the amount of AEY required would still face the same technical and ethical limitations as previously described.

Another approach to reducing the biomass could be to utilize a French pressure cell press to extract the cell wall components of the yeast cells while removing all the other unnecessary cell components. This technique may drastically reduce the biomass while maintaining all the potential advantages of utilizing yeast cells as a drug delivery vehicle, if the cell wall maintains its integrity and can shield L-ASNase from immune surveillance. However, the disruption of the cell wall during the cell press extraction procedure may compromise the L-ASNase-II shielding or inactivate the enzyme. Additionally, injecting a mixture of broken-down cell wall components may increase the number of antigens and TLR ligands that could elicit an immune response,
further increasing the risk associated with *S. cerevisiae*-based drug delivery. Complete purification of *S. cerevisiae* L-ASNase-II is also an option, but the higher $K_m$ property makes it a less attractive L-ASNase than the current standards of care. Additionally, investigation of purified *S. cerevisiae* L-ASNase-II for ALL therapy is already being conducted\textsuperscript{106}.

Since yeast cells are living organisms, one theoretical advantage to using them as a drug delivery vehicle is their ability to continue to manufacture the enzyme of interest (in this study L-ASNase) while *in vivo*. Unfortunately, living organisms that can colonize the body cannot be injected into immunocompromised cancer patients. A more technical approach to overcome this problem would be to engineer a yeast strain that had an inducible Cre-Lox system that could inactivate essential *S. cerevisiae* genes required for cell division, creating a senescent cell that can still produce the enzyme/protein of interest (in this case L-ASNase). Inducible gene expression has already been explored in *S. cerevisiae* using both the androgen and estrogen promoters\textsuperscript{149,150}. Incorporating an inducible Cre-Lox transcription regulation system on top of a hormonal-induced gene expression has also already been shown\textsuperscript{150}. Hundreds of genes essential for yeast cell division have already been identified as potential target genes for this inducible system\textsuperscript{151}. In this approach, a yeast strain with constitutive L-ASNase expression is engineered with an inducible Cre-Lox system that can permanently inactivate essential cell division genes. Once the yeast is grown up, the Cre recombinase protein is induced through exposure to the relevant hormone (e.g. estrogen). Cre recombinase then splices out essential replication cycle gene(s) that are flanked by two LoxP sites, rendering the cell senescent, but still capable of constitutive L-ASNase production. Once all cells are senescent, the yeast culture is harvested, washed, and readied for testing. Whether or not such a system could produce 100% senescent
cells is unknown. Additionally, the continuous production of L-ASNase by the senescent AEY may still not be enough of an improvement to overcome the large biomass required for effective AEY therapy. Currently, it may not be possible to reduce the biomass of AEY to make it a viable drug delivery vehicle. Any future advances in bioengineering that can increase the L-ASNase activity per cell on the order of $10^2$-$10^3$ should reopen investigation into *S. cerevisiae* as a drug delivery vehicle.

Even if the biomass issue with AEY is overcome, the lower $K_m$ property of *S. cerevisiae* L-ASNase compared to *E. coli* L-ASNase may make *S. cerevisiae* L-ASNase the inferior ALL therapeutic. To overcome this obstacle, in this study it has been shown that *S. cerevisiae* cells have the capacity to express *E. coli* L-ASNase and deplete L-ASN *in vitro*; however, the cellular location of the *E. coli* L-ASNase and the units of L-ASNase activity could not be determined. The activity of AnsB-AEY has proven difficult to determine as no L-ASNase activity is present after 15 mins, yet AnsB-AEY is able to deplete L-ASN over 48hs. A more extensive L-ASN depletion time-curve is needed to determine the onset of L-ASNase activity, the $K_m$, and the units of L-ASNase activity. How the heat-inactivated yeast cells transport L-ASN across the cell wall and membrane is also unknown. One explanation is that the L-ASN transporter proteins remain active after heat inactivation, like L-ASNase. Another possible explanation is that the cell wall and membrane become more porous after the heat treatment. To determine the cellular location, *E. coli* L-ASNase could be tagged with GFP and the location could be determined using confocal microscopy and the appropriate controls. Further investigation into amino acid leader sequences can also be done to try and express the L-ASNase on the cell wall. However, intracellular expression of L-ASNase may be an advantage for the drug delivery of L-ASNase.
Since \textit{S. cerevisiae} is a nonpathogenic organism and L-ASNase can be expressed intracellularly, removing the neoantigen (\textit{E. coli} L-ASNase) can decrease the risk of eliciting an immune response and consequently increase the half-life and safety of the drug. Although there are theoretical advantages to intracellular expression of L-ASNase, the shielding of the enzyme inside the yeast cell would likely lower the bioavailability and K\textsubscript{m} properties, making AnsB-AEY less efficacious than its AEY counterpart.

### 3.4.3 \textit{Saccharomyces cerevisiae} drug delivery for enzyme replacement therapies

This study has shown that \textit{S. cerevisiae} can, theoretically, act as a drug vehicle for interspecies enzymes. Although \textit{S. cerevisiae} is not a feasible drug delivery vehicle for \textit{E. coli} L-ASNase due to the large biomass required for an effective therapeutic dose, it may be suitable for enzyme replacement therapies such as for Gaucher disease, Fabry disease and/or phenylketonuria. In these diseases, a genetic defect prevents the patient from producing glucocerebrosidase, \(\alpha\)-galactosidase A, and phenylalanine hydroxylase respectively\textsuperscript{152-154}. Less enzyme is required for effective enzyme replacement therapy for these diseases compared to L-ASNase therapy.

Gaucher disease is treated with 30-60 units/kg of mannose-terminated glucocerebrosidase, Fabry disease with 0.2mg/kg of \(\alpha\)-galactosidase A, and phenylketonuria with 12-360 units/kg of PEGylated phenylalanine ammonia lyase\textsuperscript{155-157}. PEG-ASNase therapy requires 2,500 U/m\textsuperscript{2} per dose for children and 6,000U/m\textsuperscript{2} per dose for adults\textsuperscript{158}. For a 15kg patient, resulting dosages would be 450-900U for mannose terminated glucocerebrosidase, 3 units for \(\alpha\)-galactosidase A, 180-5400U for PEGylated phenylalanine ammonia lyase and 1625U for L-ASNase (a 15kg child has a body surface area of approximately 0.65m\textsuperscript{2})\textsuperscript{159}. All three enzyme replacement therapies have effective dosages, with at least part of the therapeutic range, lower than PEG-L-ASNase.
Thus, in Gaucher disease, Fabry disease, and phenylketonuria, it may be possible to utilize the advantages of using *S. cerevisiae* as a drug delivery vector since the enzyme replacement therapy may require less *S. cerevisiae* cells for effective dosages. Further investigation would be required to determine if using *S. cerevisiae* for enzyme replacement therapy would reach the same ethical and technical limitations AEY reached in this study.

### 3.4.4 Addressing the immunogenicity of L-asparaginase expressing yeast

The immunogenicity of repeated AEY dosing was not addressed in this study because AEY, in its current form, is not a feasible L-ASNase therapy. Determining the effect of *S. cerevisiae* cells on L-ASNase or other enzymes’ immunogenicity and subsequent side effects is essential in evaluating the future of *S. cerevisiae*-based drug delivery. Advances in the enzymatic activity to biomass ratio are less meaningful if a yeast-based vector elicits immune responses target *S. cerevisiae* for destruction. More studies evaluating the effects of repeated *S. cerevisiae* dosing must be completed to identify the benefits for *S. cerevisiae*-based drug delivery. Additionally, it must be determined how the immunogenicity of a yeast vector changes when it is constitutively expressing a neoantigen. Although there is a lot of bioengineering advancements that must take place to make AEY a viable L-ASNase therapeutic, there may be enzyme replacement therapies that could be employed using *S. cerevisiae* cells. Determining if repeated *S. cerevisiae* dosing is indeed safer than current L-ASNase and enzyme replacement therapies will be one of the most important next steps in evaluating the future of *S. cerevisiae*-based drug delivery.

In addition to lowering the biomass and determining in the safety of *S. cerevisiae*-based drug delivery, a key stakeholder needs to be considered in future studies: the patient. Advances in a
yeast-based delivery approach will have less value for patients if they reject it in favour of the current standard of care options. Putting dead microorganisms into one’s body may seem counterintuitive for patients undergoing extensive therapy. Working with the patients to learn how to address important issues surrounding safety, efficacy, and/or utility will be necessary for the success of yeast-based drug delivery. In an era of patient-centered care, we must learn how to educate and enable patients to make informed decisions about novel drug delivery.

3.4.5 Concluding Remarks

Overall, the purpose of this study was to identify if S. cerevisiae was a suitable drug delivery vehicle for L-ASNase therapy. This study has shown that S. cerevisiae constitutively expressing either the native L-ASNase-II or E. coli L-ASNase is capable of depleting L-ASN levels and inducing apoptosis in an AS-negative ALL cell line in vitro. Unfortunately, the biomass required for AEY to reach a therapeutic dose in vivo exceeds the current ethical limitations in mice, which has warranted the conclusion of this study. Additionally, the immunogenicity associated with repeated S. cerevisiae dosing remains unknown. If future advances in S. cerevisiae bioengineering can overcome the current obstacles in AEY therapy, S. cerevisiae based drug delivery should once again be explored for L-ASNase or other enzyme based therapies.
Chapter 4: The effects of neonatal *Saccharomyces cerevisiae* exposures on ALL progression

4.1 Aim
Evaluate the effect of neonatal exposures of *S. cerevisiae* on leukemogenesis in Eμ-RET mice.

4.2 Introduction

4.2.1 Problems with pediatric ALL chemotherapy
Childhood ALL survivors are also more likely to develop morbidities such as cardiac toxicity, secondary neoplasms, and neurologic toxicity, report more chronic medical conditions, report poor mental health, and have lower rates of college graduation and employment compared to their siblings\(^{160-165}\). Understanding the progression of preleukemia into leukemia can provide valuable insight into preventative health measures that can be taken to reduce the risk of developing leukemia. Although ALL cure rates are high (approaching 90%), preventing the need for cancer therapy, and the subsequent increased risk for later-life morbidities, is of great interest to improve the quality of life for the children at risk of developing ALL\(^ {13}\).

4.2.2 The role of infection in influencing pediatric leukemogenesis
Infection, ionizing radiation, pesticides, inflammation, and oxidative stress have all been linked to leukemogenesis\(^ {13,34,35,59-63}\). Of interest, mild infections, and abnormal immune responses to early-life mild infections, have long been hypothesized as a leading factor in leukemia progression\(^ {34,35}\). The epidemiological reports have found that infection can both increase and
decrease the risk of developing leukemia, with the timing of the infection playing an important role\textsuperscript{59,64-67}. In developed countries, day care attendance is used as proxy measure for exposure to mild infections since most early childhood infections result from contact with other children\textsuperscript{166,167}. Consistently, daycare attendance has been associated with a lower risk of developing ALL, suggesting that early life exposures to mild infections may decrease the risk of developing ALL\textsuperscript{168}.

4.2.3 **Indications for testing the effect of early-life *Saccharomyces Cerevisiae* exposures on leukemogenesis in Eμ-RET mice**

Recently, our lab uncovered the first mechanistic explanation for the role of infection, and timing of that infection in leukemogenesis\textsuperscript{68,69}. In Eμ-RET mice, mild infections delivered only during the neonatal period of life yield an immune response that results in antileukemic activity, delaying the onset of leukemia. Our results show neonatal exposure to attenuated *Listeria monocytogenes* (Lm) can initiate an IL-17-dependent immune response that leads to the activation of antileukemic NK cells, leading to a depletion of preleukemic cells (see Figure 4.11)\textsuperscript{69}. Since Eμ-RET mice have a constitutive transgene that continues to produce nonfunctional B cell precursors, these mice still develop leukemia; however, when the *E2A-PBX1* adoptive transfer model of leukemia was used, most of the recipients (90%) who received neonatal, mild infections achieved long-term, disease free survival. We currently hypothesize that the activated NK cells in response to infection deplete the preleukemic cells since NK cells are required for depletion and NK cells sorted from infected, neonatal spleens can directly kill leukemic cells *in vitro*. 
The role of the microbiome and nonpathogenic organisms in the development of leukemia is also currently poorly understood. It is known that the microbiota play an important role in shaping the mammalian immune system\textsuperscript{169}. As the microbiome has important interactions with the immune system and infection has been consistently linked with leukemogenesis, we wanted to further investigate the potential role of the gut microbiota on ALL progression. Recent research has begun to examine the effects of ALL chemotherapy on the composition of the microbiome, but, limited research has investigated nonpathogenic, microbiota exposures and the subsequent effects on leukemia progression\textsuperscript{114-116}. The fungal microbiome, or mycobiome, is emerging as an important component of the microbiome\textsuperscript{117-119}. Of interest, \textit{Saccharomyces cerevisiae} is a commensal organism that is generally considered nonpathogenic\textsuperscript{102,120,170}. Although, \textit{S. cerevisiae} cannot colonize the human gastrointestinal tract, exposure in early life is imminent though dietary and environmental exposures and \textit{S. cerevisiae} likely to impact the microbiome composition\textsuperscript{102,118}. As we have previously shown that mild infections can induce a neonatal, antileukemic immune response, we hypothesized that neonatal exposure to the commensal organism \textit{S. cerevisiae} can induce the same antileukemic immune responses.

In this chapter, using the same \textit{Eμ}-RET mouse model our lab used to evaluate early-life infections on leukemia progression, I will evaluate the effects of early-life \textit{S. cerevisiae} exposure on leukemogenesis. This study will provide valuable insight into whether a nonpathogenic agent, such as \textit{S. cerevisiae}, can induce the same IL-17 driven immune response to deplete preleukemic cells in \textit{Eμ}-RET mice. This information will provide insight into potential roles of microbiome exposures on leukemia development. Additionally, I will begin to evaluate the antileukemic potential of the IL-17 producing \(\gamma\delta\) T cells and explore the potential role of IL-17 in directly
activating NK cells. Understanding how to manipulate the activation of the neonatal, antileukemic NK cells may provide the basis for new immunotherapeutic approaches for ALL.
Figure 4.11 The current understanding of the mechanism underlying the depletion of preleukemic cells in Eμ-RET mice.
4.3 Results

4.3.1 Neonatal *Saccharomyces cerevisiae* exposure does not induce depletion of preleukemic cells

Attenuated *Listeria monocytogenes* (Lm) has been used by our lab to model neonatal infection responses that induce depletion of preleukemic cells and delay the onset of disease in Eμ-RET mice. To test the ability of *S. cerevisiae* to also induce the depletion of preleukemic cells, $10^4$ cfu of *S. cerevisiae* were injected IP into Eμ-RET mice age day 5-8 and mice were sacrificed 8 days later. Age matched mice were given a dose of $10^4$ cfu of Lm as a control for inducing the depletion response. The total number of preleukemic cells in the spleen was determined for each mouse. Preleukemic cells were identified as a B220<sup>int</sup>/BP-1<sup>hi</sup> population of cells present that is only present in the spleens of Eμ-Ret mice. As shown in Figure 4.12 A, no significant reduction in the number of preleukemic cells occurs in Eμ-RET mice after neonatal exposure to $10^4$ cfu of *S. cerevisiae* relative to the PBS control. Additionally, as shown in Figure 4.12 B, at $10^4$ cfu of *S. cerevisiae*, there is also no significant increase in spleen size compared to the PBS control, suggesting limited immune activation by this dose of *S. cerevisiae*. To determine if a dosage of $10^4$ cfu of *S. cerevisiae* is not sufficient to induce the depletory response, we increased the dosage of *S. cerevisiae* to $10^5$ cfu, then $10^6$ cfu, and finally $10^7$ cfu. Figure 4.12 A shows that regardless of the amount of *S. cerevisiae* injected into the mice, there is no significant depletion of preleukemic cells. To determine if a mature immune system responds differently to *S. cerevisiae*, 4-6-week-old adult mice were also injected IP with $10^5$ cfu of *S. cerevisiae*. No depletion response or increase in spleen size occurred after the *S. cerevisiae* injection in adult mice either (see Figure 4.12 C). Notably, when $10^7$ cfu of yeast is injected into neonatal mice, there is a significant increase in spleen size compared to the PBS treated control (see Figure 4.12
B). This significant increase in spleen size highlights that a threshold exists in which *S. cerevisiae* can induce a significant immune response in Eμ-RET mice, but this response does not result in the depletion of preleukemic cells. Additionally, Figure 4.12 B shows that the spleen size does not significantly increase in Lm treated mice, despite the capacity to induce antileukemic immune activity. These results reinforce our labs previous findings that mild, neonatal infectious agents are sufficient to deplete preleukemic cells.
Figure 4.12 The effect of neonatal Saccharomyces cerevisiae exposures on splenic preleukemic burden and spleen size

A) The total number of preleukemic cells (BP-1/<B220>int) in the spleens and B) the total spleen size of 2-week-old Eμ-RET mice after IP injection of PBS (n=12), 10^4 cfu (n=9), 10^5 cfu (n=10), 10^6 (n=7), or 10^7 (n=7) cfu of S. cerevisiae or 10^4 cfu of Lm (n=9). Mice were initially IP injected between day 5-8 of life and sacrificed 8 days after the injection. The total number of viable (7-AAD') splenocytes was determined by flow cytometry and used as a proxy measure for the total spleen size (One-way ANOVA). C) The total number of preleukemic cells (BP-1/<B220>int) in the spleens (left panel) and the total spleen size (right panel) of 4-6-week-old Eμ-RET mice after IP injection of PBS (n=3) or 10^5 cfu of S. cerevisiae (n=3) (Welch’s t-test).
4.3.2 Saccharomyces cerevisiae does not induce the expansion of activated γδ T cells or activate NK cells

Our lab has observed that γδ T cells and NK cells are required to produce antileukemic activity in Eμ-RET mice in response to neonatal exposure of Lm. Both γδ T and NK cells are activated after neonatal Lm exposure. To explain why S. cerevisiae does not yield antileukemic activity, the activation profile of γδ T cells and NK cells was analyzed after neonatal exposure to S. cerevisiae. 6-8-day old mice were injected IP with either PBS, $10^4$ cfu of Lm, $10^4$ cfu of S. cerevisiae or $10^7$ cfu of S. cerevisiae. The mice were sacrificed 72h after the injection and the percentage of activated (CD69+) NK and γδ T cells, as well as the total number of activated γδ T cells, was determined by flow cytometry for each condition (see Figure 4.13 A and B). Since S. cerevisiae does not induce the depletion of preleukemic cells, it was expected that S. cerevisiae would not activate γδ T nor NK cells. Indeed, as shown in Figure 4.13 A and B, Lm, but not S. cerevisiae, induces significant activation of NK cells and the expansion of activated γδ T cells. Although S. cerevisiae (both doses) can significantly increase the percentage of activated γδ T cells, to a lesser degree than Lm (24% vs. 82%, respectively), there is no significant increase in the number of CD44+ or CD27+ activated γδ T cells (see more in section 4.4). Thus, the increase in the percentage of activated γδ T cells in response to S. cerevisiae does not lead to any significant expansion of IFNγ or IL-17-producing γδ T cells. These differences further support that S. cerevisiae does not induce the same immune response as Lm.

As shown in Figure 4.12 B, a $10^7$ cfu dose of S. cerevisiae results in a significant increase in the spleen size, suggesting the induction of an immune response that does not occur at lower doses of S. cerevisiae. One contribution to the increase in spleen size is the increase in the number of
neutrophils initiated only by the $10^7$ dose of *S. cerevisiae* (see Figure 4.13 C); however, despite a significant increase in neutrophils in the spleen, there is still no depletion of preleukemic cells, suggesting neutrophils may not be required for the depletion response. Although neutrophil infiltration can be induced by IL-17, the lack of significant preleukemic depletion in mice treated with $10^7$ cfu of *S. cerevisiae* suggests neutrophils are unlikely to mediate the depletion response\(^\text{126}\). Since doses of $10^7$ cfu of yeast are not representative of the exposures to *S. cerevisiae* in early-life for mice, we stopped investigating the immune activity caused by $10^7$ cfu doses of *S. cerevisiae*. Overall, since neonatal exposure to *S. cerevisiae* does not induce the expansion of activated γδ T cells, activate NK cells or induce the depletion of preleukemic cells, we conclude that *S. cerevisiae* does not directly initiate the same neonatal, antileukemic immune activity in Eμ-RET mice in response to infectious agents, such as Lm.
A

Percentage of CD69+ cells in the spleen

B

Total number of CD69+ γδ T cells in spleen

C

Total number of neutrophils in spleen
Figure 4.13 The effect of Listeria monocytogenes and Saccharomyces cerevisiae on NK cells, γδ T cells, and neutrophils in the spleen of Eμ-RET mice

A) The percentage of activated (CD69+) NK cells and γδ cells (CD3+/γδ TCR+) in the spleens of Eμ-RET or Balb/c mice 72 after the injection of either PBS (n=9), 10^4 cfu of Lm (n=12 for γδ T cells, n=8 for NK cells), 10^4 cfu of S. cerevisiae (n=8), or 10^7 cfu of S. cerevisiae (n=7) (Two-way ANOVA).

B) The total number of activated γδ T cells (CD3+/γδ TCR+/CD69+) in the spleens of Eμ-RET or Balb/c mice 72 after the injection of either PBS (n=9), 10^4 cfu of Lm (n=10), 10^4 cfu of S. cerevisiae (n=8), or 10^7 cfu of S. cerevisiae (n=7) (One-way ANOVA).

C) Total number of neutrophils (Ly-6G+/CD11b+) in the spleens of Eμ-RET or Balb/c mice 72 after the injection of either PBS (n=4), 10^5 cfu of S. cerevisiae (n=6), 10^7 cfu of S. cerevisiae (n=4) or 10^4 cfu of Lm (n=6) (One-way ANOVA).
4.3.3 γδ T cells activated in response to infection do not have antileukemic activity in vitro

Although *S. cerevisiae* was not able to induce the depletion of preleukemic cells, we wanted to continue to investigate the role of IL-17 producing γδ T cells (γδT17) cells and IL-17 in generating antileukemic activity. Multiple subsets of γδ T cells (Vγ9Vδ2, Vδ1, and Vδ2) have shown promise as new cancer immunotherapies as they each display cytotoxicity against cancer cells. To begin evaluating the role of γδ T cells, we delivered $10^4$ cfu of Lm into Eμ-RET/IFNγ knockout mice. IFNγ production is a mechanism for cancer cell cytotoxicity for both NK and certain subsets of γδ T cells. Furthermore, our lab has recently reported that IFNγ reduces the basal number of early-life preleukemic cells and is important for delaying the onset of leukemia in Eμ-RET mice. Figure 4.14 A shows that the antileukemic immune response generated by Lm is IFNγ-independent. Thus, IFNγ produced by γδ T and/or NK cells is not the mediator of the observed antileukemic activity.

In mice, the CD27 subset and CD44 subsets of γδ T cells are associated with IFNγ and IL-17 production respectively. We evaluated the total number of activated γδ T cells for both subsets in response to infection to further illustrate the IFNγ-independent nature of the neonatal, antileukemic immune response. After infection with $10^4$ cfu of Lm, but not with *S. cerevisiae*, there is an increase in the number of activated γδ T cells in both subsets; however, the magnitude of the effect is different. The CD27$^+$ γδ T cells respond with a small increase of ≈3500 activated cells (≈5-fold increase; Figure 4.14 B), but there is no increase in the overall number of CD27$^+$ γδ T cells (Figure 4.14 C). The CD44$^+$ γδ T cells respond with a large increase of ≈250 000
activated cells (≈190-fold increase; Figure 4.16 D), and there is also a significant increase in the total number of CD44⁺ γδ T cells (Figure 4.14 E). The γδ T cell counts for both subsets after infection indicates a more dynamic expansion of activated γδT17 cells since there is no increase in the overall number of CD27⁺ γδ T cells. This difference in γδ T cell populations highlights that CD44⁺ γδ T cells, which are not associated with direct anticancer activity, are more likely to be more important intermediate mediators of the observed antileukemic activity in response to neonatal infections. One possible explanation for the increase in activated CD27⁺ γδ T cells, but no increase in the overall number of CD27⁺ γδ T cells, is that activation is an off-target effect from cytokines used to activate γδT17 and/or NK cells.

To evaluate if the γδT17 cells activated in response to neonatal infections have direct, antileukemic activity, we infected mice aged 6-8d with Lm or PBS and 72h after the infection we sorted the activated γδ T cells and the NK cells from the pooled Lm treated spleens and the NK cells from the pooled PBS treated spleens. We co-cultured the NK and activated γδ T cells overnight (16h) with the murine leukemic cell line 289 at a ratio of 50:1 (50 effector cells:1 289 cell). For two trials (n=2), we simultaneously sorted the splenic NK cells from age-matched, PBS treated mice and cultured them at the same 50:1 ratio with 289 as a control. The total number of CD44-positive γδ T cells in PBS treated mice (Figure 4.14 E) is insufficient to sort the number γδ T cells required for the proper PBS control, a limitation in this experiment. After 16h, the proportion number of viable 289 cells relative to the untreated control (0 effector cells:1 289 cell) was determined by flow cytometry. Only activated NK cells from Lm treated mice display cytotoxic, antileukemic activity (see Figure 4.15 A). As ≈90% of the activated γδ T cells (see Figure 4.15. B) in response to Lm are of the γδT17 (CD44⁺) phenotype, this data suggests that
\(\gamma\delta T17\) cells activated in response to infection do not directly exert the anticancer cytotoxicity. Overall, the infection-mediated preleukemic depletion in IFN\(\gamma\) KO mice, the more dynamic expansion of CD44\(^+\) cells over CD27\(^+\) \(\gamma\delta\) T cells in response to infection, and the absence of antileukemic activity of sorted \(\gamma\delta\) T cells \textit{in vitro} supports the \(\gamma\delta\) T cells necessary for neonatal depletion of preleukemia do not have direct leukemia-killing activity\(^{171}\).
**A**

Total BP1⁺/B220⁺ cells in the spleen

**B**

Total number of CD69⁺/CD27⁺/CD44⁺ γδ T cells in the spleen

**C**

Total number of CD27⁺ γδ T cells in the spleen

**D**

Total number of CD69⁺/CD27⁻/CD44⁺ γδ T cells in the spleen

**E**

Total number of CD44⁺ γδ T cells in the spleen
Figure 4.14 The contribution of IFNγ-producing γδ T cells in neonatal, antileukemic immune responses

A) The total number of preleukemic (BP-1+/B220−) cells in IFN-/- mice 8 days after treatment of 10⁴ cfu of Lm (n=9) or PBS (n=8) (Mann-Whitney U Test). B) The total number of activated (CD69+), IFNγ producing (left panel), C) the total number of IFNγ-producing (CD27+) γδ T cells, D) the total number of activated, IL-17 producing (CD44+) γδ T cells, and E) the total number of IL-17-producing (CD27+) γδ T cells in the spleen of Balb/c mice 72h after treatment of either PBS (n=4), 10⁴ cfu of S. cerevisiae (n=3) or 10⁴ cfu of Lm (n=6) (Kruskal-Wallis Test with Dunn post-hoc test (B) or Two-way ANOVA (C-E)).
Figure 4.15 The effect of culturing sorted, activated γδ T cells in the presence of a leukemia cell line

A) Reduction in number of viable Eμ-ret leukemia cells (289) after co-culture with purified NK cells or γδ T cells, isolated from spleens of Lm-infected or PBS-treated neonatal mice. Effector cells and 289 cultured at a ratio of 50:1. 6-9 mice spleens for each treatment group, pooled for each independent experiment. n=5 for NK cells (Lm), n=4 for NK cells (Mock), n=5 γδ T cells (Two-way ANOVA). B) The distribution of CD44+ and CD27+ activated γδ T cells in the spleen of Balb/c mice 72h after injection of 10⁴ cfu of Lm.
4.3.4 The effect of IL-17 on NK activation in Balb/c mice

The link between γδT17 cells and neonatal NK cell activation in response to infection remains unknown. Although neonatal S. cerevisiae exposures does not activate NK cells, we wanted to continue to investigate the role of IL-17 in producing antileukemic immune activity in neonatal Balb/c mice. We hypothesized that IL-17 produced by γδT17 cells directly activates the neonatal NK cells to induce their cytotoxic, antileukemic activity. To test the effect of IL-17 on neonatal NK cell activation, we cultured 8-9-day old neonatal splenocytes overnight in the presence of IL-2, IL-15, both, or unstimulated control media with and without IL-17 and observed the effect on NK cell viability and activation. Importantly, without supporting cytokines (IL-2 and IL-15), NK cells die rapidly in culture as very limited viable NK cells are present after an overnight culture (see Figure 4.16 A left panel)\textsuperscript{178}. We chose to culture the cells in the presence of IL-15 and IL-2 as these cytokines are associated with producing NK cell cytotoxicity in an IFNγ-independent manner, which is important since we already showed infection mediated antileukemic activity is IFNγ-independent (Figure 4.14 A)\textsuperscript{179}. The results in Figure 4.16 A show no significant differences (P > 0.05) in both the number of viable NK cells or the percentage of activated (CD69\textsuperscript{+}) NK cells between IL-17 and the respective control cultured splenocytes (see Figure 4.16 A). With no evidence supporting an activating effect of IL-17 on neonatal NK cells, we wanted to test if IL-17 induces or increases the cytotoxicity of neonatal NK cells cultured in the presence of IL-2 and IL-15. To test this potential role for IL-17, NK cell-enriched splenocytes were co-cultured at a ratio of 50:1 with the murine leukemic cell line 289 with media supplemented with IL-2, IL-15, and IL-17 or IL-2 and IL-15 or IL-17 or control media with no IL-2, IL-15 or IL-17. This approach for an NK cell \textit{in vitro} killing assay was established in the
methods used to produce Figure 4.15 A. The proportion of viable 289 cells relative to the control media condition was analyzed and no statistical differences (P > 0.05) in NK cell cytotoxicity were observed between the IL-2, IL-15 and IL-17 vs. IL-2 and IL-15 media conditions (see Figure 4.16 B). Additionally, no cytotoxic activity relative to the control condition was observed in NK cell-enriched splenocytes only cultured in IL-17. Combined, the results show that IL-17 does not increase the antileukemic activity of IL-2/IL-15 activated NK cells in vitro. The identical NK cell killing assay was repeated with control splenocytes (no NK cell enrichment) to control for the observed effects of IL-2 and IL-15 in killing 289 cells. Indeed, when co-cultured with control splenocytes at a ratio of 50:1, no antileukemic activity is observed in any of the treatment conditions (see Figure 4.16 C). This cytotoxic activity present only in NK cell-enriched splenocytes cultured in the presence of IL-2 and IL-15 (see Figure 4.16 D) can only be correlated with the NK-enrichment procedure as it does not yield 100% NK cells (see Figure 4.16 E). There is a possibility that a different cell type that is present in the NK cell-enriched splenocytes yields the antileukemic effect; however, the lack of the antileukemic activity in the co-culture with control splenocytes and the current literature suggest this possibility is unlikely. Since there was no observed effect of IL-17 in any in vitro experiment, we wanted to confirm the biological activity of the IL-17 being used. To confirm the activity, 0.5μg of IL-17 or PBS was injected IP into 25-30g adult Balb/c mice. 4h after the injection, the total number of neutrophils in the peritoneal cavity were determined by flow cytometry. As expected, the IL-17 treated mice see a significant increase in the number of neutrophils present in the peritoneal cavity, showing the previous assays had been using biologically active IL-17 (see Figure 4.16 F). Overall, no significant increases in NK cell counts, NK cell activation, or NK cell cytotoxicity were observed in the presence of IL-17 in these in vitro experiments.
IL-2 and IL-15 may be masking the effects of IL-17 on neonatal NK cell activation *in vitro* since the cytokines are necessary for viable NK cells in culture, but also inherently activate NK cells. To test the ability of IL-17 to activate NK cells without needing to supplement the NK cells with IL-2 and/or IL-15, we injected PBS or IL-17 IP into day-9-old mice and observed the effects on NK cell activation. 16h after the injection, the mice were sacrificed and the activation percentage of activated and the total number of NK cells in the spleen and the total number of neutrophils in the peritoneal cavity were analyzed (see Figure 4.17 A, B, and C). Neutrophil infiltration into the peritoneal cavity was used as a positive control for a successful IP injection of IL-17 into the mice (Figure 4.16 C). Figure 4.17 D contrasts the activation of NK cells in response to a Lm infection and the IL-17 injections.

There is no significant increase in the percentage of activated NK cells in the IL-17 treated mice compared to the PBS controls (see Figure 4.17 A). However, there is a significant increase in the number of NK cells in the spleens of IL-17 vs. PBS treated mice (see Figure 4.17 B). Combined, the results do not support that IL-17 alone is capable of activating NK cells *in vivo*. However, IL-17 does appear capable to increase the number NK cells in the spleen. This observation may be mediated by inducing the proliferation of NK cells and/or by increasing the recruitment of NK cells to the spleen. Currently, we do not know if these potential effects of IL-17 is direct or indirect. Although there is no positive control for IL-17 reaching the spleen in bioactive concentrations, the *in vivo* IL-17 activity was confirmed by observing a significant increase in the total number of neutrophils in the peritoneal cavity of IL-17-treated mice at the time of sacrifice (Figure 4.17 C). The estimated dose of IL-17 delivered IP exceeds the IL-17
concentration detected in the blood 72h (results not shown) after Lm infection by 4-5 orders of magnitude, if a total 2mL volume is assumed for each mouse (0.25μg/mL vs. ≈1.5pg/mL). The large amount of IL-17 compared to the concentrations detected during infection, along with a positive control for biologically active IL-17 in vivo suggests that IL-17 did reach the spleen in bioactive concentrations, but still did not result in NK cell activation.

To further test the role of IL-17 and γδT17 cells in activating NK cells, we sorted activated γδ T cells, as previously described, and injected either 1 x 10^5 or 3 x 10^5 cells IP into day 9 mice and observed the NK activation 48h later. No increase in the percentage of activated NK cells was observed after the adoptive transfer of γδ T cells, but as seen with IP IL-17 injections, there is a small, significant increase in the number of NK cells in the spleen (see Figure 4.17 E). Tail-vein IV injection would be a better route of administration for the γδ T cells, however, the size of the mouse tail vein at this age (8-9 days) is incredibly small and difficult to inject into. Overall, the lack of NK activation in the presence of IL-17 both in vitro and in vivo suggest that IL-17 does not directly activate neonatal NK cells or induce directly their cytotoxic activity. Although not conclusive, the results strongly suggest that IL-17 has an alternative function in generating antileukemic immune responses in response to neonatal infections.
Figure 4.16 The effect of IL-17 on NK activation and NK-mediated depletion of leukemic in vitro

The total number of viable NK cells (left panel) and the percentage of viable, activated (CD69+) NK cells (right panel) after overnight (16h) culture of $2 \times 10^6$ Balb/c splenocytes cultured with and without IL-17 (25ng/mL) in media supplemented with IL-2 (25ng/mL), IL-15 (25ng/mL) or both (right panel). Splenocytes were harvested from day 9 Balb/c mice 72h after the injection of either PBS (n=5) or $10^4$ cfu of Lm (n=5) (Two-way ANOVA). B) The proportion of viable 289 leukemic cells after overnight co-culture with NK-enriched splenocytes (n=3) or C) control splenocytes (n=3) in a 50:1 effector to target cell ratio relative to the unstimulated control. Cells were stimulated in media supplemented with either IL-2 (25ng/mL), IL-15 (25ng/mL), and IL-17 (25ng/mL) or IL-2 and IL-15 or IL-17 or control media (One-way ANOVA). D) Merge of C and D, highlighting that NK enriched splenocytes significantly decrease the proportion of viable 289 leukemic cells compared to control splenocytes in co-culture (Two-way ANOVA). E) The percentage of NK cells in the splenocytes used in the NK cell in vitro killing assays presented in Figure 3.5 B-D (n=3; Welch’s t-test). F) Positive control for IL-17A activity. The total number of neutrophils in the peritoneal cavity of 25-30g adult Balb/c mice 4h after IP injection of either PBS or 0.5 μg IL-17A (Welch’s t-test).
NK cells 16h after treatment:
PBS (blue) vs. IL-17A (red)

NK cells 72h after treatment:
PBS (blue) vs. Lm (red)
Figure 4.17 The effect of IL-17 on NK cell activation in vivo

A) The percentage of activated (CD69+) NK cells and B) the total number of NK cells in the spleens of Balb/c mice 16h after IP injection of either PBS (n=8) or 0.5μg IL-17A (n=11) (A: Welch’s t-test; B: Mann-Whitney U test). C) Positive control for IL-17A activity in A and B. The total number of neutrophils in the peritoneal cavity of day 9 Balb/c mice 16h after IP injection of either PBS (n=8) or 0.5 μg IL-17A (n=11). (Mann-Whitney U Test). D) NK cell flow cytometry plots of CD69 vs. side scatter-area (SSC-A), highlighting the lack of activation of NK cells after overnight IP injection of 0.5μg IL-17A (red) vs. PBS (blue) (left panel) compared to a Lm (red) infection vs. PBS (blue) (right panel) at 72h in Balb/c mice. E) The effect of adoptively transferred γδ T cells on the percentage of activated (CD69+) NK cells in the spleens of 8-9-day old Balb/c mice (left panel) and the total number of NK cells in the spleen (right panel). Either 1 x 10^5 (n=2) or 3 x 10^5 (n=3) activated γδ T cells or PBS (n=5) was injected IP into Balb/c mice at day 9 of life. Mice were sacrificed 48h after the adoptive transfer and assessed for NK cell CD69 activation. 9 mice spleens were pooled for each γδ T cell sort. For statistical analysis, the data from 1 x 10^5 and 3 x 10^5 adoptively transferred γδ T cells were combined (Student’s t-test (left panel); Mann-Whitney U Test (right panel)).
4.4 Discussion

4.4.1 Summary of results

Combined, this chapter has shown that the generally nonpathogenic agent *S. cerevisiae* is not capable of inducing the same neonatal, antileukemic immune response as a pathogenic agent, such as Lm. Additionally, compared to Lm, neonatal *S. cerevisiae* exposures does not lead to the same amount activation of NK cells or expansion of activated γδ T cells. To further understand the mechanism by which neonatal Lm exposures lead to the depletion of preleukemic cells, it was shown that the anticancer activity is generated in an IFNγ-independent manner and that activated NK cells, but not activated, CD44+ γδ T cells, yield antileukemic activity when co-cultured in the presence of the murine leukemic cell line 289. To further investigate a potential link between activated IL-17 producing γδ T cells and antileukemic NK cells, the effects of IL-17 on neonatal NK activation was observed both *in vitro* and *in vivo*. *In vitro*, IL-17 yielded no increase in NK cell activation or NK cell cytotoxicity. *In vivo*, IL-17 did not significantly activate splenic NK cells, but did significantly increase the number of splenic NK cells. The direct or indirect nature of this NK cell expansion is currently unknown. Combined, the results strongly suggest that IL-17 is incapable of directly activating NK cells by itself.
4.4.2 *S. cerevisiae* may still influence the neonatal immune system and leukemogenesis

*S. cerevisiae* does not directly induce the same neonatal, antileukemic immune responses as infectious agents in Eµ-RET mice, but still may influence leukemogenesis. The microbiome is known to influence the development of the immune system\(^{169}\). Therefore, neonatal exposures to *S. cerevisiae*, and/or other microorganisms, may not directly deplete preleukemia, but promote the development of the immune system to engage in antileukemic immune reactions in response to infection. *S. cerevisiae*’s status as a known adjuvant further supports a potential complementary role in generating effective, neonatal antileukemic immune responses rather than a direct one\(^{180,181}\). To test this possible function, we could co-deliver *S. cerevisiae* with a mild infection that does not fully deplete preleukemia (ex. murine cytomegalovirus) in Eµ-RET mice and observe the effects on preleukemic burden and onset of disease.

Correlating microbiome composition with onset of leukemia in Eµ-RET mice may provide insight into which microorganisms have the greatest influence on the immune system and subsequent disease progression. One experiment to highlight the potential role of the microbiome on leukemia progression would be to use antibiotics to remove the microbiome completely and observe the subsequent effects on leukemia onset in Eµ-RET mice. Additionally, *S. cerevisiae* may not be the most representative microorganism to test the direct role of the mycobiome in influencing leukemogenesis. *S. cerevisiae* does not colonize the human gastrointestinal tract which may limit its influence on leukemogenesis, whereas other families of yeast such as Candida and Dipodascaceae and other fungi such as Malassezia and Cladosporium are known to\(^{118}\). Moreover, each individual’s microbiome usually consists of 160 species (out of a possible \(\approx 1100\)), dominated by bacteria in the Firmicutes and Bacteroidetes phyla, highlighting the
complexity and vastness of the microbiome. Delivering neonatal exposures of each these known gut microorganisms to neonatal Eμ-RET mice may identify which, if any, species of bacteria or fungi are capable of initiating antileukemic immune responses, a time consuming and laborious task. Importantly, any species capable of generating antileukemic immune responses should also be administered in the appropriate route of exposure to observe any differences in immune responses (i.e. orally). Of interest would be testing the commensal fungus *Candida albicans* which has already been shown to elicit an IL-17 mediated immune response in response to oral administration. In this study, *S. cerevisiae* was delivered IP, which is not the best model of human *S. cerevisiae* exposure; however, the lack of immune activity in Eμ-RET mice suggests no differences would be observed with oral administration, a route associated with lower bioavailability. Certainly, lack of antileukemic immune activity caused by *S. cerevisiae* is not generalizable to the entire microbiome. Additionally, *S. cerevisiae* may impact leukemogenesis by influencing the colonization of the microbiome species capable of generating antileukemic activity.

The role of the microbiome in leukemogenesis still remains under investigated. Interspecies differences in immune responses to *S. cerevisiae* may exist between mice and humans. Thus, *S. cerevisiae* may play a more important role in generating immune activity than the results in Eμ-RET mice would suggest. More generally, a better understanding of how the composition of the human microbiome can influence the immune system and subsequent progression of preleukemia to leukemia can lead to the identification of microbiome profiles that increase the risk of developing childhood ALL. Moreover, as infection has been linked to both increased and decreased risk of infection, a better understanding of the role of the microbiome in developing
the early-life immune system may contribute to explaining these contrasting outcomes. As more is understood about microbiome profiles and leukemogenesis, they could become a risk stratification tool for future physicians to identify children with increased chance of developing ALL. For at-risk children, the implementation of fecal microbiota transplantations or other measures could possibly be used to change their microbiome and lower their risk for developing disease. Overall, these potential advantages for ALL patients highlight the need for a more comprehensive investigation into the role of the microbiome and ALL disease progression.

4.4.3 The function and contribution of γδT17 cells and IL-17 in generating antileukemic immune activity remains unknown

Our lab has shown that IL-17 produced by γδT17 cells is required for antileukemic activity; yet no direct anticancer effects of γδT17 cells or IL-17 was observed in this study. Thus, the contribution of both γδT17 cells and IL-17 in depleting preleukemic cells remains unknown. The lack of in vitro and in vivo activation of NK cells in response to IL-17 does not conclusively prove that IL-17 does not activate NK cells. These observations may be due to the multifactorial environment associated with immune responses that is needed to prime NK cells to respond to IL-17. The multitude of cytokines and pattern recognition receptor ligands present during infection is difficult to recapitulate in vitro and in vivo, absent of infection. Figure 4.18 A depicts a possible model in which more than one chemical in addition to IL-17 leads to the activation of antileukemic NK cells. Previous studies have shown that both TLR3 and TLR9 ligands can enhance NK responsiveness to cytokines, so a similar phenomenon may occur for NK cells to respond to IL-17. Experiments delivering low doses of TLR ligands prior to exogenous injection of IL-17 may increase the responsiveness of NK cells to IL-17 and explain the findings.
presented. Other experiments that could help recapitulate the multifactorial environment required to further analyze NK cell responsiveness to IL-17 are i) depleting γδ T cells prior to neonatal infection and delivering exogenous IL-17 48h after infection; and ii) infecting neonatal mice with IL-17 neutralizing antibody and culturing the NK cells 72h after infection with IL-17 to observe any responsiveness to IL-17. The first experiment could provide insight if γδ T cells only contribute IL-17 in the antileukemic immune response. If this statement is true, in theory, the appropriate amount of exogenous IL-17 delivered at the right time should restore the depletion response after γδ T cell depletion. If the depletion response is not restored with exogenous IL-17, it would suggest γδ T cells contribute more than IL-17 in generating antileukemic responses. Interpreting a negative result in this experiment would be difficult as the exact timing and amount of exogenous IL-17 required to properly recapitulate the depletion response would be difficult to determine. The latter experiment would provide insight into whether the immune milieu sensitizes the NK cells to IL-17. Although NK cells from infected spleens were cultured in the presence of IL-17 and no significant changes in activation were observed when cultured in IL-17, it is possible that the NK cell response to IL-17 is acute and masked without the use of IL-17 neutralizing antibody. This experiment would still likely be difficult to interpret as NK cell culture would still likely require cytokine supplementation with IL-2 or IL-15, which activates the cells and thus acts as a confounding variable.

Another possibility is that IL-17 does not directly or indirectly lead to the activation of NK cells, but rather has a synergistic or cooperative function. We know that NK cells gain antileukemic functionality in response to infection and that NK cells are necessary to deplete preleukemic cells. Currently, in non-small-cell lung cancer, γδT17 cells are associated as “protumour” cells
because IL-17 simulates VEGF production which results in angiogenesis\textsuperscript{186}. IL-17 has also been associated with angiogenesis in the context of other cancers, such as colon cancer\textsuperscript{171,187-189}. Additionally, IL-17 is contributes to angiogenesis in rheumatoid arthritis\textsuperscript{190}. Although angiogenesis is commonly considered a protumor function, it may be beneficial in the context of the neonatal, immune depletion of preleukemic cells. NK cells are likely to exert their cytotoxic effects through direct contact with preleukemic cells since we have shown depletion of preleukemia is IFNγ-independent. Angiogenesis may be required for the NK cells to properly survey, identify, and kill abnormal, preleukemic cells. Angiogenesis could also increase the bioavailability of other important cytokines required for NK cell activation and subsequent depletion. IL-17 has also been shown to act synergistically with several agents such as poly I:C (TLR 3 ligand), IL-22, TNF-α and Vitamin D3 to name a few\textsuperscript{191-194}. Therefore, IL-17 may also act synergistically, in the context of the neonatal immune response to infection, with an inflammatory cytokine to yield the observed antileukemic activity. More investigation is required, but the known functions of IL-17 support investigation into a cooperative or synergistic role of IL-17 in activating the NK cells required for the depletion of preleukemia. Although IL-17 when injected IP into neonatal mice did not activate NK cells, it did result in a modest increase (≈100\%) in the number of NK cells, suggesting IL-17 may be important for the proliferation of NK cells during a neonatal immune response to infection. This result compliments previous reports that IL-17 deficient mice have decreased NK cell counts\textsuperscript{195}. Additionally, this result further supports a cooperative role of IL-17 rather than a direct role in activating NK cells. My data support that the expansion of NK cells in response to IL-17 injections \textit{in vivo} is an indirect effect as no proliferative effects of IL-17 were observed \textit{in vitro} (see Figure 4.16). If IL-17 indirectly results in the proliferation of NK cells, it is possible that this
effect is masked, blocked or altered by the cytokine milieu present during infection. More investigation into the IL-17-pathway that mediates NK cell expansion is required.

Differences are known to exist between the neonatal and adult immune system\textsuperscript{196}. For example, neonates are known to have lower circulating levels of neutrophils compared to adults leading to weakened neutrophil responses\textsuperscript{197}. In another example, newborns have deficient Th1 immune responses and thus preferentially undergo Th2 responses and produce IL-23 instead of IL-12, compared to their adult counterparts\textsuperscript{198,199}. These differences highlight that it is possible IL-17 has a different, currently unknown, function in the context of neonatal immune responses. Since IL-17 and NK cells are required for the preleukemic depletion response, but the data presented suggests IL-17 does not directly activate NK cells, IL-17 may indirectly activate NK cells by stimulating another cell type to produce NK cell activating cytokines (see Figure 4.18 B). The candidate cytokines would be IL-2 and IL-15 as they are known to activate direct cell contact-NK cell cytotoxicity in an IFNγ-independent manner\textsuperscript{179}. IL-15 is the top candidate as it is known to be produced during the innate immune response compared to IL-2 which is more associated with the adaptive T cell immune responses\textsuperscript{200}. In our model, since NK cells are activated during the acute phase (within 72h) of a novel, neonatal infection, we suspect the innate immune response is more important than the adaptive response in yielding the observed antileukemic immune activity, and thus IL-15 is the top candidate for NK cell activation. Repeating the neonatal NK cell-enriched splenocytes killing assays, but culturing NK cells with only supplementary IL-2 or IL-15 (instead of both) could identify which cytokine is more important for generating neonatal NK cell antileukemic activity. Known producers of IL-15 include monocytes, dendritic cells (DCs), fibroblasts, bone marrow stromal cells, and epithelial cells\textsuperscript{201}. 
As immune cells, either neonatal monocytes or DCs would be the most promising candidates to produce IL-15 in response to IL-17. Since we have shown the antileukemic, neonatal immune response is IFNγ-independent (see Figure 4.14 A), neonatal DCs present as the more like producer of IL-15 since adult DCs can produce IL-15 in response to Type I IFNs, associated with intracellular infections. To summarize, in this possible explanation, in response to infection, IL-17 produced by γδT17 cells in response to infection acts on either neonatal DCs or monocytes (or another cell type) to produce NK cell activating cytokines, such as IL-2 or IL-15. These cytokines activate the antileukemic activity of neonatal NK cells resulting in the depletion of preleukemia. Alternatively, IL-17 may induce the production of an intermediate cytokine which then activates monocytes or DCs to produce IL-2 or IL-15. Overall, this theory is founded on the principle of a difference between the neonatal and adult in responsiveness to IL-17 and that IL-2 and/or IL-15 (or another cytokine that activates neonatal NK cells) is the final cytokine that leads to NK cell activation and subsequent preleukemic depletion. Determining if IL-15 and/or IL-2 neutralization aberrates the depletion response after infection would be an important experiment for determining if this explanation is reasonable; however, this experiment may be difficult as neutralization of these cytokines may also deplete NK cells. Additionally, since IL-17 alone does not lead to NK activation in vivo, a multifactorial response would be likely to required prime any IL-17 responders to directly or indirectly produce the NK cell activating cytokine milieu. Substantial investigation into this proposed indirect activation of NK cells by induced by infection and IL-17 is required.
Figure 4.18 Two possible mechanisms explaining the role of IL-17 in the murine antileukemic, early-life immune responses

A) A model depicting a cooperative cytokine/chemical that is required in addition to IL-17 to activate antileukemic NK cells. B) A model depicting IL-17 acting through an intermediate cell, subsequently leading to the activation of antileukemic NK cells.
4.4.4 IL-2/IL-15 mediated activation of neonatal NK cell cytotoxicity requires further investigation

In this study, IL-17 did not show any direct effects on NK cell activation or cytotoxicity, but neonatal NK cells stimulated with IL-2 and IL-15 do yield antileukemic activity in vitro. As sorted NK cells in response to infection also yield antileukemic activity, I hypothesize that infection leads to NK cell cytotoxicity through the activity of IL-2 and or IL-15. As previously mentioned, IL-15 is the top candidate as it is more associated with the innate immune response than IL-2. Two previously mentioned experiments will be important in determining the role of IL-15 in infection mediated depletion of preleukemia: i) in vivo neutralization of IL-15 during neonatal delivered infections and ii) performing NK-enriched splenocytes killing assays with only IL-2 or IL-15 as supporting cytokines in culture. If these experiments yield the hypothesized results: i) IL-15 neutralization aberrates the activation of NK cells and the neonatal, infection-induced depletion of preleukemia and ii) IL-15 alone is sufficient to induce NK cell anti-leukemic activity in vitro, the results would have important implications for the ongoing investigation and the advancement of ALL therapy.

Understanding the contribution of IL-17 in activating cytotoxic NK cells is important is because it provides insight on how to manipulate the mouse immune system to deplete preleukemia. Manipulating the mouse immune system to deplete preleukemia can then be translated to the human immune system and hopefully lead to new indications for pediatric ALL immunotherapies. If IL-15 (or IL-2) can activate NK cells to kill both preleukemic and leukemic cells, it would encourage evaluating IL-15 immunotherapy to treat pediatric ALL. IL-15 immunotherapy is already being explored and optimized for cancer and HIV patients, as well as
being tested in clinical trials. Additionally, one study has already shown that culturing of human neonatal NK cells from cord blood greatly enhances their cytotoxicity against the K562 cell line (a CLL cell line). Another study has shown that IL-15 activated human NK cells display cytotoxicity against B-ALL cells and primary ALL samples. Combined, these results provide evidence supporting that, if successful in mice, IL-15 immunotherapy could be translated to human pediatric ALL therapy. If we can use the Eμ-RET model to provide evidence that IL-15 immunotherapy can deplete both preleukemic and leukemic cells, it would further support further investigation of IL-15 immunotherapy for ALL. Even with the recent advancements with CAR T cell therapy, there are currently no standardized immunotherapies for ALL. If IL-15-activated NK cells can target leukemic and preleukemic cells, they could also potentially target ancestral ALL clones. An immunotherapy that targets ancestral clones in addition to leukemic cells should lower the risk of relapse. Thus, uncovering IL-15 (or another NK activating cytokine) as a potential immunotherapy would be of great benefit to the patient, and hopefully improve pediatric ALL therapy outcomes. With the potential implications surrounding IL-15 mediated activation of NK cells, further investigation into the contribution of IL-15 in activating NK cells in response to infection investigation is required.

4.4.5 Concluding Remarks

This study showed that S. cerevisiae cannot initiate neonatal, antileukemic immune responses and did not identify the function of γδT17 cells and IL-17 in generating these antileukemic responses. However, both results identify a need for ongoing investigation. A more comprehensive understanding of the way the microbiome influences the immune system and leukemogenesis can potentially lead to a better understanding of risk factors associated with
developing pediatric ALL. A better understanding of the role of neonatal γδT17 cells, IL-17, and NK cell activation in response to infection may lead to new immunotherapies that can target both leukemia and ancestral clones, theoretically reducing the risk of ALL relapse. Further investigation into both fields have the potential to improve ALL therapy and should be continued moving forward.
Chapter 5: Conclusion

5.1 Key Results

To address the need for improvements in ALL therapy, this thesis investigated the feasibility of using S. cerevisiae as a drug delivery vehicle for the ALL therapeutic L-ASNase, the ability of neonatal exposures of S. cerevisiae to induce immune responses that result in the depletion of preleukemic cells in Eμ-RET mice, and the role of γδT17 cells in contributing to neonatal, antileukemic activity in response to infection. L-asparaginase expressing yeast can kill asparagine synthetase-negative ALL cell lines in vitro, but the biomass required for therapeutic doses in vivo surpasses ethical limitations. Neonatal exposure to S. cerevisiae in Eμ-RET mice does not directly lead to the depletion of preleukemia. IL-17, alone, cannot directly activate the antileukemic potential of neonatal NK cells. Although negative results were obtained for these research topics, each study highlights a need for ongoing investigation.

5.2 Future Research

The large biomass required for AEY to yield a therapeutic dose of L-ASNase is currently the biggest barrier for using S. cerevisiae as a drug delivery vehicle. AEY can induce apoptosis in AS-negative ALL cell lines in vitro, so with a lower biomass, AEY could in theory improve L-ASNase therapy by lowering costs, toxicity, and immune complications. Since neonatal exposure to low doses of S. cerevisiae (less than 10^7 cfu) did not significantly increase the size of the spleens of Eμ-RET mice, the cell wall association of L-ASNase-II in yeast may indeed shield the drug from immune surveillance. At a minimum, the results support that a threshold exists at which S. cerevisiae cells do not induce an significant activation of the immune system, lowering the risk of side-effects such as the cytokine storm when using AEY. Moreover, since both adult
and neonatal *S. cerevisiae* exposures did not induce antileukemic immune activity, it is unlikely that *S. cerevisiae*-based drug delivery for ALL can also take on a duel role an immunotherapeutic that eliminate ancestral clones that persist during chemotherapy. Substantial investigation into the immunogenicity of AEY is still required to establish *S. cerevisiae* as a viable vehicle for drug delivery. Thus, any improvements in *S. cerevisiae* bioengineering that can increase the L-ASNase activity/biomass ratio should reopen investigation into utilizing yeast to deliver L-ASNase. Additionally, using *S. cerevisiae*-based drug delivery of other enzyme-based therapies for diseases such as Gaucher disease, Fabry disease and phenylketonuria should be explored as these diseases likely requires a lower activity/biomass ratio.

Further investigation into the role of microbiome in influencing the development of the mouse and human immune system is still required. Although neonatal exposures to *S. cerevisiae* do not directly lead to the depletion of preleukemia in Eμ-RET mice, early-life exposures to *S. cerevisiae*, or other microbiome organisms, may influence shaping the response of the immune system to early-life pathogens. Moreover, *S. cerevisiae* does not colonize the human gastrointestinal tract, but it is still believed to influence the dynamics of the microbiome. Since infection is a top candidate for influencing leukemogenesis, and our lab has showed that early-life, mild infections can induce the depletion of preleukemic cells, any role *S. cerevisiae* may have on influencing the development of the neonatal immune system and subsequent responses to infection is relevant. Additionally, neonatal exposures to other microbiome organisms, such as the commensal fungus *Candida albicans*, that may have a more direct role in inducing antileukemic, neonatal immune responses should be investigated. A comprehensive understanding of the role of the microbiome in leukemogenesis may uncover certain microbiome
profiles which put children at a greater risk for developing ALL. Hopefully, intervention through fecal microbiota transplantations or other means could reduce or eliminate the risk of developing ALL for these at-risk cohorts of children.

Our lab has shown that γδT17 cells and IL-17 is necessary to induce neonatal depletion of preleukemia in response to infection, yet both \textit{in vitro} and \textit{in vivo} results show that IL-17, alone, is unable to activate cytotoxic NK cells. Possible functions of IL-17 in the neonatal immune response include a cooperative or synergistic role in activating/enabling NK cell cytotoxicity or indirect activation of NK cells through another population of cells, such as neonatal monocytes or DCs. For both possible functions of IL-17, the multifactorial environment of the cytokine milieu present during infection is likely important since IL-17 alone is unable to activate NK cells. Understanding how to use IL-17 or downstream cytokines to manipulate the immune system to kill both leukemia and preleukemia can lead to new immunotherapeutic approaches for ALL. As the results presented suggest that IL-17 is a poor candidate for immunotherapy, ongoing investigation into the function of IL-17 and activation of NK cells in neonatal immune responses is essential. IL-15 presents as a top candidate for further investigation as it is a known activator of both human and murine NK cell cytotoxicity and is currently being investigated as a cancer immunotherapy. Identifying an immunotherapy that could target both leukemia and ancestral clones (preleukemia) could further improve survival rates and lower the chances of ALL relapse.
5.3 Concluding Remarks

Despite *S. cerevisiae* presenting as an organism that does not directly induce the depletion of preleukemic cells, IL-17, alone, being unable to directly activate neonatal NK cells, and *S. cerevisiae*, currently, being an unviable drug delivery vehicle for L-ASNase, these results all identify important areas for ongoing research. Investigation into the role of the microbiome in promoting or preventing leukemogenesis could lead to interventions, such as fecal microbiota transplantations that lower the risk of developing leukemia. Understanding the immunology of neonatal immune responses that generate antileukemic activity in response to infection may identify new immunotherapies indicated for ALL. Lastly, improvements in *S. cerevisiae* bioengineering could lead to *S. cerevisiae* being a viable drug delivery vehicle and possibly improve of the delivery for the ALL therapeutic L-ASNase. In conclusion, despite *S. cerevisiae* producing the negative results highlighted in this study, there are still several promising areas of research to further improve ALL outcomes\textsuperscript{13}.
References


Benzaïd I, Mönkkönen H, Bonnelye E, Mönkkönen J, Clézardin P. Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Trigger Vγ9Vδ2 T-cell


Appendices

Appendix A - Chapter 3 Flow Cytometry Gating

A.1 Initial gating for all samples

- Gate FSC-A\textsubscript{low}/FL2-A\textsubscript{hi} population to determine bead count (beads).

- Gate total cells using FSC-A\textsubscript{mid}/SSC-A\textsubscript{mid} cluster (Total Cells)

- In Total Cells, gate on single cells using FSC-H/FSC-A population (Single Cells)

- In Single Cells, gate on fixable viability dye (FVD) negative population (FVD\textsuperscript{-})

- Gate cell population of interest

- In population of interest, re-gate on viable cell population using FSC-A/SSC-A population to remove remaining cell debris (Total Alive Adjusted)
A.2 Asparaginase expressing yeast titrations

Identify viable cells (Appendix B.1)

In \textit{FVD}, gate human leukemic cells (HLA\textsuperscript{+})
A.3  Overnight, 2-day, and 5-day apoptosis and cell death assays

Identify single cells (Appendix B.1)

In *Single Cells*, gate human leukemic cells (HLA⁺)

In HLA⁺, gate dead cells (7-AAD⁺/AnnexinV⁺; Q2) and dead cells (7-AAD⁻/AnnexinV⁺; Q3)
A.4 Proliferation dye assays

Identify single cells (Appendix B.1)

In Single Cells, gate viable human leukemic cells (HLA\(^+\) \text{AnnexinV}\(^+\))

In HLA\(^+\) \text{AnnexinV}\(^+\), use FSC-A and SSC-A to gate out debris (Alive Adjusted)

In Alive Adjusted, obtain the mean fluorescent intensity of Proliferation Dye-ef670
Appendix B - Chapter 4 Flow Cytometry Gating

B.1 Initial gating for all samples

Use FSC-A and FSC-H to gate out doublets.

Use SSC-A and SSC-H to gate out doublets.

Gate 7-AAD/FSC-A\textsuperscript{med} population to identify viable splenocytes

Gate SSC-A\textsuperscript{hi}/FSC-A\textsuperscript{low} population to determine the number of counting bead events
B.2 Preleukemic cells in Eμ-RET mice

Identify single and viable cells (Appendix A.1)

Gate preleukemic cell (BP1⁺/B220⁻) population

Use RFP-negative mouse to adjust gate for preleukemic cells
B.3 γδ T cells

Identify single and viable cells (Appendix A.1)

Identify γδ T cells by gating CD3+/γδ TCR+ population

Identify γδ T cells by gating CD3+/γδ TCR+ population

Identify activated γδ T cells by gating CD69+ population

Phenotype γδ T cells by identify CD27+/CD44+ cells (Q1) and CD44+/CD27- cells (Q3)
B.4 Natural Killer cells

Identify single and viable cells (Appendix A.1)

Identify NK cells by gating CD49b⁺/CD335⁺ population

Identify activated NK cells by gating CD69⁺ population
B.5 Neutrophils

Identify single and viable cells (Appendix A.1)

Identify neutrophils by gating CD11b⁺/CDLy-6Ghi population
B.6  289-Luciferase-GFP Cells (NK cell in vitro killing assays)

Identify single and viable cells (Appendix A.1)

Identify 289 cells by gating on GFP⁺/BP-1⁺ population
Appendix C - Formulas

C.1 Formula 1 – L-asparaginase activity calculation

\[
\text{Asparaginase Activity} = \frac{[L - \text{ASN}]_{\text{initial}} - [L - \text{ASN}]_{\text{final}}}{\text{time}}
\]

C.2 Formula 2 – Determining the total number of cells of interest in a mouse spleen

Total cells of interest/spleen

\[
= \frac{\text{total beads in sample}}{\text{bead events}} \times \text{events in population of interest} \times \text{spleen multiplier}
\]

Spleen multiplier = \[
\frac{1}{\text{percentage of spleen stained for analysis on the flow cytometer}}
\]