THE IDENTIFICATION OF IMMUNE RESPONSES REQUIRED FOR DURABLE

CONTROL OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

by

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Abstract

Remarkable clinical successes have been achieved with targeted immunotherapies directed at a surface antigen of leukemia blasts in patients with relapse or chemotherapy-refractory B-ALL. This single antigen-targeted approach, however, is highly prone to tumor immune escape. Development of resistance to therapy, commonly caused by the emergence of target-negative escape variants, remains a major drawback of CD19-directed therapies for B-ALL. The efficacy of strategies that direct T cell-mediated cytotoxicity towards leukemic cells bearing non-immunogenic antigens is limited, with a lack of evidence that these interventions establish immunological memory. In this study, I use the Eµ-ret mouse model to better understand the limitations of current single antigen-targeted immunotherapies and to identify immune responses required for the achievement and, more importantly, maintenance of remission in childhood B-ALL.

My results uncovered the ability of target-directed therapy to elicit epitope spreading, enabling the generation of a secondary immune response against additional non-targeted leukemia-associated antigens that contributes to sustaining durable remission. Importantly, these results also suggest that such diversification of protective immune response is limited in an immunological setting where immune tolerance towards leukemia-associated antigens is established early in the course of leukemia progression. Furthermore, I have shown the ability of TLR agonist-mediated immune modulation to target leukemia cells in bone marrow, and induce durable immune control of primary B-ALL cells. Finally, I have demonstrated that NKT cells as a population is capable of influencing disease progression in the Eµ-ret mouse by playing a role in immunoediting.

Overall, these findings support that the generation of immune response with a broad specificity for range of leukemia-associated antigens contributes to the maintenance of remission. Furthermore, my results suggest that overcoming immune tolerance established against leukemia-associated antigens may be critical for maximizing the therapeutic benefits of immunotherapies for childhood B-ALL. Collectively, the therapeutic impact of innate immune modulation presented here in the context of B-ALL may contribute to the eradication of MRD, and thus reduce the risk of relaspse in MRD-positive patients.

Lay Summary

Acute lymphoblastic leukemia (ALL) is a type of blood cancer that occurs most commonly in children. Chemotherapy is currently the first-line treatment for ALL, but relapse or therapy-resistance remains a significant clinical challenge. For children with relapsed disease, an immune-based treatment approach offers a potentially curative option. Our immune system is capable of recognizing and generating protective immune responses against cancerous cells via a multi-step process. Defeats and/or suppression at one or more steps of this process, however, limits the optimal activation of such anti-tumor immune response, which is common in cancer patients. In this thesis, I identified immune activities that are necessary for the establishment and, more importantly, maintenance of remission in ALL. I also demonstrated how such long-term disease control can be achieved upon appropriate stimulation of immune system. Overall, this work provides a better understanding of the limitations of current immune-based treatment for childhood ALL.

Preface

A version of Chapter 3 has been published. **S. Jo**, J.H. Lee, J.J. Mattei, D.M. Barrett, P. van den Elzen, S.A. Grupp, G.S.D. Reid and A.E. Seif. (2017) Generation of multi-antigen-directed immune response for durable control of acute lymphoblastic leukemia. *Leukemia*. 32(2):539-542. Dr. Seif, Dr. Reid, and I formulated the hypothesis and designed the experiments. The Seif group (Dr. Seif, J.H. Lee, and J.J. Mattei) and I performed the experiments. Experiments in Figure 3.1b, Figure 3.3, Figure 3.5b were performed by the Seif group. Dr. Seif, Dr. Reid, and I conducted the data analysis and wrote the manuscript. Dr. D.M. Barrett and S.A. Grupp provided reagents. Dr. van den Elzen contributed to the interpretation of the experiments. Dr. Seif conducted the statistical analysis.

For Chapter 4, Dr. Reid and I formulated the hypothesis and designed the experiments. I performed the experiments. Dr. Reid and I conducted the data analysis and wrote a manuscript (in progress). Dr. van den Elzen contributed to the interpretation of the experiments. Dr. Seif assisted with statistical analyses.

For Chapter 5, Dr. Reid and I formulated the hypothesis and designed the experiment. I performed the experiments. Dr. Reid and I conducted the data analysis. Dr. van den Elzen contributed to the interpretation of the experiments.

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List of Abbreviations

ABL1	Abelson Murine Leukemia Viral Oncogene Homolog 1
ACT	Adoptive Cell Transfer
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADP	Antibody-Dependent Phagocytosis
AID	Activation-Induced Cytidine Deaminase
ALL	Acute Lymphoblastic Leukemia
Allo-HSCT	Allogeneic Hematopoietic Stem Cell Transplantation
AML	Acute Myeloid Leukemia
AML1	Acute Myeloid Leukemia (Also known as RUNX1)
APC	Antigen Presenting Cell
ARID5B	AT-Rich Interaction Domain 5B
B-ALL	B Cell Precursor Acute Lymphoblastic Leukemia
Bach2	Bric-a`-brac, Tramtrack and Broad Complex and Cap'n'collar Homology 2
BCG	Bacillus Calmette—Guerin
BCL6	B-Cell Lymphoma 6
BCP	B Cell Precursor
BCR	B-Cell Receptor
BCR	Breakpoint Cluster Region
BiTE	Bispecific T Cell Engager
BLNK	B-Cell Linker Protein (Also known as SLP65)
BTK	Bruton Agammaglobulinemia Tyrosine Kinase

CAR	Chimeric Antigen Receptor
CCND2	Cyclin D2
CD	Cluster of Differentiation
CD40L	Cluster of Differentiation 40 Ligand
cDC	Classical Dendritic Cell
CDC	Complement-Dependent Cytotoxicity
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CEBPE	CCAAT/Enhancer Binding Protein Epsilon
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CNS	Central Nervous System
CpG ODN	CpG Oligodioxynucleotides
CR	Complete Remission
CREB	Cyclic AMP-Responsive-Element-Binding Protein
CRi	Complete Remission with Incomplete Hematological Recovery
CRLF2	Cytokine Receptor Like Factor 2
CRS	Cytokine-Release Syndrome
CTA	Cancer Testis Antigen
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Associated Protein 4
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion

DMEM	Dulbecco's Modified Eagle Medium
E2A	E2A Immunoglobulin Enhancer-binding Factor E12/E47 (Also known as TCF3)
EAE	Experimental Autoimmune Encephalomyelitis
Ebf1	Early B-Cell Factor 1
EMA	European Medicines Agency
ERK	Extracellular Signaled-Regulated Kinase
ETV6	ETS Translocation Variant 6 (Also known as TEL)
FBS	Fetal Bovine Serum
Fcγ	Fc gamma
FDA	Food and Drug Administration
FOXO1	Forkhead Box Protein O1
GDH/GLDH	Glutamate dehydrogenase
GFP/luc	Green Fluorescent Protein and Firefly Luciferase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HER2	Human Epidermal Growth Factor Receptor 2
HLA	Human Leukocyte Antigen
HNSCC	Head and Neck Squamous Cell Carcinoma
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
IFN	Interferon
IFN-β	Interferon-beta
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma

Ig	Immunoglobulin
IKZF1	Ikaros Family Zinc Finger Protein 1
IKZF3	Ikaros Family Zinc Finger Protein 3
IL	Interleukin
IL-12R	Interleukin-12 Receptor
IL-2Rγ	Interleukin-2 Receptor Subunit Gamma
IL-3Rα	Interleukin-3 Receptor Subunit Alpha
IL-6R	Interleukein-6 Receptor
IL-7R	Interleukin-7 Receptor
IL-7Rα	Interleukin-7 Receptor Subunit Alpha
iNKT	Invariant Natural Killer T
Ip	Intraperitoneally
IRF3	Interferon Regulatory Factor 3
Iv	Intravenously
JAK	Janus Kinase
LIC	Leukemia-Initiating Cell
mAb	Monoclonal Antibody
MAMP	Microorganism-Associated Molecular Patterns
MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin Basic Protein
MCA	Methylcholanthrene
MEF2C	Myocyte Enhancer Factor 2C
MEF2D	Myocyte Enhancer Factor 2D

MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II
MLL	Mixed-Lineage Leukemia
MM	Multiple Myeloma
MPL	Monophosphoryl Lipid A
MRD	Minimum Residual Disease
mTOR	Mammalian Target of Rapamycin
MYC	Transcription Factor c-Myc
MyD88	Myeloid Differentiation Primary Response 88
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer
NKT	Natural Killer T
NOD/SCID	Nonobese Diabetic Severe Combined Immunodeficiency Mouse Strain
p14ARF	Alternative Reading Frame Protein Product of CDKN2A
PAP	Prostate Acid Phosphatase
Pax5	Paired Box 5 Transcription Factor
PBS	Phosphate Buffered Saline
PBX1	Pre-B-cell Leukemia Transcription Factor 1
PD-1	Program Cell Death 1
PD-L1	PD-1 Ligand 1
PD-L2	PD-1 Ligand 2

PDA	Pancreatic Ductal Adenocarcinoma
pDC	Plasmacytoid Dendritic Cell
Ph	Philadelphia
PI3K	Phosphoinositide 3-Kinase
PKB	Protein Kinase B
Poly I:C	Polyinosinic-Polycytidylic Acid
Poly-ICLC	Polyinosinic-Polycytidylic Acid with Carboxymethylcellulose and Poly-*L*-lysine
R/R	Relapse and/or Refractory
RAS	Rat Sarcoma Protein
RET	Rearranged During Transfection
RFP	Ret Finger Protein
rTCR	Recombinant T Cell Receptor
RUNX1	Runt-Related Transcription Factor 1
SCID	Severe Combined Immunodeficiency
SHP2	SH2 Domain-containing Protein Tyrosine Phosphatase 2
SLC	Surrogate Light-Chain
SLP65	SH2-Domain containing Leukocyte Protein of 65kDa (Also known as BLNK)
STAT5	Signal Transducer and Activator of Transcription 5
SYK	Spleen Tyrosine Kinase
TAA	Tumor-Associated Antigen
TAM	Tumor-Associated Macrophages
TCF3	Transcription Factor 3
TCR	T Cell Receptor

TEL	Transcription Factor TEL (Previously known as ETV6)
TGF-β	Transforming Growth Factor-Beta
Th	T Helper
TIL	Tumor-Infiltrating Lymphocytes
TIR	Toll/IL-1 receptor
TLR	Toll-Like Receptor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor Alpha
TP53	Tumor Protein 53
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
TRIF	TIR-Domain Containing Adaptor-Inducing Interferon-β
TSA	Tumor-Specific Antigen
TSLPR	Thymic Stromal Lymphopoietin Receptor
α-GalCer	Alpha-Galactosylcermide

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Dedication

This thesis is dedicated in loving memory of my grandfather,

Oh, Du Young

who valued lifelong learning.

Chapter 1: Introduction

1.1 Acute lymphoblastic leukemia

1.1.1 Overview

Acute lymphoblastic leukemia (ALL), a malignant disorder of immature lymphoid progenitors, is the most common pediatric cancer in developed societies. With over 80% of cases diagnosed between 1-16 years of age, ALL is predominately a disease of childhood [1]. In adults, ALL is less common than acute myeloid leukemia (AML), but represents a devastating disease with poorer prognosis than in children [1,2]. The hallmark of ALL is the sequential acquisition of chromosomal abnormalities and genetic lesions affecting pathways involved in differentiation, proliferation, and survival of lymphoid precursors [3–5]. Childhood ALL includes a number of subtypes defined by cell lineage (B cell or T cell), differentiation status, and cytogenetic profiles [4,6]. These subtypes differ by age distribution and clinical outcome. The incidence of the common or B cell precursor ALL (B-ALL) has a bimodal distribution with the first and second peaks occurring between 3-5 years of age and around 50 years of age, respectively [1,5]. ETS translocation variant 6 (*ETV6*)-runt-related transcription factor 1 (*RUNX1*) (also known as *TEL-AML1*) fusion and hyperdiploidy are the two most prevalent genetic alterations in pediatric B-ALL [6]. In rare cases, B-ALL presents in infants less than 1 year of age [7]. Over 80% of infant ALL cases are characterized by abnormalities in the mixed-lineage leukemia (*MLL*) gene (at 11q23) and represents a distinct leukemia with very poor prognosis [7–9]. The overall outcome for childhood ALL improved dramatically over the last five decades with advancements in efficacy of multi-agent chemotherapeutic regimens, the introduction of dose-intensification strategies based on improved risk-based stratification, and the recognition of sanctuary sites and

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incorporation of central nervous system (CNS)-directed prophylactic treatment [5,10]. Despite

achieving a significant increase in the 5-year event-free survival rate from less than 10% in the

1960s to 90% today for pediatric B-ALL, primary refractory and relapse disease remains a major

clinical challenge [5,11]. If not specified, the term 'B-ALL' will be used to refer to childhood B-

ALL with the peak incidence occurring between 3-5 years of age, which will be the main focus

for the remainder of thesis.

1.1.2   B cell development and leukemogenesis

1.1.2.1   B cell development

B cells derive from pluripotent hematopoietic stem cells (HSC) in bone marrow through a

stepwise developmental process tightly regulated by hierarchical activation of transcription

factors and selection through functional signal transductions [8]. First, HSCs differentiate into

multipotent progenitor (MPP) cells, then common lymphoid progenitors (CLP), prior to

committing to the B-cell lineage [12]. Then, from pre-pro-B cells, B cell progenitors sequentially

mature to pro-B cells, large pre-B cells, small pre-B cells, immature B cells, and finally mature B

cells [12].

At the MPP stage, the activation of transcription factors Ikaros (encoded by *IKZF1*) and

E2A are essential for promoting lymphoid-lineage-specific gene expression [13,14]. The early B-

lineage development initiates upon activation of B-lineage transcription factors Ebf1 and Pax5

downstream of E2A [15,16]. During the pro-B cell stage, signaling pathways downstream of IL-7R

initiate the rearrangement of immunoglobulin (Ig) heavy-chain variable (V), diversity (D), and

joining (J) gene segments, and a productive rearrangement results in the expression of IgM

heavy-chain [17,18]. The association of a functional IgM heavy-chain with non-polymorphic

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'surrogate' light-chain (SLC) components (λ5 and VpreB) forms the pre-B cell receptor (BCR), which enables the transition to the large pre-B cell stage [17,19]. This transient expression of a pre-BCR is associated with a proliferative burst of pre-B cells with a functional IgM heavy-chain, followed by cell cycle exit and the subsequent progression to the small resting pre-B cell stage [12]. Upon entry into the small resting pre-B cell stage, Ig light-chain V-J gene segments rearrange to enable BCR expression on the surface [19]. The acquisition of a functional BCR permits the transition to the immature B cell stage, allowing for positive selection of non-autoreactive B cells in the bone marrow [12]. Mature B cells further differentiate into various subsets (follicular B cells, marginal zone B cells, and germinal center B cells) in the periphery, where these cells depend on tonic BCR signaling for their survival [19,20]. The activation and clonal expansion of naïve B cells involve distinct signals via cytokine receptors and co-stimulatory receptors, in addition to antigen-induced BCR signaling, in the secondary lymphoid organs [19].

1.1.2.2   Cytokine receptor signaling and pre-BCR checkpoint control

B cell malignancies have been associated with a differentiation block at distinct stages of B cell development. In B-ALL, the B cell developmental process is arrested at the large pre-B cell stage. During this stage, a productively assembled pre-BCR transiently drives a clonal expansion of IgM heavy-chain[+] large pre-B cells prior to entry into the resting small pre-B cell stage, which requires the downregulation of SLC expression and the cessation of cell cycle progression [19,21]. Therefore, the expression of pre-BCR serves as the first antigen-independent checkpoint controlling the clonal selection, proliferation, and the subsequent maturation of large pre-B cells [19,22].

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Signaling pathways downstream of IL-7R (heterodimer of IL-7Rα and the common-γ chain or TSLPR) are the JAK-STAT5 and PI3K signaling cascades [23–26]. Binding of phosphorylated STAT5 together with Ebf1 on the *Pax5* promoter activates Pax5 transcription [27], which then induces the rearrangement of Ig heavy-chain and activation of pre-BCR checkpoint [28,29]. At the pre-BCR checkpoint, the majority of cells are eliminated through negative selection, unless they are rescued by survival signals emanating from a productively assembled pre-BCR [18]. The absence of a functional pre-BCR triggers the upregulation of *Bach2*, which in turn activates CDKN2A (p14ARF)- and TP53-induced apoptosis of pre-B cells carrying non-functional Ig heavy-chain rearrangements [29]. In contrast, activation of the transcription repressor BCL6 via pre-BCR provides pro-survival and pro-proliferative signal by suppressing *CDKN2A* and *TP53*, enabling the positive selection of IgM heavy-chain$^+$ pre-B cells for further differentiation [30,31]. Oncogenic lesions in B-ALL allow the evasion of this important cell-autonomous proliferation switch during early B cell development by altering the normal cytokine receptor and pre-BCR signaling pathways in favor of the leukemia development [18,32].

1.1.2.3   Evasion of pre-BCR checkpoint control

While the transcriptional repressor Bach2 is activated by Pax5, phosphorylation of Bach2 by PI3K-PKB-mTOR pathway maintains its cytoplasmic localization, preventing transcriptional activity [29,33]. Frequently, the expression of *Bach2* is downregulated in B-ALL, which is commonly associated with *Pax5* deletions, translocations, or mutations [29,34,35]. As a result, malignant clones bypass the clonal deletion mediated at the pre-BCR checkpoint, maintaining pro-survival and pro-proliferative signals.

In pre-BCR signaling, the non-receptor tyrosine kinases, SYK and BTK, are activated

downstream of PI3K and phosphorylate the adaptor protein SLP65 (also known as BLNK) [36].

SLP65 is a key cell cycle regulator that controls the transition from large cycling into small

resting pre-B cells to limit the expansion of large pre-B cells [37]. Therefore, alterations in pre-

BCR signaling via the SYK-SLP65 and SLP65-BTK axes are also evident in B-ALL [36].

Phosphorylated SLP65 downregulates the expression of SLC and IL-7R, which in turn

terminates cell proliferation and induces Ig light-chain rearrangement. Loss of *SLP65* expression

leads to constitutive activation of the JAK3-STAT5 signaling pathway, driving uncontrolled IL-

7-independent proliferation and survival at the large pre-B cell stage [38] Similarly, phosphorylated

SLP65 inhibits the activation of PKB, thereby promoting the nuclear translocation of FOXO1 [39].

FOXO1 then targets BCL6 to inhibit transcription of CCND2 and MYC, which results in

termination of proliferation and expansion prior to transition to resting small pre-B cells [31,40,41].

Inactivating mutations in SLP65 found in some cases of B-ALL release the inhibition of PKB

and JAK3, which prevents FOXO1 from activating via nuclear translocation. In the absence of

BCL6-mediated cell cycle arrest, proliferating large pre-B cells are unable to exit from cell cycle,

which is a prerequisite for the transition to the small resting pre-B cell stage [37].

RAS-ERK signaling downstream of pre-BCR is also critical for the positive regulation of

cell cycle prior to progression to small pre-B cells [42]. Activated by RAS, ERK promotes

transcription of ELK1 and CREB target genes, *MYC* or *MEF2C* and *MEF2D*, respectively,

which consequently induces cell proliferation [43,44]. Hyperactivation of the RAS-ERK pathway, as

a result of gain-of-function mutations in RAS or SHP2 (encoded by *PTPN11*), is most frequently

observed in hyperdiploid B-ALL [45,46] and at relapse [46,47].

1.1.3 Genetic basis of B-ALL

Acquired cytogenetic abnormalities are the hallmark of B-ALL [48]. Aneuploidy and recurring structural chromosomal rearrangements are important initiating events in leukemogenesis, but insufficient to establish the full leukemic phenotype [4,5]. These initiating genetic events results in perturbation of key cellular processes and contribute to the leukemic transformation of lymphoid progenitors by maintaining or enhancing unlimited capacity for self-renewal, subverting the controls for normal proliferation, blocking differentiation, and promoting resistance to death signals [5,48]. Then, the subsequent acquisition of secondary genetic events leads to clinical manifestations of leukemia [4] (Figure 1.1). Importantly, distinct subtypes and prognosis of disease are defined based on cytogenetic profiles [4,48].

Hyperdiploidy (>50 chromosomes) is one of the most frequent cytogenetic alterations in B-ALL, occurring in approximately 30% of cases [49,50]. It is characterized by a nonrandom gain of multiple chromosomes, mainly trisomies but also frequently tetrasomies [50,51]. On the other hand, monosomies and gain of more than 2 copies of a chromosome are exceedingly rare [50]. The gains may involve any chromosome, but more than 70% of cases of hyperdiploid display +X, +4, +6, +10, +14, +17, +18, or +21 [49]. Despite the high frequency of this subtype of B-ALL, the pathogenic consequences of the chromosomal gains remain largely unknown [49,50]. Clinically, however, hyperdiploid cases are associated with favorable outcome [52]. While most of the other subtypes of B-ALL are associated with specific chimeric genes or mutations, hyperdiploid cases harbor relatively few genetic abnormalities besides the extra chromosomes [50]. Aneuploidy in itself, therefore, appears to be the main driver event in hyperdiploid B-ALL with a high incidence of mutations in RAS signaling pathway and histone modifiers [50].

Hypodiploidy (<46 chromosomes) is rare, occurring in 5% to 8% of B-ALL cases, and is a strong negative prognostic factor [53,54]. It is characterized by multiple whole chromosomal losses, but hypodiploid genomes may undergo reduplication, resulting in a hyperdiploid karyotype ("masked" hypodiploid ALL) [55]. Hypodiploid ALL can be further subdivided into high hypodiploidy (40-45 chromosomes), low hypodiploidy (33-39 chromosomes), and near haploidy (23-29 chromosomes) [53]; the majority of patients with hypodiploid ALL have 45 chromosomes, and low hypodiploidy and near-haploid ALL are extremely rare (<1% of B-ALL) [53,54]. Near-haploid cases are seen primarily in pediatric patients, whereas low hypoploidy occurs in all ages [53]. Near-haploid cases harbor loss of heterozygosity resulting from monosomies or uniparental isodisomies for the majority of the chromosomes, but display a non-random retention of both parental copies of chromosomes X/Y, 14, 18, and 21 [54]. The pathogenic impact of such preferential heterodisomy remains unclear [54]. Near-haploid ALL is also characterized by chromosomal gains and, therefore, may be mistaken for a hyperploid ALL [53,54]. Most cases of near haploid ALL harbor mutations targeting Ras and receptor tyrosine kinase signaling, and often have deletion of the lymphoid transcription factor gene *IKZF3* [55].

Chromosomal rearrangements common in B-ALL disrupt genes that regulate normal hematopoiesis and lymphoid development, activate oncogenes, or constitutively activate tyrosine kinases by creating chimeric fusion genes [48,52]. The most common rearrangement in B-ALL (15 to 25% of cases) is the t(12;21)(p13;q22) chromosomal translocation that encodes *ETV6-RUNX1* (*TEL-AML1*), which results in the fusion of two hematopoietic transcription factors essential for normal hematopoiesis [5,56,57]. The expression of *ETV6-RUNX1* causes disordered early B-lineage lymphocyte development and promotes self-renewal and survival in B cell progenitors [58,59]. Generally, *ETV6-RUNX1*-positive ALL is associated with excellent prognosis [52,59]. The

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t(1;19)(q23;p13) translocation encodes a chimeric transcriptional factor *E2A-PBX1* (*TCF3-PBX1*) and is present in up to 6% of B-ALL cases[52]. This subtype is also associated with excellent prognosis, but has a higher risk of CNS relapse [52]. The expression of the *E2A-PBX1* fusion gene activates aberrant expression of *WNT-16*, which leads to the induction of an autocrine WNT/β-catenin pathway in progenitor B cells [60,61]. Such *WNT-16*-mediated growth signaling enhances proliferation and survival of B cell precursor (BCP) cells that bypasses the pre-BCR checkpoint control [19]. The t(9;22))q34;q11) translocation is observed in 3 to 5% of pediatric cases, but is the most frequent chromosomal rearrangement in adults [52]. This translocation forms the so-called Philadelphia (Ph) chromosome encoding *BCR-ABL1*, which results in a constitutive tyrosine kinase activity that provides a pro-survival signal by mimicking a constitutively active pre-BCR [62–64]. *BCR-ABL1*-positive ALL is associated with a poor prognosis mainly due to genetic instability induced, most likely, by aberrant expression of the mutator enzyme AID, which introduces DNA single-strand breaks [65]. More recently, a novel high-risk subtype of B-ALL exhibiting a gene expression profile similar to that of Ph-positive ALL, but without the *BCR-ABL1* rearrangement has been identified in up to 15% of cases [66]. The new *BCR-ABL1*-like cases harbor a diverse range of rearrangements, deletions, and sequence mutations that activate cytokine receptor and kinase signaling [67]. Deletion of *IKZF1*, rearrangements of *CRLF2*, and mutation of JAKs are commonly shown in BCR-ABL1-like cases, which results in maturation arrest [66,68,69].

Self-renewal

HSC

Multipotent
Progenitors

MPP

Ikaros (*IKZF1*)
E2A

Oligopotent
Progenitors

IL7R/CRLF2

CLP

Self-renewal
Developmental arrest

**Initiating
translocations
(in utero 'first-hit')**

Proliferation
Epigentic reprogramming

Ebf1
Pax5

IL7R/CRLF2

Pre-pro B

IL7R/CRLF2

Pro-B

Lymphoid signaling
Transcriptional signaling
Tumor suppressors

**Secondary
mutations
(after birth)**

IgH rearrangement

IL7R/CRLF2   pre-BCR

Kinase-Ras-PI2K signaling
Transcriptional coactivators
Chromatin remodeling

Committed
Precursors

Large
pre-B

pre-BCR

Small
pre-B

IgK or IgL rearrangement

BCR

Immature
B

BCR

Mature cells

Mature B

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Figure 1.1. Schematic diagram of B cell development and sequential acquisition of genetic alterations contributing to leukemogenesis

The self-renewing hematopoietic stem cell (HSC) gives rise to multipotent progenitors (MPP), which has the ability to differentiate into common lymphoid progenitors (CLP). Early B-lineage development begins with the CLP to pre-pro-B cell transition, then sequential maturation to pro-B cells, large pre-B cells, small pre-B cells, immature B cells, and finally mature B cells. 'First-hits', commonly translocations, are acquired in a lymphoid progenitor *in utero*. Additional acquisition of secondary mutations after birth contributes to an arrest in lymphoid development and perturbation of multiple cellular pathways, leading to the development of clinically overt leukemia.

1.1.4   Clonal evolution

1.1.4.1   *In utero* origin of B-ALL

Leukemogenesis is driven by clonal evolution, a process in which random mutations accumulate to create genetic diversity and sequential selections allow the expansion of subclones harboring advantageous phenotypes [2,70]. In B-ALL, genetic alterations which result in self-renewal ability, developmental arrest, and/or enhanced capacity for proliferation or survival, provide a clonal advantage and drive the emergence of covert leukemia-initiating pre-leukemic clones [71]. Acquisition of cytogenetic abnormalities, an important disease-initiating event, occurs *in utero* [72]. The oncogenic events induced by chromosomal lesions ('first-hit'), however, are insufficient to generate a fully transformed leukemic clone. B-ALL, therefore, has a clinically silent natural history prior to diagnosis, requiring postnatally acquired secondary genetic hits to drive clinical manifestations of the disease [59]. Commonly, these cooperative genetic lesions or

'second-hit' are sequence mutations and structural genetic alterations in genes involved in cell cycle control or B cell differentiation [59,73].

Several cytogenetic abnormalities can be detected in neonatal blood samples years prior to diagnosis and are retained at relapse [72,74]. Retrospective studies of monozygotic twins with concordant leukemia revealed the presence of a shared clone with a leukemia-specific chromosomal fusion (*ETV6-RUNX1*) in archived neonatal blood samples, indicative of a common fetal cell of origin for the subsequently developed leukemia [72,75]. While these twin pairs shared an identical and singular fusion gene event, other genetic alterations present, such as copy number alterations and single nucleotide variants, were different [76,77]. Importantly, the concordance rate in identical twins with the *ETV6-RUNX1* fusion is only about 10-15%, with variable incubation period and clinical outcomes [78]. Moreover, the analysis of identical twins with discordant disease showed the presence of shared *ETV6-RUNX1*$^{+}$ pre-leukemic clones in the 'healthy' co-twin. These covert pre-leukemic clones, however, lacked the additional genetic and/or epigenetic lesions that were found in the other sibling with overt leukemia carrying the same *ETV6-RUNX1* fusion [58]. In line with this finding, *ETV6-RUNX1* is detected in cord blood samples in 1% of unselected newborn babies, a frequency 100 times higher than the prevalence of clinical *ETV6-RUNX1*$^{+}$ B-ALL later in childhood [6,79]. The observations in discordant, monozygotic twins and neonatal blood spots suggest that most individuals carrying a covert pre-leukemic clone do not progress to overt ALL later in life. Collectively, these observations support the idea of prenatal disease-initiating cytogenetic alterations and the independent postnatal acquisition of additional cooperative genetic lesions that are required to drive overt or clinically evident disease [6].

1.1.4.2   Clonal origins of relapse

Recent genome-wide analyses revealed significant genetic diversity between leukemia clones [80–82]. In addition to the predominant diagnostic clone, multiple leukemia subclones are present at low levels at diagnosis [80–82]. Under therapy-induced selective pressures, this subclonal diversity evolves over time by acquiring additional genetic alternations, which lead to the eventual loss of diagnosis-specific lesions [80,83]. Hence, the relapse clone, which is commonly distinct from the predominant diagnostic clone, may emerge from cells ancestral to the primary leukemia [83].

A large genome-wide DNA copy number analysis investigating the evolutionary clonal relationship between matched diagnostic and relapse samples of B-ALL determined the approximate frequency of each of four identified pathways to relapse [83]. While less than 10% represented a genetically distinct *de novo* leukemia clone that emerged independent of diagnostic clone, over 50% of relapse clones evolved from an ancestral subclone that was present as a minor population at diagnosis, prior to the initiation of therapy. Approximately 30% of relapse clones arose directly from the predominant diagnostic clone that survived and acquired additional genetic lesions during treatment. The remainder, less than 10%, represented relapse clones that were identical to the matched diagnostic clone, were incompletely eliminated during initial treatment, and re-expanded without acquiring new genetic lesions.

Overall, B-ALL is highly likely to originate from a pool of long-lived pre-leukemic clones, which are descendants of a common fetal cell of origin, that evolve into malignant clones over time by independently acquiring secondary genetic events. As this pre-leukemic cell population acts as a reservoir of most relapse clones, therapies targeting both the dominant diagnostic clone and pre-leukemic clones may potentially reduce the incidence of relapse disease.

1.2 Experimental models of B-ALL

1.2.1 Overview

Mouse models have been widely utilized as models for the study of human diseases, including cancer. Numerous murine models that are highly representative of their human counterpart have been developed, each with their advantages and disadvantages [84]. Accordingly, careful consideration and selection of the most appropriate models are extremely important for developing *in vivo* studies. Currently, the three commonly utilized types of murine model of B-ALL are human xenografts, syngeneic adoptive transfer, and transgene-driven leukemogenesis.

1.2.2 Xenograft and syngeneic models

In human B-ALL xenograft models, human hematopoietic and/or leukemia blasts are transplanted into mice with different levels of immunodeficiency, often variants of severe combined immunodeficiency (SCID) mice, to avoid immune rejection of the human cells [85]. Xenograft models are useful to validate *in vitro* findings, as well as to evaluate therapeutic responses of patient-derived leukemia to drugs [84,85]. While the lack of an intact immune system allows interspecies engraftment of cells, such immunological deficits impede the use of xenograft models for determining the influence of immune system on ALL progression. Syngeneic models, on the other hand, allow the study of leukemia in immunocompetent hosts. This model involves genetic modification of ALL-associated genes in either primary mouse BCP cells or appropriate murine cell lines with subsequent transplantation into syngeneic recipient mice [86]. Although these models are driven by a genetic modification common in human ALL, additional germline alterations that may be irrelevant to the "second-hit" in human disease are

required to fully drive leukemogenesis [86]. Syngeneic models, therefore, cannot fully model the contributions of host microenvironment to leukemogenesis.

1.2.3   Transgenic mouse models

Multiple attempts have been made to develop genetically engineered mouse models that mimic the characteristic two-hit process of B-ALL leukemogenesis. Commonly, an ALL-associated transgene is introduced into the BCP compartments in an effort to induce the development of spontaneous disease. The *ETV6-RUNX1*-driven transgenic mouse model of leukemia, the most common subtype of B-ALL, has been rather difficult to obtain as the expression of this fusion transgene was not sufficient to drive the disease in several approaches [87,88].

The Eμ-ret mouse carries a RFP/RET fusion transgene under the transcriptional control of Ig heavy-chain enhancer [89]. This transgene, which contains the amino terminal end of the transcriptional activator RFP and the tyrosine kinase domains of the RET proto-oncogene, has a high penetrance of a late pro-B cell stage ALL in the Eμ-ret mice between 3 and 12 months of age [89,90]. The expression of the transgene, which acts as the disease-initiating event, begins *in utero*, as in children with B-ALL. Normally, RET is transiently expressed during the early development of B cells [91], but the constitutive expression of the RFP/RET transgene inhibits the normal elimination of late pro-B cells with nonproductive Ig heavy-chain rearrangements [89,90]. This leads to the selective expansion of an abnormal late pro-B cell population that is detectable in the fetal liver. Thus, the ability of RFP/RET transgene to enhance both proliferation and survival creates a high-risk population of late pro-B cells that subsequently acquire additional mutation before becoming fully transformed [89,90]. Over time, this pre-leukemic cell or leukemia-

initiating cell (LIC) population further expands and circulates throughout the body prior to the onset of overt disease, which is manifested in the development of high white cell counts, adenopathy and/or hepatosplenomegaly. While mutations in RET are frequently detected in human thyroid papillary carcinomas [92] and multiple endocrine neoplasia [93], its oncogenic activation, as in the Eµ-ret mice, is not commonly associated with the leukemogenesis of human B-ALL [94]. However, as the fetal origin of pre-leukemic cells in Eµ-ret mice mimics the two-step model of B-ALL leukemogenesis, this allowed for the investigation in the presence of an intact immune system.

In 2015, a novel transgenic mouse that conditionally expresses the *E2A-PBX1* fusion oncogene, which is presented in 5% to 7% of B-ALL cases, under the control of Cre-driving promoter (*Cd19*, *Mb1* or *Mx1*) was developed [95]. The incidence of B-ALL in these mice ranges from 5% to 50% depending on the promoter used to induce the expression of the fusion transgene [95]. Similar to *E2A-PBX1*-positive ALL in humans, the oncogenic activation of *E2A-PBX1* fusion transgene in B cell progenitors in this model leads to the acquisition of secondary genetic events, including the spontaneous loss of Pax5, required for the full transformation. Unlike the Eµ-ret transgenic model, two distinct but highly similar subtypes of B-ALL displaying a maturation arrest at the pro-B/pre-B stage of BCP differentiation are induced in the *E2A-PBX1* transgenic model [95].

1.3 Established treatments for B-ALL

1.3.1 Contemporary multi-agent chemotherapy

The recognition of B-ALL as a heterogeneous disease with multiple biologic subtypes led to tailoring of treatment intensity based on risk stratification to achieve a high cure rate, while limiting excessive toxicity and development of resistance [96,97]. Contemporary standard-of-care multi-agent chemotherapy for B-ALL typically spans 2-2.5 years and consists of four key phases: remission-induction, intensification (consolidation) with integration of CNS-directed treatment, re-induction, and continuation (long-term maintenance) [10,96,97]. Currently, minimal residual disease (MRD) is the most powerful prognostic indicator in B-ALL [98,99]. MRD is defined by a submicroscopic leukemia blast count of <5% (morphological remission), but not <0.01% (molecular remission) in the bone marrow after the restoration of normal hematopoiesis at the end of remission-induction therapy [100].

The first goal of chemotherapy is to eradicate initial blasts to achieve complete (morphological and molecular) remission (CR) with restoration of normal hematopoiesis in 96-99% of pediatric patients [11,96,97]. The backbone of chemotherapy agents administrated during 4 to 6 weeks of remission-induction therapy typically includes a glucocorticoid (prednisone or dexamethasone), vincristine, and asparaginase, with or without anthracycline (doxorubicin or daunorubicin) [11,96,101]. Anthracycline is often omitted for lower-risk patients, but not for high-risk patients, in an effort to reduce toxicity [101]. While this regimen followed by intensified post-remission therapy is usually sufficient for treating standard-risk B-ALL, patients with high- or very high-risk disease often receive additional drugs during remission-induction therapy [11,97]. For those with remission-induction failure, which is a rare but highly adverse event in children, the

eligible patients usually undergo allogeneic hematopoietic stem cell transplantation (allo-HSCT) [102].

Once patients in remission restore normal hematopoiesis and body function, a 6- to 9-month-long intensification therapy begins [97,103]. This phase aims to eradicate drug-resistant submicroscopic residual leukemia blasts (systemic MRD burden) to reduce the risk of relapse and to prevent relapse in CNS, which is a sanctuary site that has been considered difficult to penetrate with systemic chemotherapy [10,97,103]. Thus, combinations of chemotherapeutic agents, usually including agents that are different from those used in the remission-induction therapy, are used in this phase to minimize drug resistance. Commonly, intensification therapy involves both intrathecal and systemic administration of high-dose methotrexate plus 6-mercaptopurine with frequent pulses of vincristine and glucocorticoid, and uninterrupted high-dose asparaginase [10,103].

Re-induction therapy has become an integral phase of contemporary chemotherapeutic regimens as the addition of this treatment has significantly reduced the risk of relapse [103,104]. Essentially, re-induction therapy is a repeat of the remission-induction therapy using agents similar to those used during remission-induction and intensification therapy [103].

The final continuation therapy is the longest phase of treatment regimens, with a much less intensive regimen than the previous treatment phases to further reduce the risk of relapse [96]. This phase typically lasts at least 2 years and involves daily and weekly administration of methotrexate and 6-mercaptopurine, respectively, with or without pulses of vincristine and dexamethasone [11,97].

1.3.2 Allogeneic hematopoietic stem cell transplantation

In general, allo-HSCT, the most intensive form of contemporary treatment for B-ALL, is reserved for a subset of patients with very high-risk of relapse and/or remission-induction failure [102,105]. Contemporary allo-HSCT protocols have significantly reduced relapsed-related mortality, procedure-associated toxicity, and infection [106]. The role of allo-HSCT, however, has become controversial, especially with the ongoing development of effective targeted therapies. Although *BCR-ABL1*-positive B-ALL has been traditionally classified as high-risk, the incorporation of tyrosine kinase inhibitors (i.e. imatinib and dasatinib) to multi-agent chemotherapy has dramatically improved outcome, comparable or superior to allo-HSCT [107,108]. Furthermore, a level of MRD 10^{-4} prior to HSCT is strongly associated with relapse; therefore, new strategies are need for pre- and/or post-transplantation reduction of MRD burden [109,110].

The immune-mediated graft-versus-leukemia (GVL) effect has been well-recognized as the central component of curative potential of allo-HSCT for relapsed and/or refractory (R/R) B-ALL, highlighting the ability of the human immune system to mount clinically effective anti-leukemia immune response [111]. GVL effects involve the direct elimination of blasts by donor lymphocytes via the recognition of major and/or minor histocompatibility antigens, in addition to leukemia-associated antigens, expressed on leukemia cells, together with the subsequent induction of broader innate and adaptive immune activities [112,113]. The strength of GVL effect has been confirmed to be linked to the incidence and the severity of graft-versus-host disease (GVHD), which is the major life-threatening complication following allo-HSCT [111,114,115]. Similar to the mechanism of GVL activity, the donor-derived lymphocytes mediate GVHD by recognizing allo-antigen expressed on normal host tissues, leading to organ damage and dysfunction. Various strategies for enhancing the anti-leukemia activity of GVL effect, or

preventing GVHD while preserving GVL effect have been explored extensively [112,116], yet the challenge of separating GVHD from the beneficial effects of GVL effect still remains in spite of nearly three decades of efforts [112]. Currently, much effort is focused on leukemia-specific immunotherapeutics that do not rely on the manipulation of alloimmunity for therapeutic efficacy.

Subsequently, the use of donor lymphocyte infusion (DLI) post-transplantation further provided direct evidence for a critical role of immune cells in therapeutic anti-tumor responses. The successes in DLI led to substantial excitement in the growing field of adoptive cell transfer (ACT) therapy. DLI involves harvesting of leukocytes from the peripheral blood of transplant donors and then infusing back into transplant recipients, either prophylactically or as treatment for relapse, to boost the number of potent anti-tumor immune effectors [113]. DLI has shown a remarkable success in relapsed chronic myeloid leukemia (CML), whereas its therapeutic benefit is modest in acute leukemias, with resultant CRs far less common [117,118].

1.4 New generation of immune-based treatments for cancer

1.4.1 Generation of anti-tumor immunity requires a multi-step cyclic process

The generation of anti-tumor immunity requires the initiation of a series of stepwise events that allows for amplification and broadening of tumor-specific T cell responses [119]. Importantly, such responses must be maintained long enough to successfully eradicate cancer cells [119]. This cyclic process, referred as the Cancer-Immunity Cycle, has seven distinct steps that must be achieved, either spontaneously or therapeutically, to elicit an effective anti-tumor immune response [119,120]. The first step starts with the uptake of neoantigens and/or tumor-associated antigens by dendritic cells (DCs) for processing (step 1). Upon antigen encounter,

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DCs must receive immunogenic signals that promote their maturation, which allows for enhanced processing and presentation of tumor-antigen-derived peptides, as opposed to promoting peripheral tolerance to the tumor antigens [119,120]. These activation or maturation signals include proinflammatory cytokines and factors released by dying or necrotic tumor cells [119]. Next, DCs migrate to lymphoid organs to present the captured antigens on major histocompatibility complex class II (MHC-II) and class I (MHC-I) molecules to CD4$^+$ and CD8$^+$ T cells (signal 1), respectively, and provide co-stimulatory signals (signal 2) (step 2) [121]. This results in the priming of naïve T cells, and subsequent activation and proliferation of effector T cells with reactivities directed to neoantigens or tumor-associated antigens to which immune tolerance has been incompletely established (step 3) [119,120]. At this stage, the balance between immune-stimulatory and -inhibitory factors, coupled with the ratio of T effector cells versus T regulatory (Treg) cells, play critical roles in determining both the nature and final outcome of the immune response being generated [119]. During the effector phase, the activated tumor-specific effector T cells traffic to (step 4) and infiltrate the tumor bed (step 5). Finally, these effector T cells, CD8$^+$ cytotoxic T lymphocytes (CTLs) in particular, specifically recognize and bind to cognate antigens bound to MHC-I at the surface of tumor cells via their T cell receptors (TCRs) (step 6) and kill the target tumor cells (step 7) [119,121]. Dying or dead tumor cells release additional tumor-associated antigens (step 1), which enables maintenance and broadening of tumor-specific T cell responses in the ensuing rounds of the cycle [119].

Each step of the Cancer-Immunity Cycle is tightly controlled by both positive and negative regulators. While stimulatory factors promote active tumor immunity, inhibitory factors keep the process in check to prevent autoimmunity and/or dampen or arrest active immune activity [119]. In cancer patients, immune tolerance to tumor-associated antigens and/or the

immunosuppressive tumor microenvironment (TME) are significant barriers to optimal performance of the Cancer-Immunity Cycle, limiting the robustness and duration of desirable anti-tumor immune responses [122].

1.4.2   Cancer immunotherapy

1.4.2.1   Overview

The unprecedented recent clinical successes of immunotherapies are changing the treatment options for advanced-stage cancers [123]. Cancer immunotherapy is an immune-based treatment approach that generates or augments tumor immunity [124]. The goal of cancer immunotherapy is to break immune tolerance/inhibition and initiate or re-initiate a self-sustaining cycle of cancer immunity described above, enabling it to amplify and persist while avoiding the induction of severe autoimmune side effects [119]. Broadly, current immunotherapies are designed to target the following four distinct nodes of vulnerability in the cancer-immune relationships: (1) direct targeting of cell-surface tumor antigens; (2) activating tumor antigen-specific immunity; (3) enhancing the quality and quantity of immune effectors; (4) overcoming inhibitory immune suppression [125].

1.4.2.2   Direct targeting of cell-surface tumor antigens

Hematological malignancies have provided the initial proof-of-concept that targeting a surface tumor antigen with an antibody is sufficient to effectively eliminate tumor cells. In 1997, rituximab became the first monoclonal antibody (mAb) approved by the Food and Drug Administration (FDA) for the treatment of a human cancer, relapsed or refractory, low-grade (indolent) or follicular, $CD20^+$ non-Hodgkin's lymphoma [126]. Rituximab, which since has

become a standard component in the clinical management of B cell malignancies, is a genetically engineered chimeric anti-CD20 IgG1 mAb [126,127]. The target antigen CD20 is a cell-surface protein expressed almost exclusively on mature B cells, but absent on terminally differentiated plasma cells [126]. Considerable efforts over the ensuing decades have led to the development of numerous additional FDA-approved tumor-specific mAbs, including alemtuzumab (CD52), daratumumab (CD38), trastuzumab (HER2), and bispecific antibodies [120,125]. Presently, mAbs represent an extremely valuable and successful class of cancer immunotherapy for the treatment of various solid tumors, as well as hematological malignancies [128,129].

Tumor-specific mAbs have several potential mechanisms of action to kill tumor cells, both directly and indirectly. Direct actions of the antibody include blockade of receptor/ligand interactions with interruption of growth signals, agonistic activity, and induction of apoptosis via activation of a death signal, and delivery of a drug or cytotoxic agent [128,130]. Indirect killing is commonly immune-mediated, via immune effector mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent phagocytosis (ADP), and complement-dependent cytotoxicity (CDC) through the engagement of Fc gamma (Fcγ) or complement receptors on natural killer (NK) cells, macrophages, and neutrophils [125]. Antibody-induced tumor cell lysis can enhance uptake and cross-presentation of tumor antigens by DCs, leading to the generation of adaptive immune responses [128,130].

The development of bifunctional mAbs that harness the cytolytic potential of T cells have provided another mAb-based approach to overcome the limitations of an immunosuppressive TME [125]. This so-called bispecific T cell engagers (BiTEs) have dual specificities to a tumor surface antigen and to the CD3 signaling complex on T cells [125]. By physically linking T cells to tumor cells, BiTEs allow T cells to activate and proliferate, irrespective of their natural antigen

specificity, and target tumor cells via granzyme- and perforin-mediated tumor cytotoxicity [131].

As the efficacy of BiTEs depends on passive recruitment of CTLs to the tumor milieu and

subsequent tumor cell lysis, presence of functional immune effector cells is crucial for anti-tumor

activity [125].

1.4.2.3 Activating tumor antigen-specific immunity

The striking discovery that non-viral human tumor cells bear antigens that can be

recognized by autologous T cells in the context of MHC molecules provided the possibility of

developing therapeutic cancer vaccines to boost such pre-existing spontaneous tumor-specific

responses and/or induce *de novo* activity [132,133]. One of the major challenges to the development

of effective cancer vaccines is identifying the optimal antigens to use. Currently, tumor antigens

are defined under three broad classifications: (1) tumor-specific antigens (TSAs); (2) tumor-

associated antigens (TAAs); (3) cancer testis antigens (CTAs). TSAs are novel immunogenic

neoantigens uniquely expressed by malignant cells, but not encoded in normal host genome, that

arise as a consequence of tumor-specific somatic mutations [134]. Because TSAs are not subject to

central tolerance, this class of tumor antigens constitutes an attractive targets with minimal risk

of inducing autoimmunity [135]. On the other hand, TAAs are proteins encoded in the normal

genome as differentiation antigens or aberrantly expressed normal proteins that are also

overexpressed on tumor cells [132,135]. As TAAs are normal proteins to which immunological

tolerance have been established, the T cell repertoire specific to these antigens is limited [136].

While TAAs generally have lower TCR affinity compared to TSAs, generation of autologous T

cell immune responses against these self-antigens nevertheless run the risk of inducing serious

autoimmune toxicity [136]. Finally, CTAs are encoded by germline genes which have normal

expression predominantly in testis, fetal ovaries, and trophoblasts, but are aberrantly expressed in a large range of human cancers [135,137]. Central tolerance towards CTAs is incomplete due to their highly restricted tissue expression pattern, therefore, this class of tumor antigens are immunogenic and have considerable potential to be used as tumor vaccines [137].

The search for optimal targets for immunotherapy is ongoing and among the plethora of tumor antigens that have been identified, vaccines for non-viral cancers have largely utilized TAAs and CTAs that are common to a particular cancer type [138,139]. Although the majority of high-affinity self-reactive T cells are likely to be eliminated through the mechanisms of central tolerance, an adequate T cell repertoire remains to be exploited for therapeutic purposes [136,139,140]. Nevertheless, loss or downregulation of antigen expression, dysfunctional antigen processing and presentation, and difficulty maintaining robust post-vaccine responses can greatly limit the efficacy of therapeutic cancer vaccines irrespective of the source of target epitopes (e.g. tumor antigens, peptides, or whole-tumor cells) [122]. So far, sipuleucel-T (Provenge®) is the first and the only therapeutic cancer vaccine approved by the FDA, which is for the treatment of metastatic, asymptomatic stage IV prostate cancer [141,142]. Sipuleucel-T is an autologous DC-based cancer vaccine that targets the prostate differentiation antigen prostate acid phosphatase (PAP), in which the clinically meaningful benefit is an increase in overall survival (average of 3-4 months) rather than tumor eradication [141]. Overall, the lack of cancer eradication is explained by the suboptimal vaccine designs due to the general lack of understanding of the mechanisms of immunization, especially the role of DCs, and the presence of an immunosuppressive TME [120,139]. With the recent advances in next-generation sequencing and *in silico* epitope prediction, the identification of TSAs has been transformed from time consuming and laborious to a more rapid process [143]. Currently, TSA-based vaccines utilizing target antigen in the form of DNA, RNA, or synthetic

long peptides are emerging designs of therapeutic vaccines with improved capacity to induce both CD4^{+} and CD8^{+} effector and memory T cell responses [139].

1.4.2.4 Enhancing the quality and quantity of immune effectors

Building on the successes of DLI in the management of post-transplant leukemic relapse, as aforementioned, various strategies of ACT therapy have been developed to better expand and increase the number of functionally competent anti-tumor effector cells [125,130]. The concept of ACT is to generate robust immune-mediated anti-tumor responses by (re)introducing a large number of activated tumor-specific T cells that exhibit unimpeded anti-tumor function, thereby harnessing the natural ability of T cells to specifically recognize and eliminate target cells [144]. ACT involves isolation of T cells from tumor-bearing patients, *ex vivo* expansion, and manipulation of these autologous tumor-specific T cells, either naturally occurring or genetically engineered, and subsequent reinfusion into the patient. [145–147].

ACT therapy using tumor-infiltrating lymphocytes (TILs) can induce long-lasting complete regression of metastatic melanoma, which has high number of spontaneous tumor-specific lymphocytes [147,148]. In this case, TILs are T cells that were isolated from surgically resected metastatic tumor deposits and *ex vivo* expanded in the presence of IL-2 for optimal activation prior to reinfusion [148]. The impressive high response rates and durable complete regressions following a single infusion of TILs for melanoma have demonstrated the curative potential of tumor-specific T cells for advanced cancer in human [148]. This approach, however, is logistically complex and requires large surgical samples in order to isolate and expand sufficient number of TILs for reinfusion, but most importantly tumors enriched with tumor-specific T cells [148]. Owing to the characteristic presence of spontaneous T cell priming and immune infiltration

into tumor sites [149–151], the application of TIL therapy has been effective against melanoma, but not other forms of cancer [152].

To extend the benefits of ACT to other types of cancer that lack spontaneous immune activation and are devoid of TILs, genetic engineering has been employed to modify circulating lymphocytes to exhibit potent anti-tumor activity by redirecting specificity of these cells to tumor antigens with high affinity [146,147]. To date, the most striking success of ACT therapy has been achieved with anti-CD19 chimeric antigen receptor (CAR) T cells for the treatment of B-ALL and lymphoma (discussed in detail later) [146,153]. CARs are genetically engineered receptors with a single-chain variable fragment of mAb as an extracellular antigen recognition domain, linked to the intracellular signaling domains of the TCR and a co-stimulatory signaling domain, including but not limited to CD28, 4-1BB, and/or OX40. Upon binding to their target antigen, CAR transduced T cells get activated and proliferate *in vivo*. CAR T cells, therefore, are designed to redirect their specificity to and induce cellular cytotoxicity against cells expressing target antigen independent of MHC restrictions [154,155]. In principle, CAR T cell therapy is applicable to any cancer for which suitable cell-surface target antigens have been identified. The choice of target antigen recognized by the CAR is critical for both the efficacy and safety of CAR T cell therapy [156]. Ideally, the target antigen should be expressed exclusively on tumor cells and not on normal cells to prevent or minimize the risk of on-target, off-tumor toxicity [153]. For example, CD19 is expressed on most B cell malignancies, but also on normal B cells [157]. Long-term toxicity of B cell aplasia, therefore, is an expected on-target, off-tumor activity of CD19-targeted CAR T cells therapy, but is tolerable [146] as patients are clinically managed with life-long infusions of immunoglobulin [125]. In addition to on-target, off-tumor toxicity, there remain several major challenges to the broad application of CAR T cell therapy: (1) loss of target antigen

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expression leading to development of resistance; (2) modest clinical benefit in solid tumors due to an immunosuppressive TME that is suboptimal for T cell function and lack of targetable sufficiently specific tumor surface antigens; (3) difficulty of industrialization due to labor intensive and expensive nature of autologous T cell manufacturing [124,156].

1.4.2.5   Overcoming inhibitory immune suppression

The FDA approval of anti-cytotoxic T lymphocyte associated protein 4 (CTLA-4) antibody, ipilimumab, and anti-program cell death 1 (PD-1) antibodies, pembrolizumab and nivolumab, for the treatment of metastatic melanoma in 2011 and 2014, respectively, was a milestone in the development of cancer immunotherapy [140]. Importantly, the clinical successes achieved with ipilimumab in patients with late-stage metastatic melanoma provided the proof-of-concept for the therapeutic benefits of targeting immune checkpoints to modulate endogenous anti-tumor T cell responses [158]. By the mid-90s, the complexity of T cell activation was much better understood. By then, it was realized that the activation of T cell-mediated immune responses occurs in parallel with the activation of T cell-intrinsic inhibitory pathways called immune checkpoints to prevent excessive and uncontrolled immune responses [140]. The mechanisms of immune checkpoints operate at the cell surface of T cells via immune-checkpoint receptors, including, but not limited to, CTLA-4 and PD-1, to suppress T cell function and dampen T cell immune responses [159].

CTLA-4 is expressed exclusively on T cells and acts early in T cell activation in secondary lymphoid organs [160,161]. CTLA-4 shares the ligands CD80 and CD86, expressed on APCs, with CD28, a constitutively expressed T cell co-stimulatory receptor [160,161]. Upon activation following the co-stimulatory signals, CTLA-4 translocates to the surface of T cells to

basically counteract the activity of CD28 by binding to CD80 or CD86 expressed by APCs with a much higher affinity than CD28 [160,162]. Although its mechanism of action is the subject of considerable debate, the expression of CTLA-4 effectively limits the activation of T cells by preventing TCR/CD28 co-ligation by outcompeting CD28 for CD80 and CD86 [160] and possibly by depleting these ligands via *trans*-endocytosis [163]. The development of fetal autoimmune disorders caused by massive lymphoproliferation in CTLA-4-deficient mice revealed the critical negative regulatory role of CTLA-4 in T cell activation and expansion [164,165]. The loss of CTLA-4, therefore, suggested its central role in regulating autoimmunity and peripheral tolerance, thereby constraining pre-existing T cell-mediated anti-tumor activity as well [166–168]. Additionally, via constitutive expression on Treg cells [169], CTLA-4 also facilitates the key role of Tregs in maintaining peripheral tolerance and immune homeostasis [170,171].

PD-1 is another well-studied immune-checkpoint receptor expressed on antigen-stimulated T cells [172]. In contrast to CTLA-4, PD-1 acts later in the T cell response. The central role of PD-1 is to suppress the effector T cell responses in peripheral tissues at the time of an inflammatory response to infection in order to limit T cell-mediated collateral tissue damage and autoimmunity [159,173,174]. PD-l has two known ligands that are expressed on the surface of target cells in response to local inflammatory cytokines, in particular interferon-gamma (IFN-γ) [175]. PD-1 ligand 1 (PD-L1) is expressed on a wide range of cell types, including activated hematopoietic cells, epithelial cells, and tumor cells; PD-1 ligand 2 (PD-L2) is predominately expressed on activated APCs [172,176]. Unlike CTLA-4, the ligation of PD-l on the cell surface interferes with signaling downstream of TCR to block T cell proliferation and to attenuate cytokine release and cytotoxic effector activity, which ultimately renders T cells anergic [177–179]. The inhibitory effects of PD-1 can be overcome by providing co-stimulatory signal through

CD28 or indirectly through IL-2, which augments cytokine production and cell survival [179].

Normally, the expression of PD-1 is transiently induced following immune activation. Chronic antigen exposure, in the context of chronic viral infections and cancer, however, can lead to persistently high levels of PD-1 expression [180,181]. Excessive expression of PD-1 on T cells can induce a state of reversible immune dysfunction termed exhaustion [181,182]. Besides PD-1, PD-L1 can also bind to CD80 expressed on T cells to deliver additional inhibitory signals to induce and maintain T cell exhaustion [180,183]. High expression levels of PD-L1 on malignant cells, evident in most melanoma, ovarian, and lung cancers, are recognized as an important immune-evasive strategy that can functionally impair tumor-specific T immune responses [159,184,185].

The use of blocking or antagonist mAbs, such as anti-CTLA-4 mAb and anti-PD-1 mAbs, to overcome tumor-induced negative regulation in the context of triggering anti-tumor immune responses is termed "checkpoint blockade" [161,186]. When effective, the use of checkpoint blockade, especially against solid tumors with high mutation loads [124,187], can restore existing endogenous anti-tumor immunity, inducing long lasting clinical responses [188–190]. This therapeutic effect, however, fundamentally relies on the presence of spontaneous T cell priming and infiltration of endogenous tumor-specific T cells into tumor sites, prior to treatment, with potential to mount anti-tumor immune activity in response to such T cell-stimulatory therapies [149,191]. So far, immune checkpoint inhibitors have translated to durable anti-tumor effects only in a small subset of metastatic melanoma patients as monotherapies, with the response rates ranging from about 20% for anti-CTLA-4 mAb to under 45% for anti-PD-1 mAb [188]. The clinical application of immune checkpoint inhibitors in hematological malignancies is currently under investigation [192]. Owing to the presence of relatively low number of potentially immunogenic somatic mutations, B-ALL has a rather low neoantigen burden [124,193]. Neither the incidence of

neoantigen presentation nor the presence of endogenous leukemia-specific T cells is likely be high in B-ALL, therefore, the use of immune checkpoint inhibitors alone may be inadequate to induce meaningful therapeutic effects [124].

1.4.3   Novel immunotherapies for relapse and/or refractory B-ALL

Hematological malignancies are at the forefront of the effective application of immunotherapeutic approaches, particularly tumor-targeted mAb-based and ACT therapies [124,125]. In 2017, three groundbreaking and distinct immunotherapies for R/R B-ALL received full FDA and/or European Medicines Agency (EMA) approval: inotuzumab ozogamicin, blinatumomab, and tisagenlecleucel [194]. So far, targeted immunotherapies directed at surface antigens of leukemia blasts have shown remarkable efficacy in patients with R/R B-ALL.

Inotuzumab ozogamicin is an anti-CD22 mAb conjugated to calicheamicin, a potent DNA-binding cytotoxic anti-tumor antibiotic [195]. The target, CD22, is a cell-surface glycoprotein expressed in more than 90% of patients with B-ALL and is not shed into the extracellular matrix [195–199]. Upon binding to CD22, the conjugate complex is rapidly internalized and calicheamicin is released [195]. Calicheamicin binds to the minor groove of DNA and induces double-stranded breaks, leading to subsequent apoptosis [200]. Overall, a significantly higher CR and CR with incomplete hematological recovery (CRi) were achieved with inotuzumab ozogamicin compared to standard intensive chemotherapy[201]. Both progression-free and overall survival were significantly longer with inotuzumab ozogamicin [201]. Veno-occlusive liver disease, however, was the major adverse event associated with inotuzumab ozogamicin [201]. The rate of veno-occlusive liver disease was especially higher in patients who had undergone HSCT before or after receiving inotuzumab ozogamicin [201,202]. Fortunately, with an unprecedented increased in the

number of newly approved therapies for R/R B-ALL, treatment options alternative to inotuzumab ozogamicin are available for patients with a high baseline risk of veno-occlusive liver disease. In June 2017, inotuzumab ozogamicin received EMA approval as monotherapy for the treatment of adults with CD22-positive R/R B-ALL and adults with Ph-positive-CD22-positive R/R B-ALL who have failed treatment with at least one tyrosine kinase inhibitor [203].

Blinatumomab is a bispecific CD19-directed CD3 T-cell engager antibody construct [204]. Upon binding, blinatumomab links $CD3^+$ cytotoxic T cells with $CD19^+$ B cells, which enables the patient's endogenous T cells to induce perforin-mediated death of the $CD19^+$ target cell [204,205]. Expressed in more than 90% of B-ALL blasts, CD19 is a B-lineage specific cell-surface signaling protein expressed from the earliest stages of B cell development until being lost on maturation to plasma cells [199,206]. Similar to inotuzumab ozogamicin, blinatumomab significantly improved the CR rate (34% versus 16%) and median overall survival (7.7 months versus 4.0 months) compared to standard-of-care chemotherapy [207]. Since receiving accelerated approval for the treatment of Ph-negative-R/R B-ALL in December 2014 [208,209], blinatumomab received full FDA approval for the treatment of both children and adults with R/R B-ALL, including adults with Ph-positive B-ALL, in July 2017 [194,210].

The landmark FDA approval of tisagenlecleucel in August 2017 for the treatment of R/R B-ALL in children and young adults (≤25 years) marked the culmination of advances in protein and genetic engineering [194,211]. Becoming the first genetically engineered ACT therapy to be approved by the FDA, tisagenlecleucel, is an autologous CD19-directed CAR T cell therapy [194,212,213]. CR rates, with MRD-negative bone marrow, as high as 90% have been reported with tisagenlecleucel [214–217]. In the pivotal ELIANA clinical trial of tisagenlecleucel, the relapse-free probability at 6 months was 75%, while the overall survival at 6 months was 89% and 79% at 12

months [217]. Supraphysiologic T cell proliferation, a hallmark of CAR T cell therapy, contributes to both efficacy and two common and potentially life-threatening toxicities, cytokine-release syndrome (CRS) and neurotoxicity [155,215,217–219]. Aggressive supportive care is necessary for all patients experiencing CAR T cell therapy-associated toxicities, where early management and treatment of hypotension and concurrent infections, respectively, are critical [220]. Anti-IL-6R antibody tocilizumab was approved simultaneously with tisagenlecleucel for the treatment of CAR T cell-associated CRS [194,217,221,222]; and systemic corticosteroids are reserved for neurotoxicities and CRS not responsive to tocilizumab [220].

1.4.4 Relapse after CD19-directed CAR T cell therapy

Despite the remarkable clinical outcomes, relapse after tisagenlecleucel remains a challenge and two main patterns of relapses, either CD19-positive or CD19-negative, have been reported [155,223]. Typically, CD19-positive relapses, which retain the surface expression of CD19, result from poor expansion and persistence of the engineered T cells [157,215,224]. CR can be obtained in CD19-postive relapses with additional infusion of CAR T cells [216,224], therefore, further optimization of CAR T cell therapy to prolong T cell persistence can potentially reduce the incidence of this type of relapse significantly. On the other hand, CD19-negative relapses are not associated with loss of CAR T cells, but rather with loss of the target epitope [214,215]. Several novel mechanisms of resistance driving the emergence and/or enrichment of CD19-negative escape variants have been uncovered: (1) deletion or mutation of *CD19* locus [224]; (2) selection for pre-existing alternatively spliced CD19 isoforms lacking the CAR-recognized epitope [206,224]; (3) clonal evolution and outgrowth of pre-existing rare CD19-negative clone [225,226]; (4) induction of lineage-switching [227].

Targeting a single antigen inevitably carries the risk of introducing selection pressures that lead to the outgrowth of escape variants, which consequently enables the development of resistance to therapy. Therefore, novel strategies to offset antigen loss, including simultaneously targeting multiple antigens on blasts, are currently under investigation [155,156,223]. CAR T cells co-targeting CD19 and CD123 (IL-3Rα) have been reported to both treat and prevent antigen loss in clinically-relevant preclinical model of CD19-negative leukemia escape [225]. CD20 and CD22 are two other promising targets being evaluated for dual targeting strategies to avoid CD19-negative escape [156,223,228]. Although the concept of this dual targeting approach is straightforward, further validation is required prior to clinical application. With a limited pool of clinically validated antigens and the constraint of suitable epitope selection, pairing epitopes that can be both safely and effectively targeted with CAR T cells may prove challenging [229]. Importantly, if the mechanisms of resistance emerge from leukemia blasts adapting to strong selective pressure of therapy, targeting two antigens may be insufficient to avoid escape variants, especially considering the high clonal heterogeneity of B-ALL [223].

1.5   Strategy to induce a broad therapeutic anti-tumor immune response

1.5.1   Overview

Currently, immunotherapeutic strategies exploiting effector T cell functions are in the spotlight for their capacity to directly recognize and eliminate tumor cells, holding tremendous potential for advanced-stage cancer treatment beyond hematological malignancies [159,230]. Elimination of cancer cells by T cells, however, is only one step in the multi-step process of the Cancer-Immunity Cycle [119], as described earlier. The majority of patients do not respond to cancer immunotherapy, while relapse occurs among the few who respond [231]. The so-called

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"magic bullet", therefore, has not yet been found, despite considerable global efforts since the term was first coined by Paul Ehrlich in 1900 [232]. As a promising alternative to directed- or T cell-based therapies, non-specific immunostimulatory strategies that broadly target multiple immune cell types simultaneously may allow for the induction of *de novo* and/or reactivation of pre-existing endogenous anti-leukemia immune responses directed to diverse range of TAAs, including leukemia-associated antigens. This broad, polyclonal anti-tumor immune response may ultimately overcome immune tolerance against leukemia-associated antigens, as well as prevent tumor antigen escape in the setting of surface antigen-targeted immunotherapies.

Systemic administration of high-dose IL-2 demonstrated that a purely immunostimulatory manipulation of autologous T cells is sufficient to mediate complete durable regressions of metastatic melanoma and renal cancers in the mid 1980's [233]. Treatment with high-dose IL-2, however, not only has low response rates, but is also associated with a significant risk of serious systemic inflammation, greatly limiting its broad application [148,234]. Similarly, IFN-alpha (IFN-α) monotherapy, which induces modest anti-tumor activity in patients with metastatic melanoma, has limited utility in the treatment of stage IV melanoma due to low response rates and the cumulative toxicities [234]. Therefore, new strategies capable of inducing broad immune effects, including optimally coordinated release of multiple cytokines, with a minimal risk of high toxicities may offer both effective and universally applicable immunotherapeutic approach for achieving durable control of a wide range of cancers.

In essence, the generation of effective anti-tumor responses needs to reach two primary phases: the priming phase that leads to the activation of *de novo* tumor-specific T cells; and the effector phase that leads to the recognition and elimination of cancer cells by these tumor-specific T cells [119,121]. The identification of an inflamed microenvironment containing infiltrating

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T cells in many cancer patients fueled the development of therapeutic strategies to overcome dominant effects of tumor-mediated immune suppression [235]. Meanwhile, a lack of spontaneous immune activation is observed in a subset of patients with a non-T cell-inflamed TME, which is devoid of T cell infiltrate and often characterized by the absence of innate immune engagement [151]. Therefore, approaches that stimulate or enhance certain features of adaptive immune system have been a main focus of immunotherapy with only more recent approaches targeting innate immunity. Interestingly, powerful synergistic effects have been observed in a number of combination immunotherapies that target both innate and adaptive immunity [236–238]. These studies indicated that the engagement of rapidly responsive innate immunity in concert mediates a front-line defense against tumor, providing a supportive window for tumor-specific and effector memory responses to be successfully initiated, but also plays an important role in promoting and amplifying such tumor-eradicating immune responses. Therefore, leveraging the potent immunostimulatory capacity of innate immunity that complements and/or enables tumor-specific adaptive immune responses is gaining a renewed interest, especially in the context of combination immunotherapy, leading to novel strategies to elicit a more integrated immune response against cancer [237,239–241].

1.5.2 Targeting Toll-like receptors in cancer immunotherapy

TLRs are the best characterized family of germline encoded pattern recognition receptors that play an important role in initiating the first line of host defense against invading pathogens [242,243]. These receptors have broad specificities for a range of evolutionarily conserved small molecular motifs derived from various microbial pathogens, including bacteria, viruses, protozoa, and fungi, called microorganism-associated molecular patterns (MAMPs), [242–244]. The

ligation of TLRs activates the downstream signaling pathway that leads to the production of

proinflammatory cytokines, type 1 IFNs, potent antimicrobial factors, and chemokines involved

in leukocyte recruitment, thereby inducing inflammatory and antimicrobial innate immune

responses [245–247]. Importantly, TLR signaling mediates key innate immune events that bridge to

antigen-specific adaptive immunity. TLR-mediated functional maturation of APCs, particularly

DCs, induces increased expression of co-stimulatory molecules (e.g. CD40/CD80/CD86) and

MHC-II molecules and production of inflammatory cytokines, such as IL-12 [242,248]. Once

matured, DCs acquire enhanced antigen-presenting capacity, while losing their capacity for

endocytosis, and migrate from the peripheral tissues to draining lymph nodes to prime naïve T

cells for the subsequent generation of antigen-specific adaptive immune response [242,249].

Although the majority of known TLRs direct the development of T helper type 1 (Th1)-polarized

immune responses, the phenotypes of effector T cells that differentiate from naïve T cells are

influenced greatly by the pattern of cytokines induced by the specific TLR ligations [250] (Figure

1.2).

The positive correlation between infection and spontaneous tumor regression has been

reported since the beginning of the 18th century [251]. The anti-tumor effects of microbial products

were further recognized in with a relatively effective use of Coley's toxin, a mixture of killed

Streptococcus pyogenes and *Serratia marcescens* [252,253]. Nonetheless, the use of Coley's toxin

has been discontinued in the early 1960s, mainly in the wake of the controversial thalidomide

case (thalidomide was withdrawn 11 years after its approval due to its highly teratogenic activity)

[254,255]. It was later discovered that Coley's toxin mediates its therapeutic effects by activating

TLR2 and TLR4 [256]. It is now well-understood that TLRs recognize not only MAMPs, but also

non-microbial molecules, termed damage-associated molecular patterns (DAMPs) [257]. These

host-derived endogenous ligands are released or exposed by stressed, dying or dead cells, including cancer cells, during infection or cellular injury to convey a danger signal, and trigger non-infectious inflammatory responses [239,242,257–260]. Thus, the interplay between cancer-derived DAMPs and TLR represents an alternative approach for the induction and/or enhancement of therapeutically relevant anti-tumor immune responses.

The ability to enhance DC maturation via upregulation of co-stimulatory signals, leading to increased cross-priming of antigen-specific T cells, is the key function that has led to harnessing TLRs in cancer immunotherapy [261]. Accumulating evidence supports that TLRs contribute to overcoming tolerance to tumor antigens and improve anti-cancer immunity, without the need for increasing antigenicity, by enhancing immunogenicity and/or the susceptibility to immune attack [260–263]. Importantly, strategies for targeting TLRs to trigger the activation of tumor-specific adaptive immune responses offer a non-targeted approach with potential for diverse application in various forms of malignancies, as pre-identification of target or tumor antigens is not required.
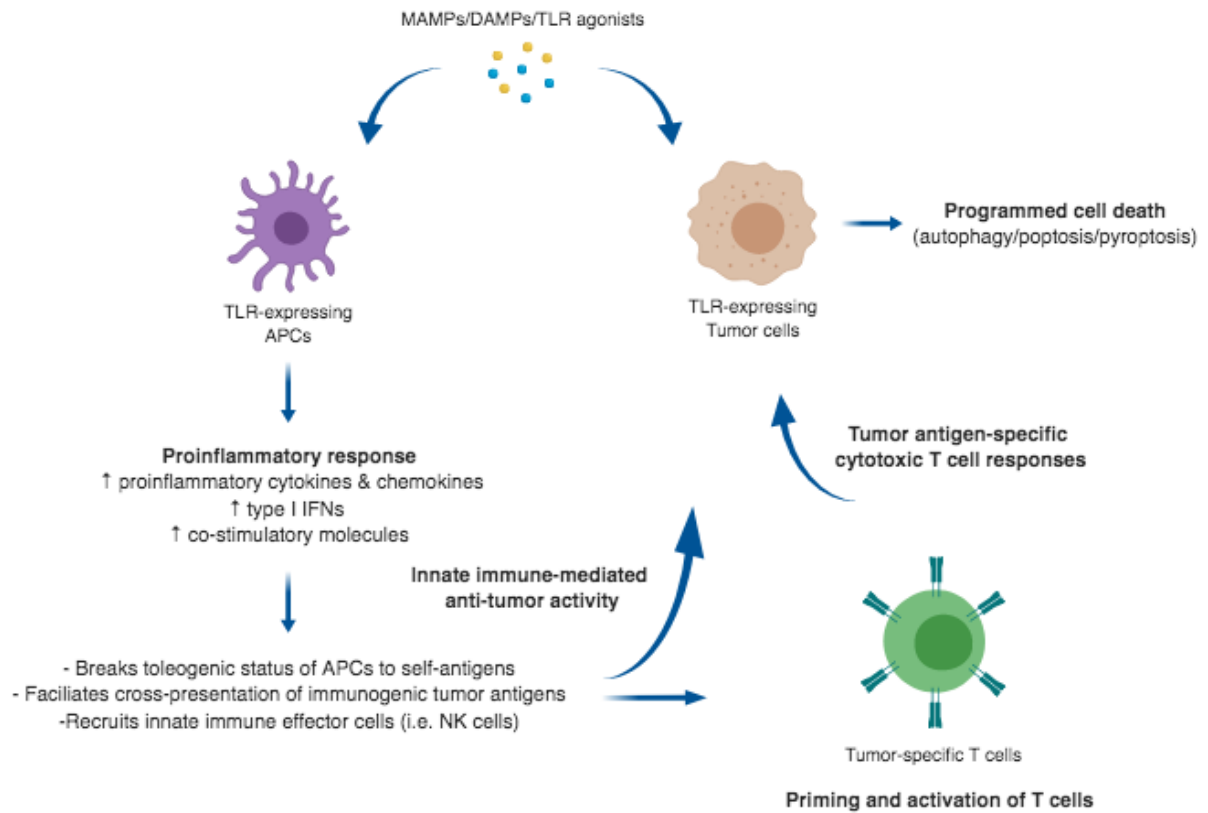
Figure 1.2. The role of TLRs in anti-tumor immunity

Upon the binding of PAMPs, DAMPs, or TLR agonists, activated TLRs induce proinflammatory response, which subsequently leads to the activation of tumor antigen-specific T cell responses, and/or programmed cell death in cancer cells.

1.5.2.1   Structure and signaling of Toll-like receptors

Presently, 10 human TLRs (TLR1-TLR10) and 12 murine TLRs (TLR1-9, TLR11-13), have been identified [244,264,265]. TLRs are type I transmembrane proteins each composed of an ectodomain with leucine-rich repeats for the recognition and binding of ligands, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain for initiating signal transduction [266]. TLRs that recognize lipid or protein ligands are expressed on the cell surface (TLR1-2, TLR4-6, TLR10), while those that detect nucleic acids are localized to the endosomes (TLR3, TLR7-9, TLR11-13) [244]. Upon ligand binding, TLRs dimerize and undergo conformational changes that allow for the differential recruitment of specific TIR domain-containing adaptor proteins [266]. This leads to the activation of two major signaling cascades, namely the myeloid differentiation primary response 88 (MyD88)-dependent and -independent pathways. All TLRs, except for TLR3, recruit the adaptor protein MyD88 and activate the MyD88-dependent pathway that leads to activation of the nuclear factor kappa B (NF-κB) pathway and the mitogen-activated protein kinase (MAPK) signaling pathway for the transcription of proinflammatory cytokine genes [267]. TLR4 is also capable of recruiting TIR-domain containing adaptor-inducing interferon-β (TRIF), while TLR3 exclusively utilizes this adaptor protein to activate the Myd88-independent pathway, also referred to as the TRIF-dependent pathway, to activate interferon regulatory factor 3 (IRF3), which is required for the induction of type I IFN, particularly IFN-beta (IFN-β) [246].

1.5.2.2   Recent progress of clinical development of TLR agonists for cancer therapy

Agonists for TLRs come from a wide range of sources, both natural and synthetic, with varying degrees of immunostimulatory effects [244]. Interestingly for some of these agonists, their anti-cancer potential was well-recognized several decades before they were identified to function as TLR agonists [268]. For example, bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis* that operates as a mixed TLR2/4 agonist initially developed as a vaccine against tuberculosis, was found to be safe and highly effective for the treatment of bladder carcinoma as early as the mid 1970s [268]. In 1990, the FDA approval of BCG as a standalone therapy for adult bladder cancer was the culmination of the clinical development of BCG as an therapeutic agent for cancer treatment [268]. Since then, a vast amount of preclinical and clinical evidence has been generated, indicating the ability of TLR agonists to exert potent immunostimulatory effects in oncological settings [255,256,268,269]. Nonetheless, only two additional agonists have been approved by the FDA and equivalent agencies worldwide for use in cancer patients so far; TLR7 agonist imiquimod as a monotherapy for basal cell carcinoma and TLR4 agonist monophosphoryl lipid A (MPL) as a vaccine adjuvant for cervical cancer [256,269]. By 2012, the progress of clinical development of TLR agonists for oncological indications was stalled, where a steady decrease was observed in the number of newly launched clinical trials involving TLR agonists [255,256,269]. The limited availability of clinical grade of reagents and their rather disappointing clinical results as monotherapies have contributed to a decline in the interest for TLR agonists [255,256,269]. Although some groups sought alternative sources of TLR agonists, including clinically approved commonly used prophylactic vaccines, the clinical interest in TLR agonists remained low [255]. This trend, however, has been reversed as of the second quarter of 2014 [256,260,269], following the FDA approval of anti-PD-1 mAbs for the treatment of metastatic

melanoma [140]. Since then, several key preclinical studies have reported a synergistic effect of TLR agonists and immune stimulatory or inhibitory checkpoint antibodies in combination therapies [236,270,271]. The Guiducci group demonstrated that the combined treatment with low intratumoral doses of TLR9 agonist CpG oligodioxynucleotides (ODN) and anti-PD-1 rapidly induces durable rejection of several anti-PD-1 non-responder mouse carcinoma cell lines and generates systemic immunity to untreated distant-site tumors [270]. Such a control of tumor growth correlated with a rapid infiltration and expansion of tumor-specific CD8^{+} T cells, indicating the ability of locally injected CpG ODN to overcome resistance to PD-1 blockade by changing the tumor microenvironment towards one that is favourable to the induction of systemic anti-tumor immunity. Similarly, the Cohen group demonstrated that local treatment with TLR7 or TLR9 agonist in combination with anti-PD-1 primarily acts on tumor-associated macrophages (TAMs) to subsequently generate tumor-specific CD8^{+} T cell immune responses, which led to suppression of primary tumor growth and prevention of metastasis in head and neck squamous cell carcinoma (HNSCC) models [271]. More recently in 2018, the Levy group reported that CpG ODN in combination with an activating antibody against OX40 is sufficient to break tumor immune tolerance in a spontaneous model of breast cancer via the induction of tumor antigen-specific T cell immune response [236]. With these preclinical studies providing a strong rationale for clinical evaluation of combination therapy, more than 50 clinical trials have been initiated within the last 4 years to evaluate the safety and therapeutic efficacy of TLR agonists in cancer patients, mostly in combination with other therapeutic modalities [236,260]. The majority of these trials involves the FDA-approved agonists BCG and imiquimod, and the experimental TLR3 agonist poly-ICLC (Hiltonol™), but the use of CpG ODN (SD-101), given intratumorally, is being increasingly tested in patients with advanced solid tumors (e.g. lymphomas, metastatic

melanoma, and HNSCC) in combination with checkpoint inhibitors and/or radiation therapy [236,260]. Agonists, such as BCG, imiquimod, and CpG ODN, are investigated in combination with conventional chemotherapy, targeted anti-cancer agents, or other forms of immunotherapy, while poly-ICLC is generally employed to adjuvant a peptide-based anti-cancer vaccine or DC-based interventions [236,260]. The results of these recently initiated clinical trials may pave the way to fully exploit the therapeutic potential of TLR agonist for the treatment of wide-range of cancers.

1.6   Hypotheses

As discussed, despite achieving CRs in the majority of patients, the efficacy of strategies that direct T-cell-mediated cytotoxicity towards leukemic cells bearing non-immunogenic antigens, such as CD19-targeted CAR T cells and bi-specific T-cell engagers, is limited with no evidence that these interventions generate long-term immune memory [215]. The primary goals of my thesis work are to better understand the failures of current single antigen-targeted immunotherapies and to identify the immune activities that contribute to remission length and reduce the incidence of B-ALL recurrence. The overall hypothesis of this work is that *the generation of a protective T cell-mediated immune response with a broad specificity for range of leukemia-associated antigens is required for durable control of B-ALL*. More specific hypotheses are outlined in the proceeding chapters as follows.

The potential contribution of a diverse anti-leukemia immune response, directed beyond the target antigen following single antigen-targeted therapies, to durable remission has yet to be demonstrated in the context of B-ALL. The work described in Chapter 3 was designed to provide a new platform for investigating epitope spreading in ALL during neoantigen-driven remission in the mouse model of B-ALL. This work evaluated the hypothesis that *epitope spreading after a*

target antigen-specific response enables the generation of a diversified immune response that contributes to durable control of ALL. I found the ability of a neoantigen-targeted immune response to generate long-term protection against B-ALL by unmasking previously non-immunogenic leukemia-associated antigens, which is abrogated by tolerance to such antigens.

Extending on the previously reported ability of CpG ODN to induce T cell-mediated protection against outgrowth of transplanted syngeneic ALL cell lines [272], the work described in Chapter 4 investigated the therapeutic potential of additional endosomal TLR agonists for the treatment of B-ALL. This chapter was designed to test the hypothesis that *TLR agonists have differential capacities for inducing innate immune responses required for the productive activation of leukemia antigen-specific T cells.* I found the superior ability of CpG ODN to elicit both a rapid innate immune-mediated and T cell-mediated anti-leukemia responses. In addition, I found evidence implicating bone marrow as a potentially key target site for establishing durable remission of ALL.

Unlike for other types of blood cancers, the understanding of a role for the CD1d–natural killer T (NKT) cell axis in the control of B-ALL is lacking. The work described in Chapter 5 is designed to test the hypothesis that *NKT cells inhibit B-ALL progression by acting as key early players constraining the survival of pre-leukemic cells*. I aimed to determine the role of NKT cells during disease progression in an age-dependent manner. I demonstrated that the absence of type 1 NKT cells accelerates leukemia onset, and that the therapeutic activation of iNKT cells using α-GalCer during early stage of disease in Eµ-ret mice achieves IFN-γ-dependent depletion of LICs. Additionally, I found the evidence of immunoediting by type 2 NKT cells, in the absence of type 1 counterparts, in the Eµ-ret mouse model. Importantly, my results indicate that

the neoantigen-driven protective immune response via epitope spreading is limited in the absence

of NKT cells.

Chapter 2: Materials and methods

2.1 Mice

C.FVB-Tg(CAG-luc,-GFP)L2G85Chco/Fath transgenic (GFP/luc-transgenic), CD1d-knockout BALB/c (CD1d$^{-/-}$), IFN-γ-knockout BALB/c (IFN-γ$^{-/-}$), wild-type (wt) BALB/c, Rag-1-deficient BALB/c (RAG1$^{-/-}$), and NOD/SCID (NOD/LtSz-scid/scid) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained as in-house breeding colonies. Jα18-knockout (Jα18$^{-/-}$) C57BL/6 mice, previously gifted to Dr. Peter van den Elzen, were backcrossed 10 generations onto a BALB/c background. CD1d$^{-/-}$, IFN-γ$^{-/-}$, Jα18$^{-/-}$, and GFP/luc-transgenic mice were then crossed with wt Eμ-ret transgenic mice, which were generously provided by Dr. Stephan Grupp (University of Pennsylvania, Philadelphia, PA), to generate stable gene-knockout colonies carrying the Eμ-ret transgene. Hemizygous Eμ-ret mice on an otherwise wild-type BALB/c background were maintained by in-house breeding Eμ-ret sires with BALB/c dams obtained from Jackson Laboratories. Eμ-ret-transgenic, GFP/luc-transgenic, and Eμ-ret/GFP/luc double transgenic BALB/c colonies were maintained at both the Children's Hospital of Philadelphia (CHOP) and BC Children's Hospital Research Institute (BCCHR) animal care facilities under specific pathogen-free conditions. All other mice were maintained at BCCHR under specific pathogen-free conditions. For experiments using colony mice, animals were randomized to treatment arms with a goal of maintaining a similar age and sex distribution among arms. All experiments were performed in accordance with the Canadian Council of Animal Care and Institutional Animal Care Committee and Use Committee-approved protocols at University of British Columbia (A15-0187) and CHOP.

2.2   Cells

Cell lines 289 and t309, derived from spontaneous primary leukemias arising in Eµ-ret mice, were provided by Dr. Stephan Grupp (University of Pennsylvania). These cell lines were transduced with a self-inactivating lentiviral construct encoding green fluorescent protein and firefly luciferase (GFP/luc) to generate stable GFP/luc-expressing variants [273]. Primary leukemias were harvested from the spleen and/or bone marrow of spontaneously arising leukemia in Eµ-ret mice. Leukemia-involved spleen and bone marrow were processed with Tris-Buffered Ammonium Chloride (TAC; pH: 7.2) to lyse red blood cells. The characteristic Eµ-ret BCP leukemia cell phenotype (B220^{int}/BP-1^{hi}) was used to identify and quantify leukemic cell populations in all cases by flow cytometry [89,90]. 4T1 breast cancer cell line cultures were purchased from the American Type Culture Collection (Manassas, VA). Stably transduced GFP/luc-expressing primary human B-ALL cells (96-ALL-GFP/luc and IR812-GFP/luc, generously provided by Dr. David Barrett (University of Pennsylvania, Philadelphia, PA) were generated as previously described [273]. All cell lines were cultured at 37°C with 5% CO_2 in complete Dulbecco's Modified Eagle Medium (DMEM; Gibco, LifeTechnologies, Carlsbad, CA) supplemented with heat-inactivated fetal bovine serum (FBS; 20%), penicillin/streptomycin (Gibco, LifeTechnologies; 100U/mL; 100 µg/mL), MEM non-essential amino acids (Gibco, LifeTechnologies; 1X), HEPES (Gibco, LifeTechnologies; 10 mM), 2-mercaptoethanol (Sigma-Aldrich; 50 µM), L-glutamine (Gibco, LifeTechnologies; 2 mM). Cell lines 289 and t309 were also supplemented with 250 pg/mL mouse IL-7, which was obtained from Sigma-Aldrich (St. Louis, MO).

2.3 Adoptive transfer studies

Mouse leukemia cells (each figure legend states specific number, type and source of cells), suspended in total volume of 100 μL phosphate buffered saline (PBS), were adoptively transferred into recipients by tail vein injection. Recipient mice were then followed for time to disease onset. Unless otherwise stated, this general protocol was used in all adoptive transfer studies.

2.4 Survival studies

Disease progression was monitored by assessment of LIC or blast counts in peripheral blood. Disease onset was defined by the presence of palpable/enlarged lymph nodes, hindleg paralysis, or white blood cell count of >15,000/μL in peripheral blood. For recipients of GFP/luc-positive leukemia cells, *in vivo* bioluminescence imaging was performed to assess disease burden.

2.5 Disease burden assessment

Red blood cell lysis was performed on 45μL of peripheral blood collected from mice using a 7-minute incubation followed by a 5-minute incubation in TAC. Cells were then washed in PBS and re-suspended in 100 μL of PBS with 2% FBS. Cells were stained for evaluation by flow cytometry using the following antibodies purchased from BioLegend (San Diego, CA): B220/CD45R (Clone: RA3-6B2), BP-1/Ly-5 (Clone: 6C3), 7-AAD. LIC and blasts were identified based on their characteristic $B220^{int}/BP-1^{hi}$ phenotype. CountBright beads (Invitrogen, Carlsbad, CA) were used to calculate absolute counts of LICs or leukemia blasts by flow

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cytometry. Unless stated, this general protocol was used in all subsequently described assessments of *ex vivo* disease burden.

2.6   *In vivo* bioluminescence imaging

Mice bearing luciferase-tagged leukemia cells were injected with 150 μL of 1% D-luciferin in PBS (GoldBio, St. Louis, MO) intraperitoneally 5 minutes prior to imaging. Disease burden was followed every 2 to 3 days using bioluminescence. The endpoint was defined by the radiance of cells $>1 \times 10^9$ p/scm^2. All live imaging was performed on an Ami-X (Spectral Instruments Imaging, Tucson, AZ) and analyzed using AMIView.

2.7   *In vivo* antibody-based cell depletion and receptor blockade

NK cells (by asialo-GM1, Cedarlane, Burlington, NC) or CD4 and CD8 T cells (by clones GK1.5 and 53-6.72 respectively, BioXcell, West Lebanon, NH) were depleted *in vivo* with 200 μg antibody/dose administered intraperitoneally. CTLA-4 antibody blockade (R&D Systems, Minneapolis, MN) was given at 200 μg/dose intraperitoneally on days 7, 10 and 14 after ALL adoptive transfer.

2.8   IFN-γ ELISPOT analysis

After adoptive transfer of 1×10^6 GFP/luc-modified or unmodified 289 cell lines into wild-type BALB/c mice, T cells were isolated from spleens of recipients on day 9 or day 20, and from naïve control mice using EasySep™ Mouse T Cell Isolation Kit (STEMCELL Technologies Inc, Vancouver, BC, Canada) according to the manufacturer's instructions. GFP/luc-modified and unmodified 289 cell lines were treated with mitomycin C (10 μg/mL;

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Sigma-Aldrich, St. Louis, MO) in complete DMEM and incubated at 37°C for 2 hours. Cells were then washed with media and 3 more times with PBS. 1 x 10^5 or 5 x 10^4 T cells were co-cultured in complete DMEM with mitomycin C-treated GFP/luc-modified and unmodified 289 cell lines on IFN-γ-coated ELISPOT plates (eBioscience, San Diego, CA) at 2:1 responder to stimulator ratio in triplicates and incubated overnight at 37°C.

2.9 Reverse transcription polymerase chain reaction

RNA was isolated from mouse primary B-ALL cells using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) and qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA) was used for first strand synthesis. Subsequent PCR was performed using *Taq* polymerase (New England Biolabs, Ipswich, MA). The primers used were: *Tlr3*F – TCGGATTCTTGGTTTCAAGG; *Tlr3*R – TTTCGGCTTCTTTTGATGCT; *Tlr7*F – GGAGCTCTGTCCTTGAGTGG; *Tlr7*R – CAAGGCATGTCCTAGGTGGT; *Tlr8*F – GGCACAACTCCCTTGTGATT; *Tlr8*R – CATTTGGGTGCTGTTGTTTG; *Tlr9*F – TCGCTTTGTGGACTTGTCAG; *Tlr9*R – GGCTCAGGCTAAGACACTGG.

2.10 Direct and immune-mediated effects of TLR agonists on primary B-ALL cells

Mouse primary B-ALL cells were labeled with PKH26 (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. PKH-labeled B-ALL cells (1 x 10^4 cells) were plated in U-bottom 96-well plate and stimulated with Class B CpG ODN (1826; 5 μg), R848 (10 μg), or poly I:C HMW VacciGrade™ (50 μg) in 200 μL media/well for 16 hours at 37°C with 5% CO_2, in the presence or absence of splenocytes harvested from wild-type mice. Cells were cultured in complete DMEM with 250 pg/mL IL-7. All TLR agonists were purchased from InvivoGen (San

Diego, CA). Cells were stained with the following antibodies to determine viability of target cells (PKH26^{+}) and 7-aminoactinomycin D (7-AAD). All antibodies were purchased from BioLegend (San Diego, CA)

2.11 TLR agonist treatment studies

Dose selection for CpG ODN (100 μg) was based on published protocols [236,272]. No significant difference in efficacy was observed with intraperitoneal and intravenous injection of TLR agonists (Figure 2.1), so intraperitoneal injection was used throughout the study to avoid potential difficulties associated with repeated tail vein injections.

Starting on day 7, mice were injected intraperitoneally with 100 μg of CpG ODN (1826), R848, or poly I:C HMW VacciGrade™ in 200 μL PBS. For single-dose experiments, mice were sacrificed 3 days after the treatment for evaluation of disease burden in spleen and bone marrow. For 3-dose experiments, mice were given 2 more treatments 4 days apart and were either sacrificed at day 21 for evaluation of disease burden in spleen, bone marrow, and peripheral blood by flow cytometry or monitored for disease onset.

Whole spleen and bone marrow were extracted and peripheral blood was collected from these mice. Red blood cell lysis was performed on a single-cell suspension of splenocytes and bone marrow using a single 4-minute incubation in TAC. Peripheral blood samples were processed as described previously. Cells were then washed in PBS and splenocytes and bone marrow cells were re-suspended in 1 mL of PBS with 2% FBS. 5% of total splenocytes, 10% of total bone marrow cells, and peripheral blood samples were stained for evaluation by flow cytometry using the protocol described previously. This protocol was also used for subsequently described assessments of splenic disease burden.
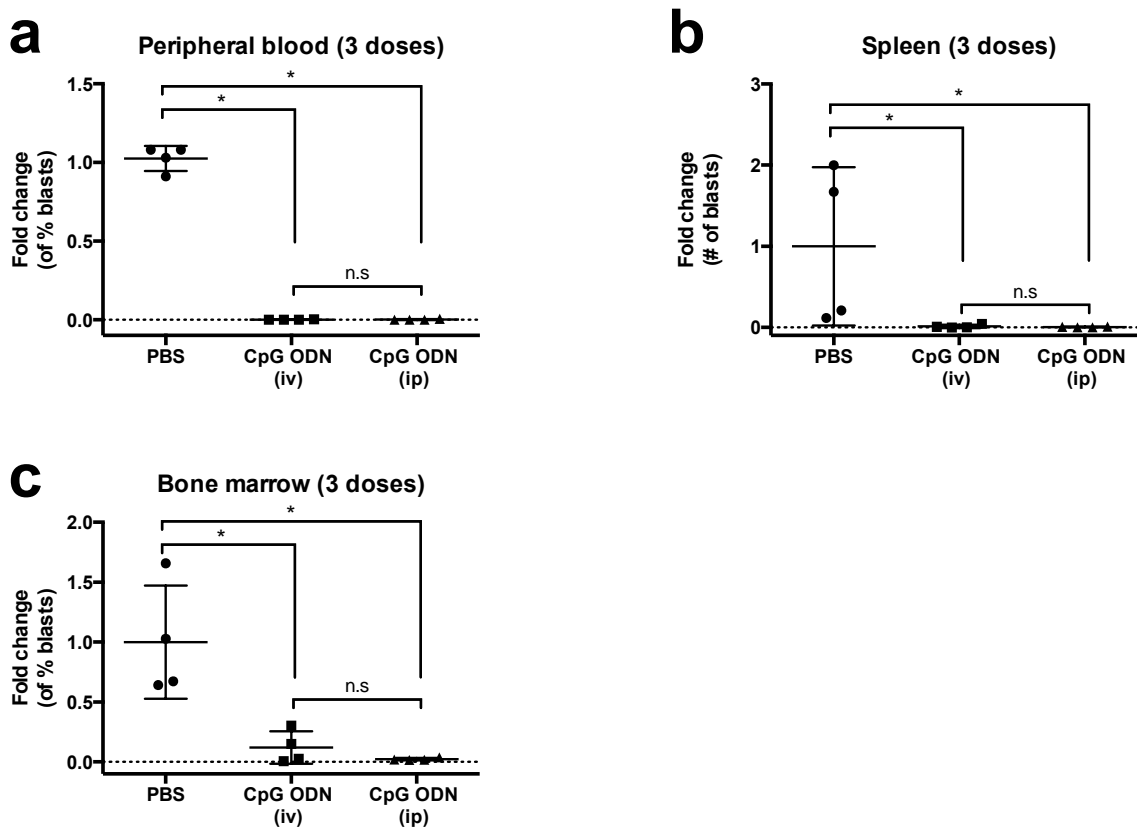
Figure 2.1. Comparison of *in vivo* efficacy of CpG ODN-induced depletion of primary B-ALL between different routes of administration

Wild-type BALB/c mice bearing syngeneic primary B-ALL cells were administered with 3 doses of indicated TLR agonists intravenously (iv) or intraperitoneally (ip) and evaluated for disease burden in peripheral blood (a), spleen (b), and bone marrow (c) six days after the last treatment. Results shown are from one independent experiment; n = 4 mice per group. Mann-Whitney test, bars represent mean±S.D.; *$p < 0.05$. n.s = not significant

2.12 *In vivo* human leukemia sensitivity to TLR agonists

1×10^5 human primary leukemia cells (96-ALL-GFP/luc or IR812-GPF/luc), suspended in total volume of 100 μL PBS, were injected via tail vein into 4-6 weeks old NOD/SCID mice. Between day 21 and 24, when the average systemic radiance had reached above 1×10^7 p/scm^2,

mice were injected with 3 doses of the indicated TLR agonist or PBS over 8 days. Disease burden was followed using bioluminescence. Change in disease burden was determined by measuring the difference in bioluminescence immediately prior to the first treatment and 4 days after the last treatment.

2.13 Serum cytokine analysis

Serum collected from wild-type mice 16 hours after a single TLR treatment was stored at -80°C until analysis for cytokine and liver-specific enzyme production. Serum concentration of IL-6, IFN-γ, IL-12p70, and TNF-α were measured with the MDS "V-Plex Custom Proinflammatory Panel 1" (Meso Scale Discovery, Rockville, MD), according to manufacturer's instructions.

2.14 Cellular immune responses to TLR agonist administration in ALL-bearing mice

The number and activation status, based on changes in the surface expression of CD69, CD40, CD80, CD86, and MHC-II, of immune cells in spleen and bone marrow of ALL-bearing wild-type mice were evaluated following TLR agonist treatment by flow cytometry. Immune cell subsets were defined based on following surface phenotypes: $CD335^+$ (NK cells); $CD11c^{int}B220^+CD11b^-Ly-6C^{int}MHC-II^+$ (activated plasmacytoid DCs); $F4/80^+CD11b^+ CD11c^-$ (Macrophages); $CD11b^+CD11c^+B220^-MHC-II^+$ ($CD11b^+$ classical DCs); $CD8a^+CD11b^+ CD11c^-$ $B220^-MHC-II^+$ ($CD8a^+$ DCs); $CD3^+B220^-$ and $CD4^+$ or $CD8^+$ ($CD4^+$ and $CD8^+$ T cells). All antibodies were purchased from BioLegend (San Diego, CA). CD335 (Clone:29A1.4), CD11b (Clone: M1/70), CD11c (Clone: N418), Ly-6C (Clone: HK1.4), MHC-II (I-A/I-E; Clone: M5/114.15.2), F4/80 (Clone: BM8), CD3 (Clone: 17A2), CD4 (Clone: RM4-5), CD8a (Clone:

53-6.7), To minimize non-specific binding of antibodies to FcγR, cells were pre-incubated with anti-mouse CD16/32 (Clone: 93; BioLegend) prior to performing surface staining. CountBright beads (Invitrogen, Carlsbad, CA) were used to calculate absolute cell numbers by flow cytometry.

2.15 Splenic LIC burden assessment

Wild type, CD1d$^{-/-}$, and Jα18$^{-/-}$ Eμ-ret were sacrificed at 14 days of age and whole spleen were harvested from these mice. A single-cell suspension of splenocytes were processed and stained for evaluation by flow cytometry.

2.16 *In vivo* α-GalCer administration

α-GalCer (KRN 7000; Cayman Chemical Company, Ann Arbor, MI) was reconstituted prior to administration according to the manufacturer's instructions. 4 μg of α-GalCer of vehicle (5.6% sucrose, 0.75% L-histidine, and 0.5% Tween 20) in total volume of 200 μL in PBS containing 0.5% Tween 20 was injected into wild-type and IFN-γ$^{-/-}$ Eμ-ret intraperitoneally at 2 weeks or 3 weeks of age. 7 days after treatment, whole spleen was harvested from these mice to assess LIC burden and the number of iNKT cells. Processed splenocytes were first stained with PBS57 (an α-GalCer analog)-loaded or empty-CD1d tetramers (NIH Tetramer Core Facility), then with the following antibodies purchased from BioLegend (San Diego, CA): TCR-β (Clone: H57-597), B220/CD45R (Clone: RA3-6B2), BP-1/Ly-5 (Clone: 6C3), 7-AAD. CountBright beads were used to calculate absolute counts of LICs and iNKT cells.

2.17 Statistical methods

Kaplan-Meier curves generated for spontaneous and leukemic cell adoptive transfer survival studies were analyzed by log-rank tests. Differences in bioluminescence over time were measured by repeated measures ANOVA. Antigen loss by time to relapse was analyzed by Fisher's exact test, selected due to concern for non-parametric distributions in small numbers of events. ELISPOTs were analyzed by 2-way ANOVA with Bonferroni post-hoc comparisons. Analyses of change in the expression of co-stimulatory molecules *in vitro* and *in vitro* cytotoxic assays were performed using a one-way ANOVA (Dunn's multiple comparisons test). *In vivo* burden analyses of LICs and leukemic cells in the spontaneous and adoptive transfer experiments, respectively, were performed using Mann-Whitney test or a one-way ANOVA (Dunn's multiple comparisons test) in any case where more than two groups were being compared. 2-way ANOVA (Tukey's multiple comparisons test) were used any time more than one variable was involved. Statistical analyses were performed using Prism 5 for Mac OS X (GraphPad, San Diego, CA). Specific n values for each experiment are listed in figure legends. All graphs depict the combined results from at least two independent experiments.

2.18 Services

Flow cytometry work was conducted at BCCHR Flow Core Facility. Data acquisition was performed on the BD Fortessa X-20 and LSR-II flow cytometers (Becton Dickinson, Franklin Lakes, NJ) and data analysis was performed using FlowJo V.10.1r7 (Treestar, Ashland, OR).

Chapter 3: A multi-antigen-directed immune response is required for durable control of acute lymphoblastic leukemia

3.1 Overview and rationale

Epitope spreading is emerging as an essential process for the magnification of effective adaptive immune responses, as the diversification of epitopes broadens the range of antigens that can be recognized by both T and B cells [274–276]. First described in experimental autoimmune encephalomyelitis (EAE), a murine model of autoimmune disease, epitope spreading was described as the broadening of an immune response initially directed against a single dominant epitope to include subdominant epitopes on the disease-inducing myelin basic protein (MBP) [277,278]. Subsequent studies of peptide- and RNA-based cancer vaccines indicated that the specificity of T cell immune response induced by vaccination expands to additional non-targeted tumor antigens, supporting the relevance of epitope spreading to therapeutic responses induced by immunotherapies [279–283].

Similarly, epitope spreading is increasingly proposed as a secondary mechanism underlying immune-mediated durable control of cancer following single antigen-targeted and checkpoint blockade-mediated interventions. In two recent case reports from the first-in-human studies of mesothelin-specific mRNA CAR T (CARTmeso) cells in patients with mesothelin-expressing solid malignancies, clinical evidence for a broad anti-tumor immune response was provided by the development of novel anti-self antibodies [284]. The induction of such a polyclonal anti-tumor humoral immune response was consistent with CARTmeso-cell-mediated tumor destruction leading to the release of self-antigens that are cross-presented in a classical process of

B cell epitope spreading [276,284]. Evidence of epitope spreading was also reported in patients with metastatic melanoma who achieved durable tumor regression following adoptive transfer of autologous melanoma-specific polyclonal CTLs combined with anti-CTLA-4 mAb [285]. These patients demonstrated a significantly increased reactivity to the melanoma-associated proteins and developed response to additional non-targeted tumor antigens [285]. The contribution of epitope spreading to durable remission following single antigen-targeted therapies, such as tisagenlecleucel (CD19-directed CAR T cells) and blinatumomab (CD19-directed BiTE), has yet to be demonstrated in the context of B-ALL.

Encouraged by these findings, I initiated an investigation of the role of broad spectrum T cell responses against ALL-associated antigens in durable protection against recurrence. To study the ability of target-directed therapy to induce secondary immune responses against non-targeted antigens with high consistency and reproducibility, we developed a neoantigen-driven remission model using the Eμ-ret transgenic mouse model of B-ALL. As the subsequently described work will illustrate, I found the ability of a neoantigen-targeted immune response to generate long-term protection against B-ALL was dependent on the unmasking of previously non-immunogenic ALL-associated antigens. While immune tolerance to such leukemia-associated antigens abrogated durable control of disease, CTLA-4 blockade partially restored the protective immune response.

3.2 Neoantigens induce T cell-mediated immune control of syngeneic ALL

To reproduce the very high rates of initial response following single antigen-directed therapies, syngeneic ALL cell lines (289 and 309) derived from primary leukemias in Eµ-ret mice [89,90,273] were modified to express model antigens GFP and luciferase, which act as neoantigens in wild-type BALB/c mice. While the majority of wild-type BALB/c recipients of unmodified ALL cells rapidly progressed to lethal leukemia between 20 and 35 days after injection, less than 15% of mice receiving GFP/luc-modified ALL cells succumbed to disease and the rest achieved durable remission (Figure 3.1a). To determine the kinetics and cell dependency of the immune control of GFP/luc-modified ALL cells, either NK cells or CD4 and CD8 T cells were depleted in the recipients at the time of leukemia adoptive transfer. PBS-treated mice and NK-depleted mice both developed disease detectable by bioluminescent imaging by day 7, which was then subsequently cleared by day 10 (Figure 3.1b). On the other hand, T cell-depleted mice were unable to control GFP/luc-modified ALL, indicated by high bioluminescence, and succumbed to disease within 3 weeks. The control of GFP/luc-modified ALL cells, therefore, followed primary immune response kinetics and depended on T cells, but not on NK cells.
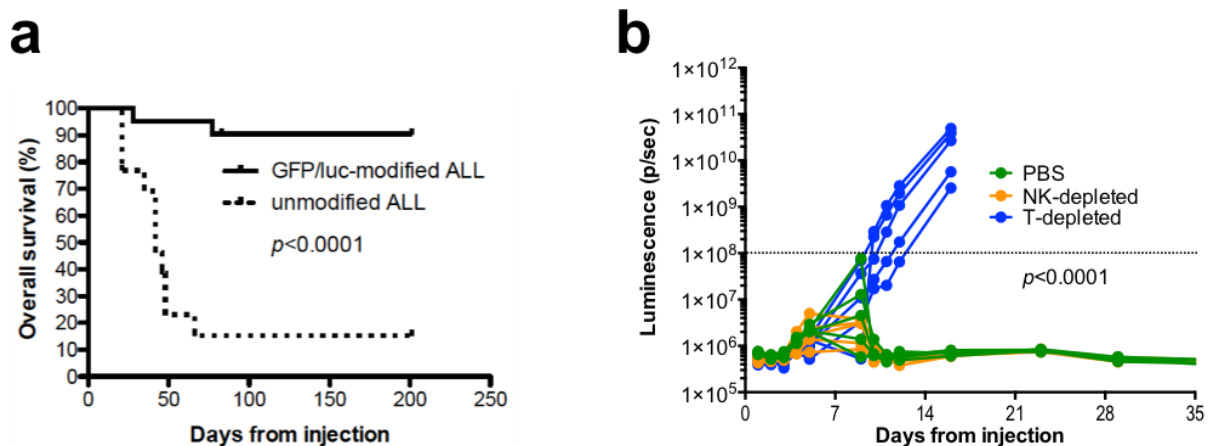
Figure 3.1. Neoantigens induce T cell-mediated immune control of syngeneic ALL

Wild-type BALB/c mice were adoptively transferred with either unmodified or GFP/luc-modified syngeneic cell line (289 or 309) and were followed for overall survival. N=34; GFP/luc-modified and unmodified ALL cells were adoptively transferred into 21 and 13 recipients, respectively, pooled from two independent experiments. Log-rank test (a). Mice were treated with PBS, asialo-GM1 (NK-depleted), or CD4/CD8-depleting antibodies (T-depleted) at the time of adoptive transfer of GFP/luc-modified ALL cell lines. Dashed line at 1 x 10^8 p/sec corresponds roughly to disease burden detectable in peripheral blood. N=25; 5 mice per arm using cell line 309-GFP/luc. Two-way ANOVA. Dashed line at 1 x 10^8 photons/s corresponds roughly to disease burden detectable in peripheral blood (b)

3.3 Immune tolerance to neoantigens abrogates protection against ALL

To confirm the initiating role of immune response directed against the GFP/luc
neoantigens for the protection against ALL outgrowth, GFP/luc-modified ALL cells were
adoptively transferred to transgenic BALB/c mice (C.FVB-Tg(CAG-luc,GFP)L2G85Chco/Fath)
in which GFP and luciferase are expressed as self-antigens to which immune tolerance is
established. GFP/luc-transgenic mice were unable to reject GFP/luc-modified ALL and, unlike
wild-type BALB/c mice, rapidly succumbed to disease, indicating that immune tolerance to
initiating neoantigens prevents the induction of a protective response (Figure 3.2).
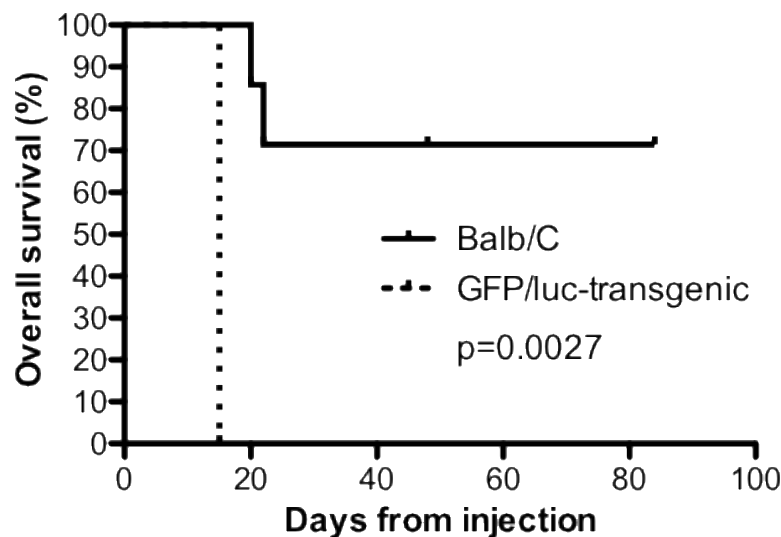
Figure 3.2. Tolerance to GFP/luc abrogates protection against GFP/luc-modified ALL
Wild-type BALB/c or GFP/luc-transgenic mice were adoptively transferred with GFP/luc-
modified ALL cell line (289 or 309). Mice were followed for overall survival. N=10; GFP/luc-
transgenics, n=3; Wild-type BALB/c, n=7. Log-rank test.

3.4 Loss of target antigen is more common in late relapses

Similar to the relapse cases commonly reported following CD19-targeted immunotherapies [155,223], two distinct patterns of relapse were observed among the wild-type BALB/c recipients of GFP/luc-modified ALL cells that failed to achieve durable remission. Mice that either failed to control disease or had early relapses, defined as reoccurrence before day 60 from adoptive transfer, contributed to 41% of total deaths in the study, whereas late relapses (>60days) accounted for the remainder of deaths (Figure 3.3). Notably, leukemias in mice that failed to achieve remission or had early relapses were more commonly GFP/luc-positive (27% versus 14%). Conversely, GFP/luc-negative leukemia, which is caused by loss of target antigen expression, developed more commonly in mice that relapsed after day 60 than mice that fail to achieve remission or had early relapses (45% versus 14%).
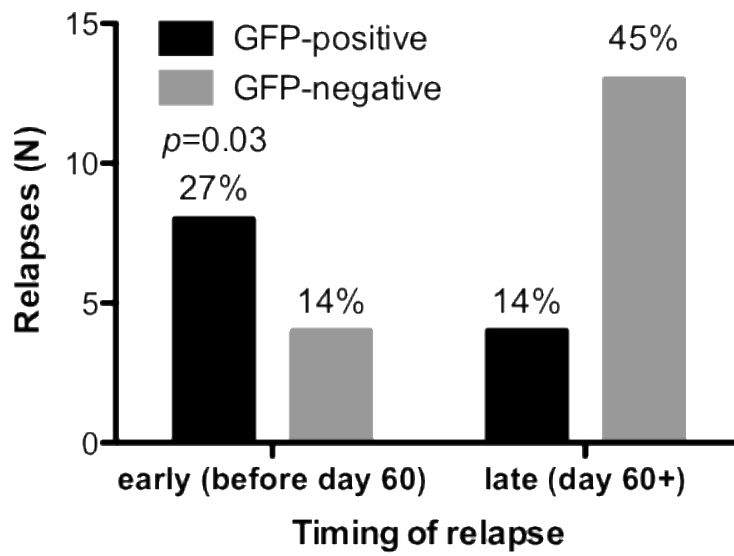
Figure 3.3. Neoantigen expression by progressing leukemia

Y-axis represents all deaths among wild-type BALB/c recipients of GFP/luc-modified ALL (289 or 309) across multiple studies. N=31 deaths among 106 total mice inclusive of 21 individual studies. Fisher's exact test; Odds ratio 6.5; 95% confidence interval 1.26-33.59. Percentages indicate contribution to total deaths in the study by category

3.5 Neoantigen-directed primary immune responses induce secondary responses to non-targeted ALL antigens

After validating that the neoantigen-driven remission model is a reproducible and appropriate platform to investigate the immune activities that contribute to remission length and reduce the incidence of immune escape, the specificity of the protective T cell response in this model was evaluated. Wild-type BALB/c mice in spontaneous remission following adoptive transfer of GFP/luc-modified ALL cells (289-GFP/luc) or syngeneic 4T1 breast cancer cells expressing GFP/luc were rechallenged with either primary ALL cells or unmodified cell line (289). Mice rechallenged after exposure to GFP/luc-modified ALL cells were protected from outgrowth of both unmodified primary ALL cells and 289 cells, while all leukemia-naïve mice died of unmodified ALL by day 35 (Figure 3.4). Although wild-type mice eliminated GFP/luc-expressing 4T1 cells and achieved remission, they were unable to reject rechallenge with primary ALL and succumb to disease with similar kinetics to naïve mice. Together, these results indicate that the diversification of the T cell response to additional leukemia-associated antigens, and not immunogenic retroviral neoepitopes common to many mouse cell lines [286], had been induced during the primary response against GFP/luc-modified ALL cells.
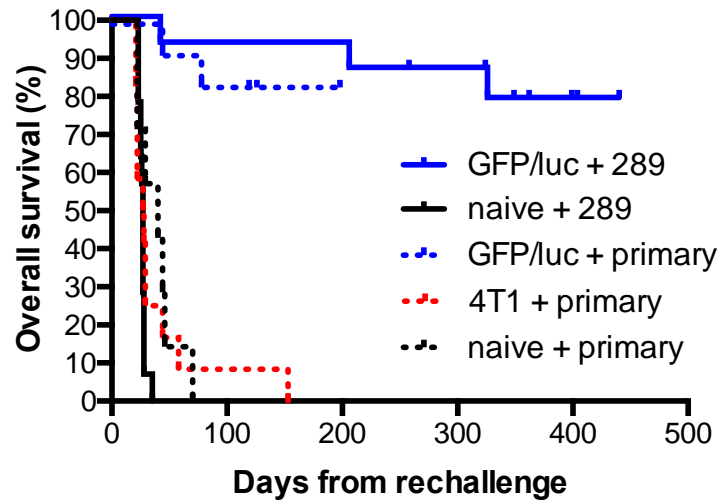
Figure 3.4. Neoantigens induce epitope spreading that is specific to additional ALL antigens

Leukemia-naïve wild-type BALB/c mice or wild-type mice that went into remission following adoptive transfer of 289-GFP/luc were (re)challenged with unmodified primary ALL or 289 cell line. Wild-type BALB/c mice that developed spontaneous remission after challenging with GFP/luc-expressing 4T1 breast cancer cells were similarly rechallenged with unmodified primary ALL cells. Mice were followed for overall survival. N=65; GFP/luc-modified ALL-cleared + unmodified 289, n=15; Naïve BALB/c + unmodified 289, n=14; GFP/luc-modified ALL-cleared + primary ALL, n=12; GFP/luc-expressing 4T1-cleared + primary ALL, n=12. Results shown are pooled from three independent experiments. Log-rank test; *p*<0.0001

3.6   Epitope spreading is induced early during the primary response against neoantigens

To confirm the specificity of the diversified T cell immune and to determine the timing of such diversification, an ELISPOT assay was performed to measure IFN-γ production by T cells, obtained at two different time points, in response to either unmodified or GFP/luc-modified ALL target cells. T cells isolated from spleens of GFP/luc-modified or unmodified ALL-bearing mice and naïve mice were co-cultured with GFP/luc-modified or unmodified ALL cells on IFN-γ-coated ELISPOT plates. Mice challenged with GFP/luc-modified ALL cells had a robust T cell response against GFP/luc-modified ALL targets at both day 9 and day 20 after adoptive transfer of ALL cells (Figure 3.5a). Additionally, there were more T cells producing IFN-γ against unmodified ALL target cells at day 20 than at day 9. Neither naïve mice nor mice challenged with unmodified ALL cells produced significant IFN-γ when co-cultured with either unmodified or GFP/luc-modified ALL target cells. These results suggest that although the response to GFP/luc-expressing cells precedes the broadening of the primary response, epitope spreading occurs early. To further demonstrate the therapeutic impact of this early diversification of primary immune response on leukemia, wild-type BALB/c mice were challenged with 1:1 mix of GFP/luc-modified and unmodified ALL cell lines. Mice receiving unmodified ALL rapidly developed disease and succumbed by day 35, while mice receiving GFP/luc-modified ALL cells or a 1:1 mixture of GFP/luc-modified and unmodified ALL (mixed) were protected from leukemia progression and achieved durable remission (Figure 3.5b).
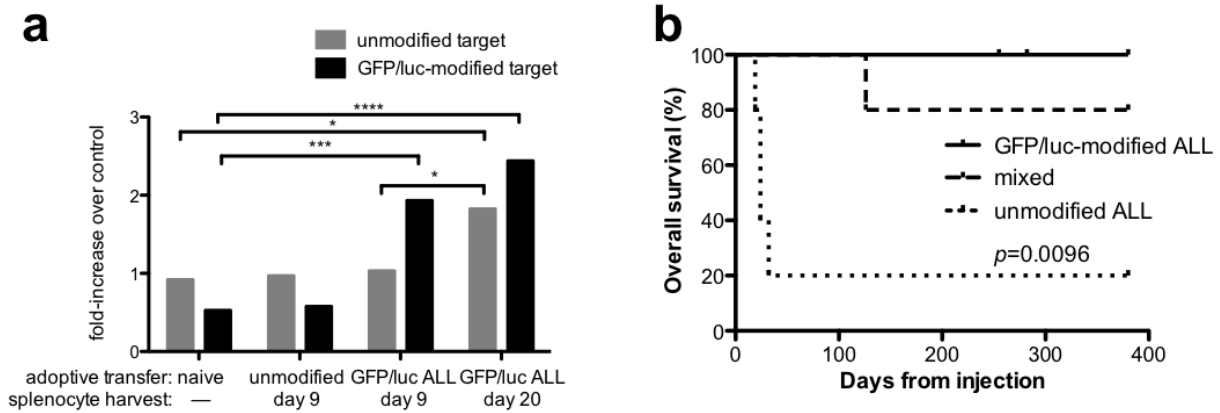
Figure 3.5. T cell recognition of multiple ALL antigens is achieved promptly following induction of neoantigen-directed primary response

T cells isolated from spleen of GFP/luc-modified or unmodified 289-bearing mice and naïve mice were co-cultured with GFP/luc-modified or unmodified 289 cells at 2:1 responder (T cells) to stimulator (ALL cells) ratio on IFN-γ-coated ELISPOT plates overnight. Cell cultures were plated in triplicates. Representative data from one of three experiments. Bonferroni post-hoc comparisons test; *$p<0.05$, ***$p<0.001$, ****$p<0.0001$ (a). Wild-type BALB/c mice were adoptively transferred with 1:1 mix of GFP/luc-modified and unmodified 289 cell line (mixed), GFP/luc-modified or unmodified 289 cell line and were followed for overall survival. N=15; 5 mice per arm. Log-rank test (b)

3.7 Tolerance to ALL antigens abrogates neoantigen-induced epitope spreading

Most children with B-ALL harbor abnormal pre-leukemia clones which are detectable at birth as leukemogenesis begins *in utero* [72]. Therefore, in contrast to a transplantable model in which recipient mice are naïve to leukemia-associated antigens, children with ALL are first exposed to such neoantigens during fetal development. Thus, leukemia-associated antigens are present during a time of extensive immune tolerance induction. Similarly, leukemia in Eμ-ret mice develops following additional genetic changes within an abnormal BCP population that arises *in utero*. To evaluate how tolerance to antigens associated with early-occurring disease initiating events affects the generation of protective responses via epitope spreading, healthy Eμ-ret mice were challenged with GFP/luc-modified ALL. Unlike GFP/luc-transgenic mice that succumbed to GFP/luc-modified ALL as quickly as to unmodified ALL (Figure 3.2), Eμ-ret mice were able to respond to the GFP/luc antigens and achieve a remission state with similar kinetics to wild-type BALB/c (Eμ-ret$^-$) mice that are naïve to Eμ-ret transgene-associated antigens (Figure 3.6). While this response conferred a significant survival advantage on Eμ-ret recipients of GFP/luc-ALL compared to those recipients of unmodified ALL (median survival of 49 days and 24.5 days), respectively, Eμ-ret recipients of GFP/luc-ALL did not achieve durable remission seen with wild-type BALB/c mice (Figure 3.7).

As GFP/luc is a highly immunogenic neoantigen, a relatively weakly expressed endogenous protein, CD1d, was utilized as the target of a primary response to determine if the diversified T cell response against leukemia-associated antigens is limited to xenogeneic (GFP/luc) antigen-driven responses. Most CD1d$^{-/-}$ mice rejected ALL derived from a mouse expressing normal CD1d (CD1d$^+$ ALL), but not ALL derived from a CD1d$^{-/-}$ mouse (CD1d$^-$ ALL)

(Figure 3.8). CD1d$^{-/-}$ Eμ-ret$^+$ mice challenged with CD1d$^+$ ALL have prolonged survival compared to CD1d-naïve mice challenged with CD1d$^-$ ALL (median survival 56 versus 23 days); however, they all eventually succumb to disease. There was no difference in survival among CD1d-tolerant BALB/c mice receiving CD1d$^+$ or CD1d$^-$ ALL with median survival of 28.5 and 30 days, respectively.
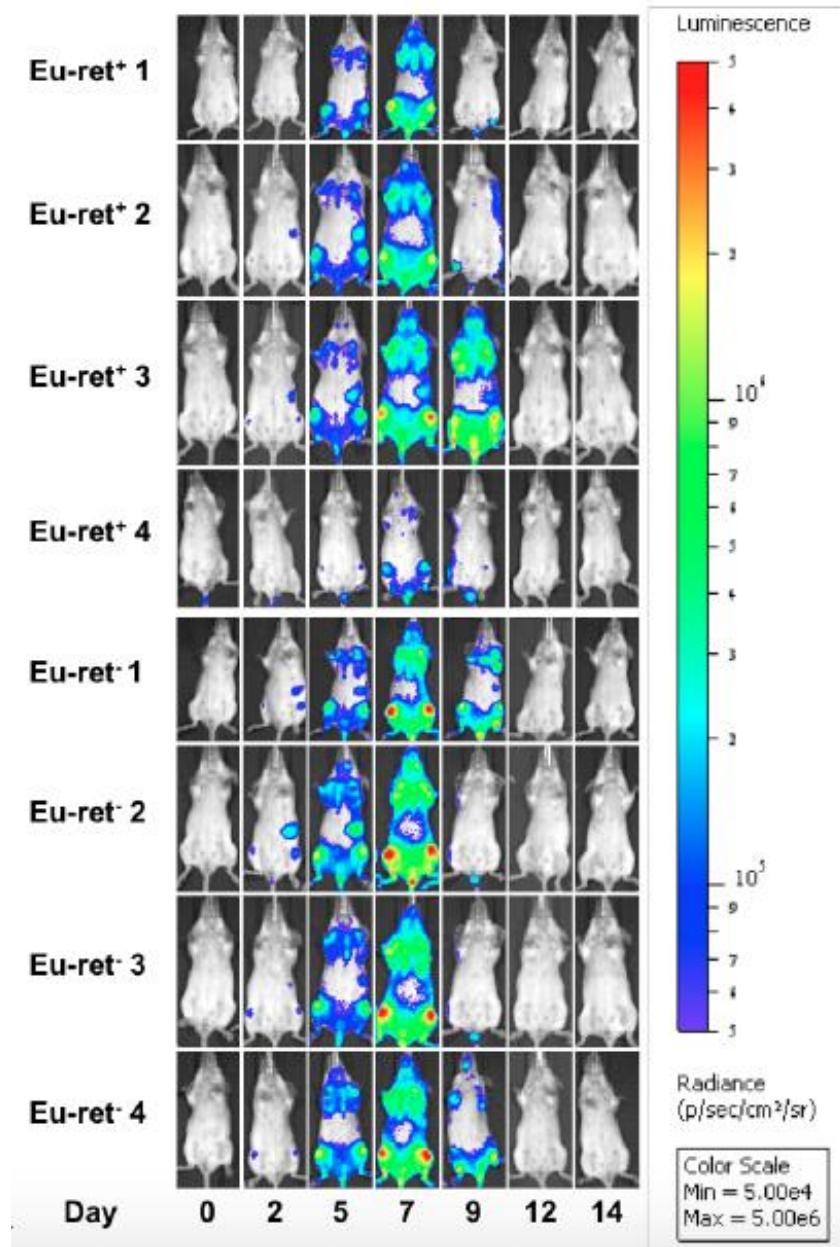
Figure 3.6. Eμ-ret mice develop an immune response to GFP/luc-modified ALL

3-4-week-old healthy Eμ-ret transgenic (Eμ-ret[+]; top 4) and wild-type (Eμ-ret[-]; bottom 4) mice were adoptively transferred with GFP/luc-modified 289 cell line. Mice were imaged every 2 to 3 days to determine the disease burden as measured by bioluminescence. N=8; 4 mice per arm. Representative study of 3 studies
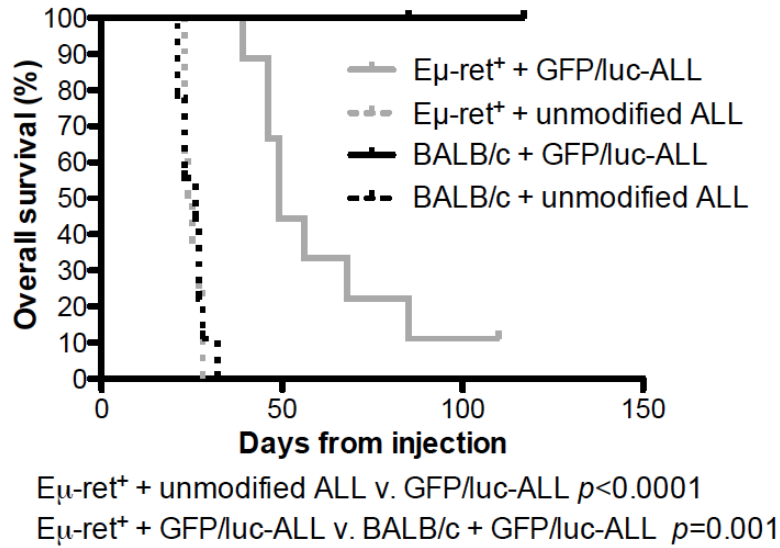
Figure 3.7. Tolerance to ALL antigens abrogates neoantigen-induced epitope spreading

3-4-week-old healthy Eμ-ret transgenic (Eμ-ret[+]) and wild-type BALB/c mice were adoptively transferred with either GFP/luc-modified or unmodified 289 cell line and were followed for overall survival. Eμ-ret[+] + GFP/luc- ALL, median survival = 49 days, range 39–110; Eμ-ret[+] + unmodified ALL, median survival = 24.5 days, range 23–28. N=35; 9 mice per arm, except for Eμ-ret[+] + unmodified ALL, n=8. Log-rank test
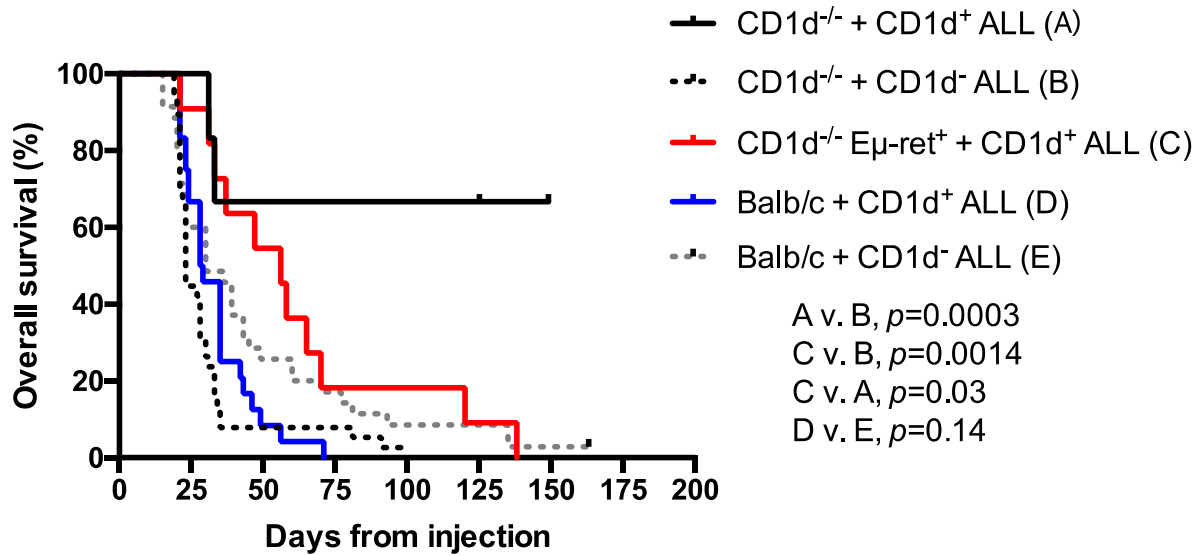
Figure 3.8. A syngeneic protein can function as a neoantigen in an antigen-naïve mouse
Wild-type BALB/c, CD1d$^{-/-}$, and 3-4-week-old healthy CD1d$^{-/-}$ Eμ-ret transgenic (CD1d$^{-/-}$ Eμ-ret$^+$) mice were adoptively transferred with either primary ALL derived from a mouse expressing normal CD1d (CD1d$^+$ ALL) or a CD1d$^{-/-}$ mouse (CD1d$^-$ ALL) and were followed for overall survival. CD1d$^{-/-}$ + CD1d$^-$ ALL (B; n=38), median survival = 23 days; CD1d$^{-/-}$ Eμ-ret$^+$ + CD1d$^+$ ALL (C; n=11), median survival = 56 days; BALB/c + CD1d$^+$ ALL (D; n=24), median survival = 28.5 days; BALB/c + CD1d$^-$ ALL (E; n=35), median survival = 30 days. N=114; CD1d$^{-/-}$ + CD1d$^+$ ALL (A), n=6. Log-rank test

3.8   Checkpoint blockade partially restores neoantigen-driven protection in ALL antigen-tolerized mice

Despite their ability to respond to the GFP/luc antigens, the significantly reduced survival observed in Eµ-ret mice compared with their wild-type BALB/c counterparts indicates immunological tolerance to leukemia-associated antigens may hinder diversification of immune response required for achieving durable remission. Given the capacity of CTLA-4 blockade to generate diverse antigen-specific immune responses [285,287], the impact of this checkpoint inhibitor was investigated in the Eµ-ret transgenic mouse model. Again, Eµ-ret recipients of GFP/luc-ALL ultimately succumbed to disease, however, treatment with anti-CTLA-4 antibody (200 µg/dose) doubled the median survival (Figure 3.9). The median survival of anti-CTLA-4 mAb-treated Eµ-ret recipients was 80 days, while that of PBS-treated controls was 37 days.
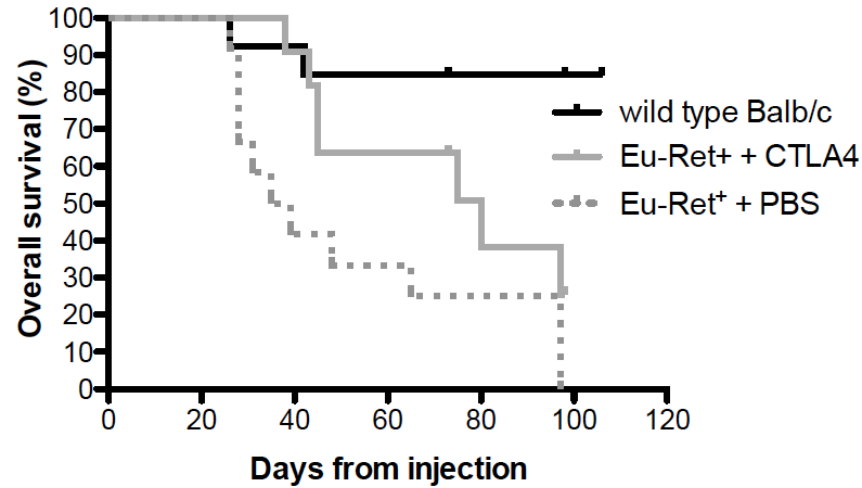
Eu-ret+ median survival: CTLA4 80d v PBS 37d, p=0.053

Figure 3.9. Checkpoint blockade enhances neoantigen-driven protective immune responses in Eμ-ret transgenic mice

3-4-week-old healthy Eμ-ret transgenic (Eμ-ret+) and wild-type BALB/c mice were adoptively transferred with GFP/luc-modified 289 cell line. On days 7, 10, and 14 after ALL adoptive transfer, Eμ-ret recipients were given PBS or CTLA-4 antibody blockade intraperitoneally and were followed for overall survival. N=36; BALB/c, n=13; Eμ-ret+ + CTLA-4, n=11; Eμ-ret+ + PBS, n=12. Pooled from three independent experiments. Log-rank test.

3.9   Discussion

CD19-directed CAR T cell therapy has become the paradigm of successful use of genetic engineering of T cells in cancer, promoting ACT from the fringes to the mainstream of cancer therapy [288]. Despite its unprecedented success, CAR T cell therapy has also uncovered the emergence of antigen-loss variants as a mechanism of immune escape limiting the efficacy of single antigen-targeted treatments. Currently, loss of CD19 expression due to the strong selective pressures introduced by the infused engineered T cells is the major cause of resistance to CD19-directed CAR T cells [224]. Unlike CD19-positive relapses, which occur within the first 3 months after infusion and are commonly associated with early loss of CAR T cells, CD19-negative relapses occur in the sustained presence of detectable CAR T cells [155,215]. Thus, rather than prolonging the persistence of CAR T cells *in vivo*, which in essence prolongs the exposure of tumor cells to selective pressures, new strategies that broaden the spectrum of T cell response against ALL-associated antigens initiated by CAR T cells have more potential to minimize the risk of immune escape, thereby reducing the incidence of relapse [289]. Therefore, better understanding of T cell-mediated immune activities necessary for achieving durable remission is required for the maximal potential of CAR T cell-based ACT to be realized.

The results outlined in this chapter demonstrate the ability of a neoepitope-targeted immune response to generate long-term protection against B-ALL by expanding immune activity to previously non-immunogenic, and as yet unidentified, tumor antigens via epitope spreading. The initiating role of the immune response directed against the GFP/luc immunogenic neoantigens is revealed by the failure to achieve remission in neoantigen-tolerized mice. Notably, the finding that the development of GFP/luc-negative disease is more common in late rather than early relapses in our neoantigen-driven remission model suggests that once immune control is

achieved, loss of the immunogenic antigen is required for immune escape in most cases. These

late relapse cases also suggest that, in at least a subset of recipient mice, leukemic blasts are not

entirely eliminated by the initial immune response, but held in equilibrium by ongoing long-term

immune surveillance until an immune evasive clone emerges.

Our results suggest that while immunogenic neoantigens are required to initiate ALL-

directed T cell responses, protection is associated with the generation of additional responses to

other leukemia-associated antigens, a diversification process which occurs quickly after the

primary response. The significantly reduced survival of Eμ-RET mice compared with their wild-

type BALB/c counterparts, however, demonstrates that immune tolerance established towards

antigens associated with early-occurring disease-initiating events may hinder neoantigen-induced

epitope spreading, further highlighting the importance of a broad immune response against

multiple ALL antigens to achieve long-term remission. In line with this, the sustained presence

of cells harboring leukemia-associated translocations in healthy neonates and adults is consistent

with the lack of an effective immune response against the neoantigens generated by gene fusions

[79,290]. We also demonstrated that this epitope spreading-dependent durable protection is not

limited to a xenogeneic antigen-driven response. While our results do not demonstrate the

mechanism of action, the partial restoration of a protective immune response by CTLA-4

blockade in our tolerized model demonstrates the ability of checkpoint blockade to enhance

immune responses against ALL, providing additional rationale for combining antigen-directed

immunotherapies with checkpoint inhibitors to maximize clinical benefit. While the evidence of

epitope spreading in patients who have achieved durable immune-mediated cancer control

following single antigen-targeted or checkpoint blockade-mediated interventions has been

increasingly reported[275], my results indicate that the therapeutic efficacy of epitope spreading is

limited in an immunological setting where immune tolerance to leukemia-associated antigens has been established early in the course of leukemia progression, which is commonly the case in the clinical setting. Nonetheless, the clinical data and our results suggest that the generation of *de novo* tumor-directed killing combined with the induction of anti-tumor immune against a broad range of TAAs is necessary for the success of current target-directed cancer immunotherapies. To date, there are no published data demonstrating epitope spreading in patients who have received single antigen-targeted therapies for leukemia. The subset of patients that remain in remission without CD19-negative relapse, however, could possibly reflect the mechanistic role of epitope spreading in propagating diversified secondary immune responses that persist, thereby reducing the risk of resistance induced by antigen loss.

The demonstration that induction of T cell responses to non-immunogenic epitopes contributes to durable protection in the ALL setting, which was also recently reported in the KPC mouse model of pancreatic ductal adenocarcinoma (PDA) [291], has clear clinical implications. In contrast to adult epithelial tumors, which are neoantigen-rich as result of high mutational burden, ALL and PDA are both characterized by low mutational burden and show little evidence of immunoediting [193,291,292]. The relatively small number of secondary genetic events needed to drive full transformation in ALL, which results in a low number of neoepitopes that can be targeted immunologically, likely limits the potential range of epitope spreading. However, our study indicates that enhanced protection by epitope spreading to otherwise immunologically quiescent endogenous antigens is achievable in ALL and could potentially generate the immunological memory that sustains remission. This suggests that the immunogenicity of specific changes induced by secondary genetic events, whether via mutation or dysregulated expression, might influence the effectiveness of targeted immunotherapies.

Notably, the neoantigen-driven remission model utilized in this study provides a basis for future investigations of epitope spreading in the ALL setting with high consistency and reproducibility as the choice of experimental model greatly influences the sensitivity of detection of diversification of immune responses. The use of immunocompetent wild-type recipient mice that are naïve to either transgene or TAAs, although otherwise syngeneic, will likely overstate the protective benefit, while the use of immunodeficient xenograft models will fail to reveal potentially enhanced therapeutic immune activity induced by epitope spreading. Recently, a rapid induction of T cell dysfunction by progressive leukemia that nullified the efficacy of a therapeutic vaccine in a syngeneic transplantable TCF3/PBX1-driven murine model of pre-B cell ALL has been reported [293]. Contrary to a new report from long-term follow-up of CD19-directed CAR T cell therapy in B-ALL that revealed that patients with a low disease burden prior to treatment had a significantly enhanced remission duration and survival than did patients with a higher disease burden [294], even minimal levels of ALL present during early leukemia progression reduced the functionality of CAR T cells generated from TCF3/PBX ALL-bearing donors [293]. Given the partial enhancement of protective immune responses with CTLA-4 blockade, progressive, rather than rapid, functional impairment of T cells due to prolonged exposure to leukemia-associated antigens may also contribute to the failure to achieve remission in the Eµ-ret mouse model.

Chapter 4: Early bone marrow response predicts outcome of systemic administration of endosomal TLR agonist against syngeneic B-ALL

4.1   Overview and rationale

Contemporary risk-adapted multi-agent chemotherapy with integration of presymptomatic CNS prophylaxis has led to a dramatic improvement in survival for children with B-ALL [10]. Nonetheless, the prognosis for those with relapsed or therapy refractory disease has not improved much over recent decades, and remains a significant clinical challenge [295]. The early response to initial remission-induction therapy, whether determined by bone marrow or peripheral blood assessment, has long been used for prediction of outcome in B-ALL [296,297]. More recently, monitoring of MRD has become routine clinical practice in frontline treatment of B-ALL, particularly in the pediatric setting [298]. The assessment of MRD provides an estimate of the reduction of disease burden in bone marrow at various time-points after therapy. To date, the presence of MRD at the end of remission-induction therapy is the strongest predictor of relapse in both pediatric and adult B-ALL [98,99,299].

Leukemia cells present a diverse range of antigens, which can elicit leukemia-specific T cell responses, as evident by identification of leukemia antigen-specific CTLs in patients [300–304]. Thus, leukemias appear to often progress under some form of immune control that was inadequate to prevent the development of overt disease. B-ALL exhibits the low mutational burden common to other pediatric cancers which may result in a paucity of neoantigens available for recognition by host T cells [124,187]. A transient exposure or release of a broad range of leukemia antigens as a result of chemotherapy-mediated cell death, however, could significantly

increase the availability of antigens required for triggering effective anti-leukemia immune responses as part of its intended therapeutic effect [251,305]. If such chemotherapy-induced cell death is immunogenic (characterized by release of DAMPs by dying cells), the initial remission-induction therapy may further contribute to the elicitation of therapeutically relevant leukemia antigen-specific immune responses associated with immunological memory [306–308]. Combining the beneficial effects of chemotherapy with leukemia antigen-specific immune response, therefore, may lead to synergistic clinical activity. Intensive and prolonged chemotherapy, however, affects not only blast cells, but also normal hematopoiesis and function of immune system [309]. Although chemotherapy causes severe B cell depletion that resolves only gradually, the T cell compartment recovers rapidly and remains relatively intact functionally during chemotherapy in children with standard-risk or intermediate-risk ALL [309–311]. Given the characteristic of MRD, a relatively low-level residual disease localized in bone marrow, chemotherapy-induced damage to immune system may be offset by a high effector-target ratio potentially achieved by marked reduction in the leukemia load. Use of an immune stimulant to induce robust leukemia antigen-specific immune responses capable of sustaining remission following chemotherapy may achieve effective immune-mediated control of MRD-positive disease.

Despite the limited neoantigenicity and weak immunogenicity of B-ALL blasts [312–315], we previously reported the ability of CpG ODN, an agonist for TLR9, to induce anti-leukemia activity that achieved T cell-dependent durable protection against outgrowth of transplanted syngeneic ALL cell lines [272]. CpG ODN also reduced the burden of primary human B-ALL in mouse xenografts, primarily via induction of NK cell-mediated cytotoxicity [316]. Our recent demonstration that endosomal TLR agonists generate distinct effects on pre-leukemic BCP cells

[317], coupled with the divergent effects of TLR agonists on the immunogenicity of primary human B-ALL cells [314,316,318], suggest that there are significant variables that contribute to the outcome of TLR stimulation in the context of BCP cell malignancy. These findings formed the basis for my comparative evaluation of the endosomal TLR agonists, poly I:C (TLR3), R848 (TLR7/8), and CpG ODN (TLR9), for their ability to elicit control of primary ALL cells, and identification of immune modulation required for achieving long-term protection in Eμ-ret transgenic mouse models of B-ALL [89,90] in an organ-specific manner.

As the subsequently detailed work illustrates, I found that a single-dose systemic administration of endosomal TLR agonists is sufficient to induce rapid innate immune-mediated depletion of transplanted B-ALL cells in an organ-specific manner. Among the endosomal TLR agonists, CpG ODN, which achieved a complete elimination of leukemia cells particularly in bone marrow with highest consistency, conferred the most significant enhancement of long-term control of B-ALL. Overall, our findings support that systemic administration of CpG ODN in the context of B-ALL may feasibly contribute to eradication of bone marrow MRD.

4.2   Direct and indirect cytotoxic effects of endosomal TLR agonists on B-ALL *in vitro*

Recent studies have shown that the expression of TLRs is not restricted to normal cells but is also found in various malignant cells, including B-ALL cells [314,319–321]. TLR agonists, therefore, can exert both direct and indirect (immune-mediated) effects on cancer cells. In spite of *TLR9* expressions on B-ALL cells, CpG ODN-induced reduction in ALL burden is via immune-mediated effects rather than direct cytotoxicity of CpG ODN to leukemic blasts [272]. Having confirmed the expression of TLR3 and 7-9 on primary Eμ-ret BCP ALL cells (

Figure 4.1), I performed a 16-hour cytotoxicity assay in which primary B-ALL cells were stimulated with CpG ODN, R848, or poly I:C in the presence or absence of syngeneic splenocytes to compare direct and immune-mediated cytotoxic effects of TLR agonists on B-ALL cells. All three TLR agonists exerted a minimal direct cytotoxicity on these cells as measured by changes in cell viability (Figure 4.2). In the presence of splenocytes, B-ALL viability was reduced but only achieved significance with R848. In line with previous reports, these findings suggest that any anti-leukemic activity induced by endosomal TLR agonists *in vivo* is likely to be mediated via immune cell activation that is independent of direct toxicity on ALL cells.
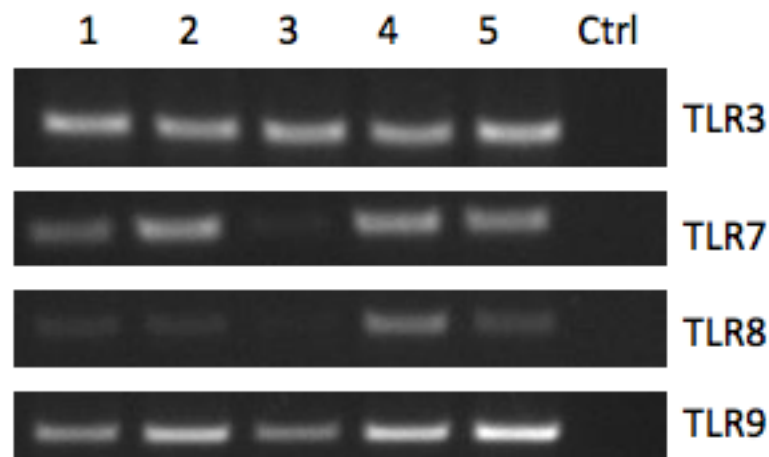
Figure 4.1. TLR3 and TLR7-9 are expressed on mouse primary B-ALL cells
Primary B-ALL cells harvested from spleen of five Eµ-ret mice (1-5) were analyzed for the mRNA expression of TLRs 3 and 7-9 using standard reverse transcription polymerase chain reaction. The negative control indicated contained no RNA in the RT-PCR reaction.
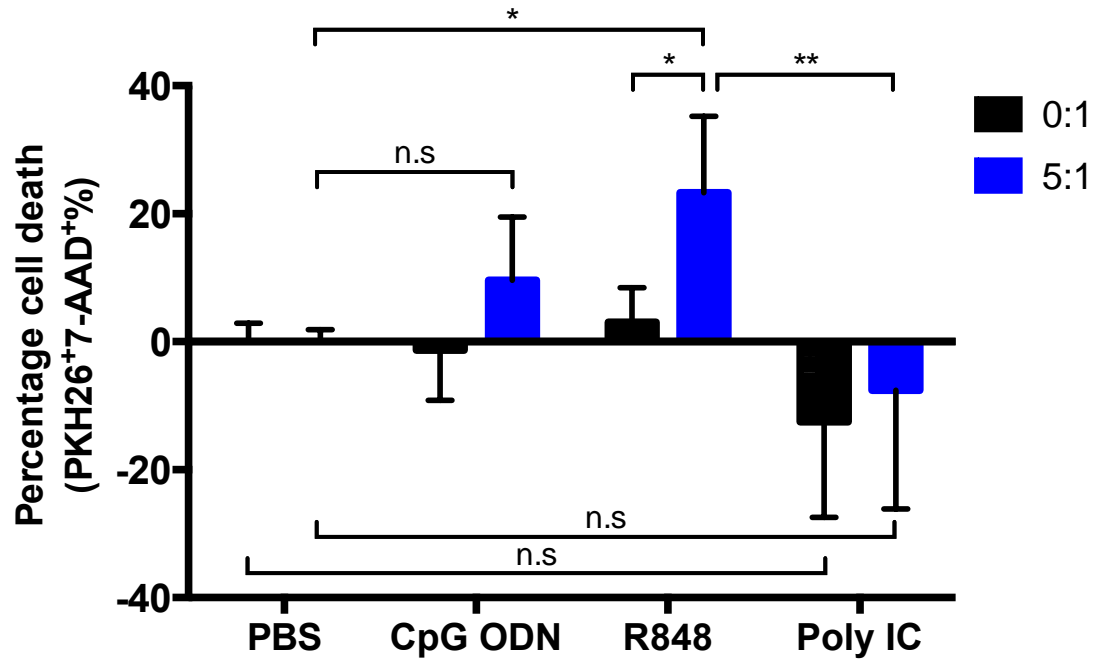
Figure 4.2. Direct and immune-mediated cytotoxic effects of endosomal TLR stimulation on mouse primary B-ALL cells *in vitro*

Direct and immune-mediated cytotoxic effects following 16 hours of endosomal TLR stimulation on primary B-ALL cells, pre-labeled with PKH26, in the presence or absence of splenocytes harvested from wild-type BALB/c mice at 5:1 effector to target ratio. Results shown are pooled from four independent experiments. Tukey's multiple comparisons test, bars represent mean±S.D.; *$p<0.05$, **$p<0.01$. n.s = not significant

4.3   Endosomal TLR-induced innate immune activity is sufficient for rapid depletion of B-ALL cells

As rapidity of response to the initial therapy has long been served as a useful surrogate for outcome in B-ALL [296,297], I evaluated the early treatment response to systemic administration of endosomal TLR agonists in B-ALL-bearing wild-type and RAG$^{-/-}$ BALB/c mice (lacking T, B, and NKT cells) by assessing disease burden in an organ-specific manner. At day 7 after challenge with syngeneic primary BCP ALL cells, a period sufficient for bone marrow engraftment of transferred BCP ALL cells, mice were randomized to receive a single dose of CpG ODN (100 µg), R848 (100 µg), poly I:C (100 µg), or PBS intraperitoneally. Three days after the treatment, spleen and bone marrow were harvested from mice and assessed for disease burden. A single dose of TLR agonist was sufficient to stimulate anti-ALL immune activity capable of depleting B-ALL cells in the spleen of both wild-type and RAG1$^{-/-}$ mice, with CpG ODN inducing the strongest response in both strains (Figure 4.3a). However, a significant reduction in disease burden in bone marrow was only achieved with CpG ODN treatment; its occurrence in both wild-type and RAG1$^{-/-}$ mice confirms the independence of this early immune activity from recombination-dependent immune cell subsets (Figure 4.3b). As a trend towards reduction in disease burden was observed in the bone marrow of R848- and poly I:C-treated mice, titration experiments were performed for dose optimization. While the response in spleen did not further improve with higher dose (200 µg), a statistically significant and a trend towards increased depletion of B-ALL were observed with R848 and poly I:C, respectively, in bone marrow of treated mice (Figure 4.4a-d).

To validate the potential clinical relevance of this pattern of immune stimulation in the diverse niche capable of supporting human ALL, I treated NOD/SCID mice xenografted with

83

primary human ALL with TLR agonists. While each TLR agonist trended towards reduced

systemic disease burden compared to PBS-treated control mice, again only CpG ODN treatment

led to significantly reduced disease burden systemically, and in bone marrow at low dose (Figure

4.5a-b).
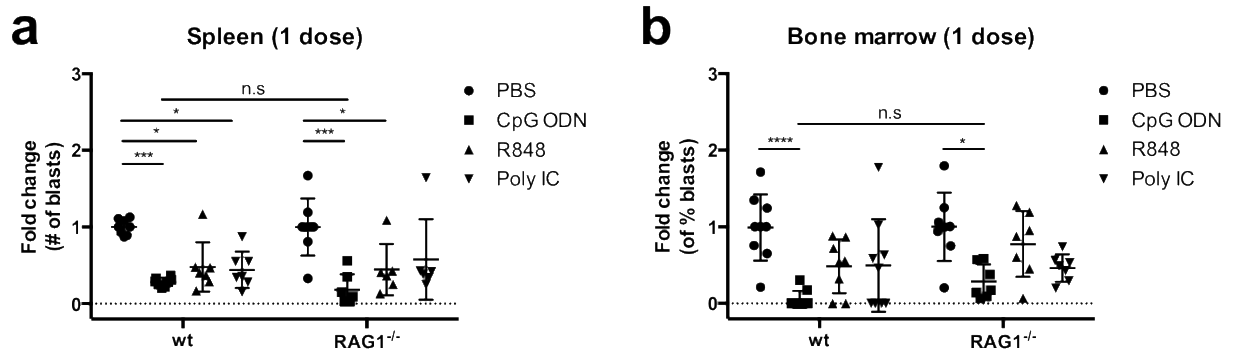
Figure 4.3. Endosomal TLR-mediated innate immune activity is sufficient to initiate a rapid control of mouse leukemia progression

Wild-type and RAG$^{-/-}$ BALB/c mice bearing syngeneic primary B-ALL cells randomly received a single dose of indicated TLR agonists on day 7. Three days after the treatment, the disease burden in the spleen (a) and bone marrow (b) were evaluated. Results shown are pooled from four independent experiments; PBS-treated wild-type (n=8) and RAG$^{-/-}$ BALB/c (n=8); CpG ODN-treated wild-type (n=8) and RAG$^{-/-}$ BALB/c (n=7); R848-treated wild-type (n=7) and RAG$^{-/-}$ BALB/c (n=6); Poly I:C-treated wild-type (n=7) and RAG$^{-/-}$ BALB/c (n=6). Tukey's multiple comparisons test, bars represent mean±S.D.; *p<0.05, ***p<0.001, ****p<0.0001. n.s = not significant
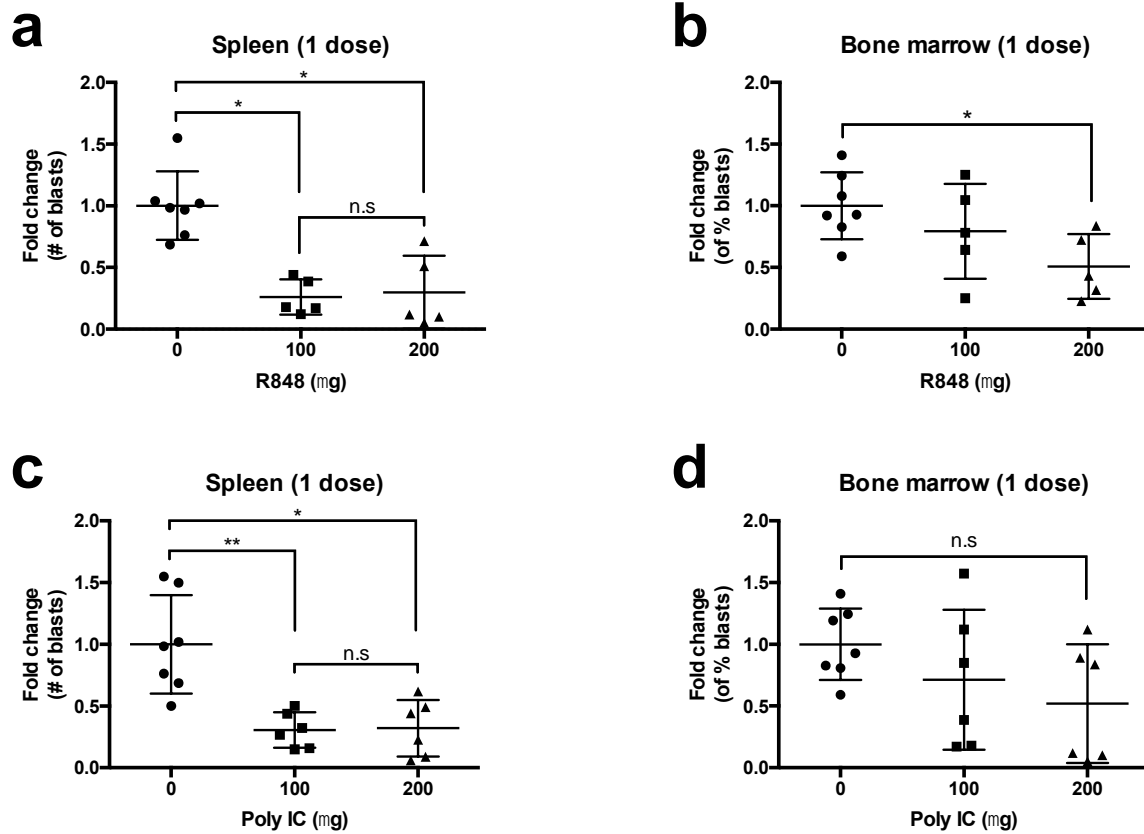
Figure 4.4. A dose-dependent efficacy of single-dose administration of R848 and poly I:C
Wild-type BALB/c mice bearing syngeneic primary B-ALL cells or 289 cell line received a
single dose (100 μg or 200 μg) of indicated TLR agonists. Three days after the treatment, the
disease burden in the spleen and bone marrow of poly I:C- (a-b) and R848-treated mice (c-d)
were evaluated. Results shown are pooled from two independent experiments; PBS-treated mice
(n=7); Poly I:C-treated mice (n=6/dosage); R848-treated mice (n=5/dosage). Dunn's multiple
comparisons test, bars represent mean±S.D.; *$p<0.05$. n.s = not significant

a Systemic (3 doses)

b Bone marrow (3 doses)
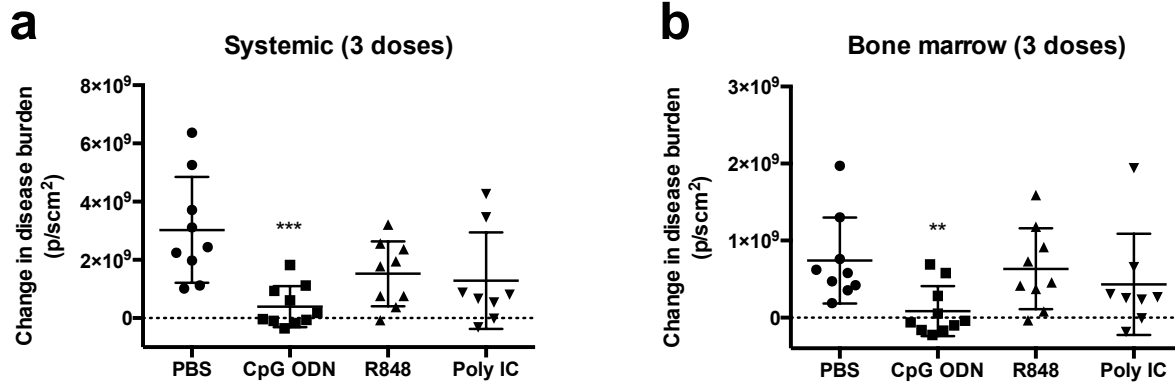
Figure 4.5. TLR-mediated innate immune response is sufficient to initiate control of human leukemia progression

NOD/SCID mice bearing human primary ALL expressing GFP/luc were treated with the indicated TLR agonists. Disease burden systemically (a), and in bone marrow (b) were evaluated by *in vivo* bioluminescence imaging. Results shown are pooled from four independent experiments. PBS-treated NOD/SCID (n=9); CpG ODN-treated NOD/SCID (n=10); R848-treated NOD/SCID (n=9); Poly I:C-treated C57BL/6 (n=8). Dunn's multiple comparisons test, bars represent mean±S.D.; *$p<0.05$, ***$p<0.001$.

4.4 Immunostimulatory effects of a single, systemic dose of endosomal TLR agonists in B-ALL-bearing mice

To determine whether immunostimulatory effects associated with endosomal TLR-induced early depletion of B-ALL cells provides sufficient signals necessary for priming of leukemia antigen-specific immune responses associated with immunological memory, we then evaluated the organ-specific immunostimulatory effects of systemic administration of single-dose endosomal TLR ligands in B-ALL-bearing mice. A rapid upregulation of various activation markers on macrophages ($F4/80^+CD11b^+CD11c^-$) were exclusively observed in CpG ODN-treated mice (Figure 4.6a-d); while the expression levels of CD40 and CD80 were only upregulated in the spleen, MHC-II molecules and CD86 were upregulated in both spleens and bone marrow of CpG ODN-treated mice. The strongest activation of NK cells ($CD69^+CD335^+$) was observed again in both bone marrow and spleens of CpG ODN-treated mice (Figure 4.7a). CpG ODN and poly I:C, but not R848, triggered activation of both $CD4^+$ T cells ($CD3^+CD4^+CD69^+$) (Figure 4.7b) and $CD8^+$ T cells ($CD3^+CD8^+CD69^+$) (Figure 4.7c) in the spleens of treated mice. Moreover, the number of activated pDCs ($CD11c^{int}B220^+CD11b^-Ly-6C^{int}MHC-II^+$) rapidly increased in spleen of all treated-mice; the highest and lowest increase were observed in CpG ODN- and poly I:C-treated mice, respectively (Figure 4.7d). Finally, the number of $CD11b^+$ cDCs ($CD11c^+B220^-MHC-II^+$) was increased only in the bone marrow of CpG ODN-treated mice (Figure 4.7e).

To determine the cytokine response associated with the observed cellular responses as well as reduced disease burden, I measured the presence of key pro-inflammatory cytokines in peripheral blood after a single dose of TLR agonist. After 16 hours, mice treated with each TLR agonist secreted TNF-α, IL-6, IFN-γ, and IL-12p70 (Figure 4.8a-d), but in varying amounts;

only CpG ODN-treated mice produced statistically significant levels of these pro-inflammatory cytokines.
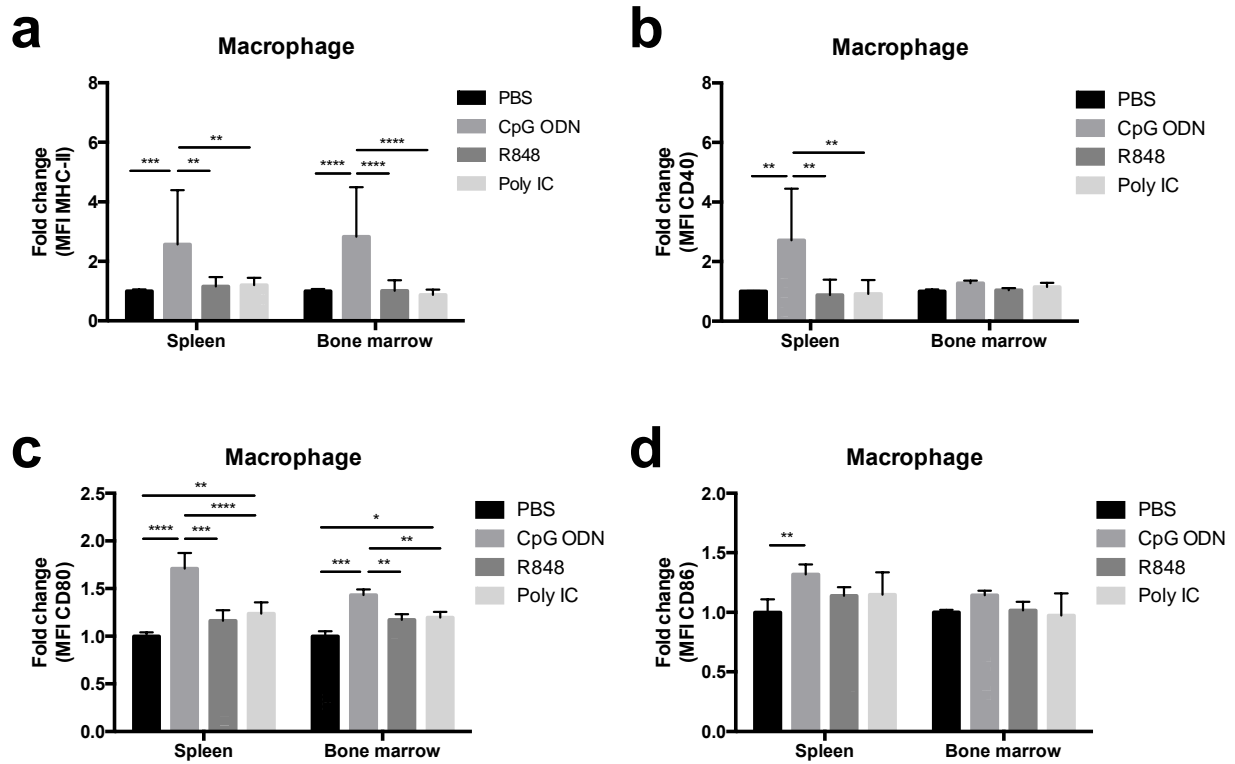
Figure 4.6. CpG ODN induces a rapid upregulation of MHC-II and co-stimulatory molecules on macrophage of mice bearing B-ALL

The expression of activation markers on macrophages three days after a single-dose treatment with endosomal TLR agonists in B-ALL-bearing wild-type BALB/c mice. The fold change in the expression levels of MHC-II (a), CD40 (b), CD80 (c) and CD86 (d) on macrophages bone marrow and spleen were measured. The geometric MFI is shown. Tukey's multiple comparisons test, bars represent mean±S.D.; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Results shown are pooled from four independent experiments; PBS-treated mice (n=15); CpG ODN-treated mice (n=14); R848-treated mice (n=15); Poly(I:C)-treated mice (n=17)

a NK cells

b CD4+ T cells (Spleen)

c CD8+ T cells (Spleen)

d Plasmacytoid DCs (Spleen)

e cDCs
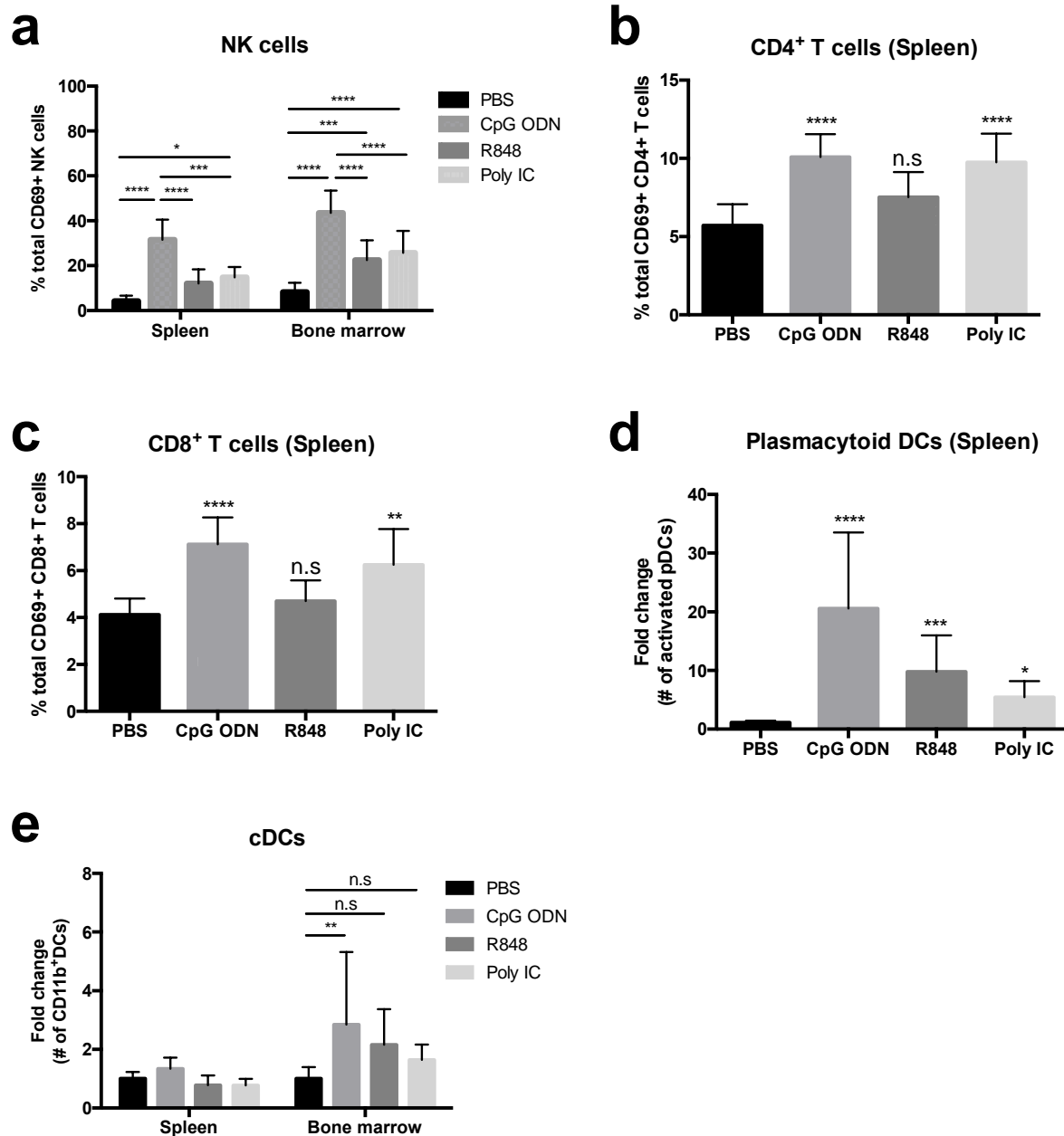
Figure 4.7. Low-dose CpG ODN is sufficient to exert potent immunostimulatory effects in mice bearing B-ALL

Three days after a single-dose treatment with endosomal TLR agonist, the percentage or number of activated innate immune cells in spleens and/or bone marrow of wild-type BALB/c mice bearing syngeneic primary BCP ALL cells were measured. The percentage of activated NK cells among total viable NK cells in spleen and bone marrow (a). The percentage of activated CD4+ T

cells (b) and CD8$^+$ T cells among total viable CD4$^+$ T and CD8$^+$ T cells, respectively, in spleen. Fold change in the absolute number of activated pDCs in spleen (d) and CD11b$^+$ cDCs (e) in spleen and bone marrow. Tukey's (a and e) and Dunn's (b-d) multiple comparisons tests, bars represent mean±S.D.; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Results shown are pooled from four independent experiments; PBS-treated mice (n=15); CpG ODN-treated mice (n=14); R848-treated mice (n=15); Poly(I:C)-treated mice (n=17). n.s = not significant
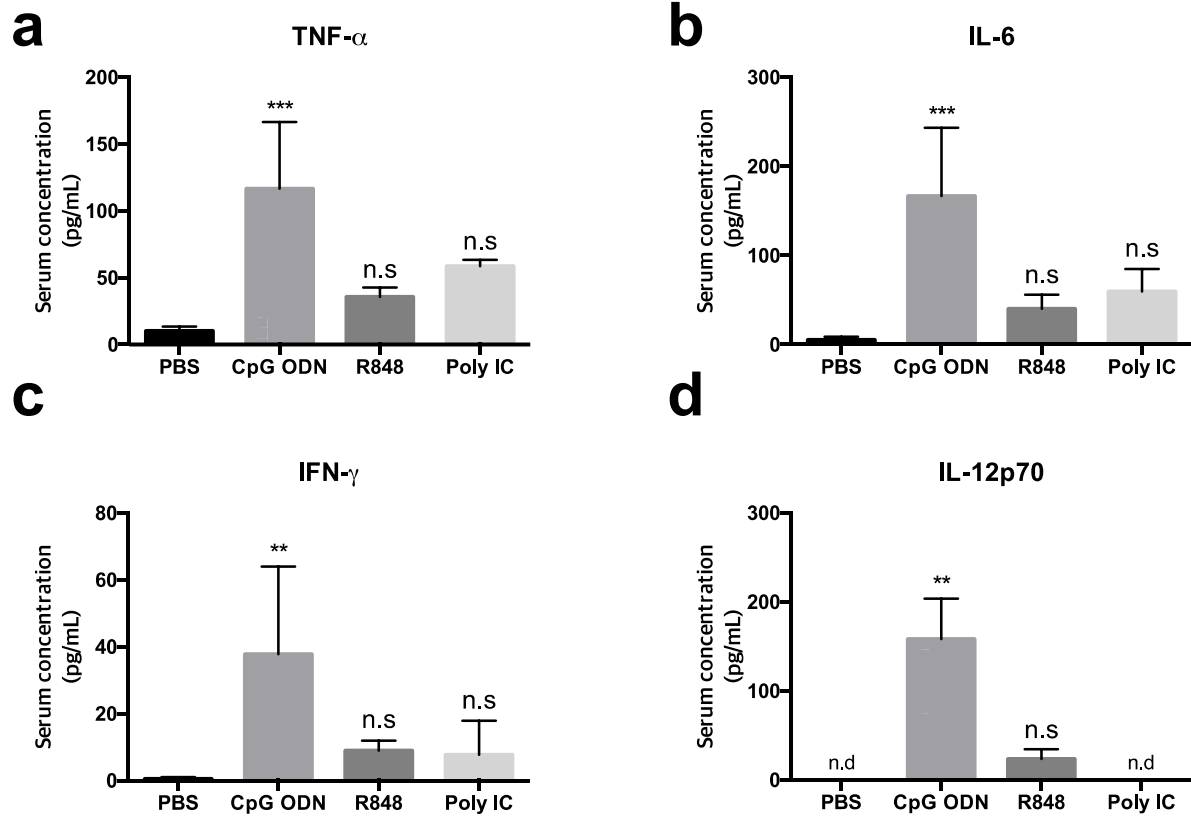
Figure 4.8. CpG ODN induces a robust pro-inflammatory cytokine response

Serum concentration of TNF-α (a), IL-6 (b), IFN-γ (c), IL-12p70 (d) were measured in wild-type BALB/c mice 16 hours after treatment. Results are shown in averages of duplicate wells; n = 4 mice per group for PBS- and R848-treated and n = 5 mice per group for CpG ODN- and poly I:C-treated wild-type BALB/c. Dunn's multiple comparisons test, bars represent mean±S.D.; **$p<0.01$, ***$p<0.001$. n.d = not detected

4.5 Systemic administration of endosomal TLR agonists achieves control of ALL progression *in vivo*

A 3-dose CpG ODN treatment regimen is sufficient to achieve long-term protection against outgrowth of transplanted syngeneic ALL cell lines [272]. To further investigate the prognostic significance of early bone marrow response to endosomal TLR agonists in terms of remission achievement, as well as the overall efficacy of TLR-induced anti-ALL immune activity against primary B-ALL, I administrated TLR agonists to B-ALL-bearing wild-type mice every 4 days for 3 doses starting at day 7 after the leukemia injection. For half of the mice in each group, disease burden in peripheral blood, spleen, and bone marrow were evaluated six days after the last treatment, which may serve as a reliable surrogate for outcome. Each TLR agonist achieved significant reduction of disease burden in all three sites, but did so to varying degrees (Figure 4.9a-c); R848 and poly I:C treatment failed to match the near-complete elimination of BCP ALL cells achieved with CpG ODN treatment, most prominently in bone marrow. The remaining mice in each treatment group were followed for disease progression. In line with our previous reports using BCP ALL cell lines, CpG ODN treatment conferred a significant survival advantage, in which over 50% of treated mice achieved long-term disease-free survival (Figure 4.9d). Although significantly smaller than in CpG ODN-treated mice, a modest increase in disease-free survival was observed in poly I:C- and R848-treated mice, with median survival of 42 days and 40 days, respectively, compared to that of 28.5 days of PBS-treated control mice.
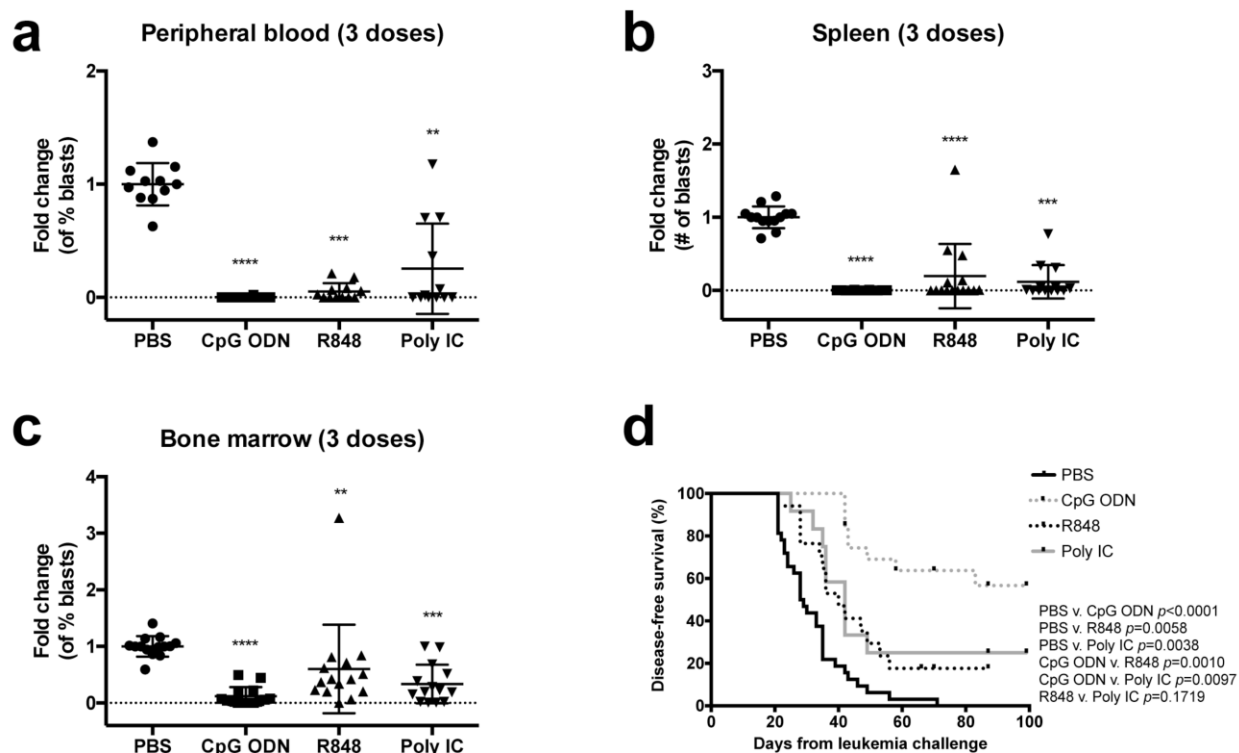
Figure 4.9. TLR-mediated immune stimulations induce protective immune responses in mice bearing established leukemia

Wild-type BALB/c mice bearing syngeneic primary B-ALL cells were randomly treated with indicated TLR agonists and evaluated for disease burden in peripheral blood (a), spleen (b), and bone marrow (c) six days after the last treatment, or for survival (d). Results shown are pooled from five independent experiments. PBS-treated mice (n=11), median survival = 28.5 days; CpG ODN-treated mice (n=16); R848-treated mice (n=16), median survival = 40 days; Poly I:C-treated mice (n=16), median survival = 42 days. Dunn's multiple comparisons test, bars represent mean±S.D.; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (a-c). Log-rank test (d)

4.6 Discussion

Since the FDA approval of checkpoint inhibitors, several key preclinical studies have reported a synergistic effect of TLR agonists and immune stimulatory or inhibitory checkpoint antibodies in combination therapies [236,270,271]. Many new clinical trials, mostly applying TLR agonists in combination with other therapeutic agents, are underway [260]. We previously reported the ability of CpG ODN, an agonist for TLR9, to induce anti-leukemia activity that achieved T cell-dependent durable protection against outgrowth of transplanted syngeneic B-ALL cell lines. Given MRD is a low-level disease localized in bone marrow, induction of robust leukemia antigen-specific immune responses associated with immunological memory may offer an effective immune-mediated control of MRD-positive disease.

The results outlined in this chapter demonstrate that a single-dose systemic administration of endosomal TLR agonists is sufficient to induce rapid innate immune-mediated depletion of transplanted BCP ALL cells in an organ-specific manner. While dying and killed leukemia cells in this context may become the source of antigens for the subsequent protective adaptive response, not all forms of cell death are immunogenic and lead to the priming of leukemia antigen-specific immune response associated with the establishment of immunological memory [322]. The robust induction of cellular and pro-inflammatory cytokine responses under our experimental conditions provides evidence that the immunostimulatory effects exerted by CpG ODN produces necessary signals for priming productive T cell response required for durable control of BCP ALL. After the 3-dose regimen, a significant reduction of disease burden was achieved in both peripheral blood and spleen with all three treatments. Notably, a complete eradication of leukemia cells in bone marrow was observed following CpG ODN treatment with a high consistency. Despite the capacity of each endosomal TLR agonist to induce an early

reduction in leukemia burden leading to extended disease-free survival, CpG ODN conferred the most significant enhancement of long-term control of BCP ALL. The inability of poly I:C and R848 to achieve durable protection despite the effective depletion of B-ALL cells from spleen and peripheral blood implicates induction of immune activity in the bone marrow as a key requirement for sustained protection.

The rapid *in situ* destruction of blasts by systemic administration of TLR agonists is likely to result in the release of additional leukemia-associated antigens recognizable by immune effector cells. Nevertheless, the minimal induction of cellular and cytokine responses following R848 and poly I:C treatments imply the insufficient induction of immunostimulatory signals to promote cross-priming under our experimental settings. The ability of R848 and poly I:C to act as immunostimulatory adjuvants, however, is well-established [260,323,324]. Given the observed improvement in bone marrow response with higher dose (200 μg), the use of suboptimal dose (100 μg), therefore, may have limited the magnitude of immunostimulatory effects exerted by R848 and poly I:C under our experimental conditions.

Overall, the findings presented in Chapter 4 provide further support for the need for a combined induction of rapid innate immune-mediated anti-leukemia response and the subsequent T cell-mediated immune response for initial and durable control of B-ALL, respectively. While this study identified several key immune components associated with durable CpG ODN-induced remission, the full pathway for CpG-ODN-induced anti-leukemia immune activity remains to be identified. Results presented in Chapter 3 highlights breaking immune tolerance established early in life towards B-ALL antigens as a major clinical challenge to sustaining remission. One limitation to the adoptive transfer setting utilized in this study, therefore, is the use of recipients naïve, rather than immune tolerized, to B-ALL-associated antigens. Further

validation of the therapeutic effects of CpG ODN in more stringent models of ALL that correspond to patient settings, such as Eμ-ret mice, will have more clinical relevance. The poor antigen presentation capacity of B-ALL blasts, in concert with rapid induction of T cell dysfunction by progressive leukemia may contribute to the failure of immune therapy in many ALL patients [293,312,315]. Together with the results presented in Chapter 3, particularly the ability of CTLA-4 blockade to partially break immune tolerance in the Eμ-ret mouse model, the potent immunostimulatory activity of CpG ODN demonstrated in this chapter provide potentially complementary therapeutic approaches to overcome T cell functional impairments, as well as immune tolerance to leukemia-associated antigens. Collectively, these results provide additional support for investigating checkpoint inhibitors in combination with TLR agonists to maximize the therapeutic efficacy.

Chapter 5: The influence of natural killer T cells on acute lymphoblastic leukemia progression

5.1 Overview and rationale

Polymorphisms in genes of adaptive and innate immunity both have been associated with increased risk or reduced latency of B-ALL [325–328]. To date, ionizing radiation is the only accepted causal exposure for childhood B-ALL[6,11]. However, epidemiologic evidence currently supports that B-ALL is a consequence of an abnormal immune response to common infections due to lack of immune priming in a population at risk during early childhood [6,329]. In this context, genetic polymorphisms may account for immunological defects that promote dysregulated immune responses early in life which may influence leukemia progression through their pro-leukemic effects on early-occurring LICs [6,329]. For example, the ability of basal IFN-γ to significantly delay disease onset in the Eμ-ret mouse model by directly inhibiting the early-life proliferation of LICs has been identified [292]. While IFN-γ plays a central role in immunoediting in various solid tumor models [330,331], this basal IFN-γ-induced proliferation-inhibitory activity is restricted to the pre-leukemic phase of disease [292]. The early direct effects of basal IFN-γ, however, do not preclude the possibility that cytokine levels influence ALL kinetics at multiple points, including during later stages of disease progression, possibly via leukemia-editing mechanisms. Polymorphisms in several other cytokine genes, such as transforming growth factor-beta (TGF-β), IL-10, and IL-12, are also associated with ALL risk [325–328]; however, their influence on ALL development, in the context of potential roles for the early-life immune environment or immunoediting, are less well-defined.

Natural killer T (NKT) cells are CD1d-restricted T cells that possesses both cytolytic and immunostimulatory properties and mediate anti-tumor activity via multiple mechanisms [332,333]. These cells have innate-like capacity to rapidly respond to antigen and secrete a wide range of cytokines, including IFN-γ, TNF-α, IL-2, IL-4, IL-10, IL-13, IL-17, IL-21, IL-22, and CM-CSF in large amounts [333–336]. NKT cells represent a heterogenous population of lipid-reactive T cells and are divided into two major subsets based on their TCR repertoire and lipid reactivity. Type I or invariant NKT (iNKT) cells, the most widely studied subset of NKT cells, express a germline encoded invariant TCRα chain (Vα24-Jα18 in humans; Vα14-Jα18 in mice) paired with a limited repertoire of TCRβ chains (Vβ11 in humans; Vβ2, 7, or 8 in mice) [337,338]. Analogs of alpha-galactosylcermide (α-GalCer), a glycolipid derived from the marine sponge *Agelas mauritianus* [339], are potent activators of iNKT cells [340]. Type II NKT cells are CD1d-restricted T cells that do not recognize α-GalCer [341]. These cells do not display restricted invariant TCRα chain usage and exhibit a more diverse TCR repertoire [341,342].

CD1d, a non-polymorphic MHC-I-like, β2-microglobulin-associated surface protein, is normally expressed predominately by hematopoietic cells, especially those of myelomonocytic and B-cell lineages [343–345]. Functionally distinct from MHC-restricted conventional T cells, NKT cells primarily recognize both endogenous and exogenous glycolipid antigens presented by CD1d. Numerous hematological malignancies, including juvenile myelomonocytic leukemia and AML [346], chronic lymphocytic leukemia (CLL) [347,348], and T-ALL [349], express CD1d molecules. CD1d-positive tumor cells presenting endogenous glycolipids are subject to direct NKT-cell cytotoxicity via perforin/granzyme exocytosis, TNF-related apoptosis-inducing ligand (TRAIL) or Fas-Fas ligand interactions during immunosurveillance [346,350–352]. With the exception of tumors originating from tissues and cell types outside of the hematopoietic system that normally

express CD1d molecules (e.g. hepatocytes and intestinal epithelial cells) [353,354], the majority of non-hematopoietic solid tumor cells either do not express CD1d or express it at very low levels [355,356]. Accordingly, the dominant anti-tumor activity of iNKT cell is via indirect mechanisms involving IFN-γ-mediated transactivation of DCs and downstream cytolytic effectors, such as NK and CD8$^+$ CTLs [357,358]. Although CD1d-negative tumors are not direct targets, iNKT cells contribute to controlling tumor growth by selectively targeting immunosuppressive cells, such as tumor-associated macrophages, CD1d$^+$ myeloid-derived suppressor cells, and IL-10-producing neutrophils, within TME that support tumor cell growth [359–362].

Numerical and functional defects in iNKT cells have been reported in patients with hematological cancers [363–373]. In patients with AML, the frequency of iNKT cells in the blood has been found to correlate inversely with tumor load and positively with prognosis [374]. Reduction in the number of iNKT cells is often concomitant with reversible functional defects, such as poor responsiveness to α-GalCer and loss of IFN-γ production, in some cancers [367,375–378]. In adult B-ALL cases, reduction in frequency of type 1 NKT cells with IL-21-related functional impairment in response to α-GalCer stimulation has been reported, which positively correlated with their reduced capacity to promote an IL-21-dependent effector CD8$^+$ T cell responses [378]. It remains unclear whether defects in iNKT cells contribute to tumor development or are caused by tumor-mediated immune suppression.

In recent years, the important contribution of NKT cells to immunosurveillance against hematological cancers, such as B and T cell lymphomas, CLL, and multiple myeloma (MM) has been well-established [369,379–382]. Their role in immunosurveillance and in the natural history of B-ALL, however, remains largely unknown. Given the broad and rapid cytokine-producing capacity of NKT cells, which allows these cells to modulate immune responses, I investigated

the potential influence of NKT cells on early-life ALL progression and immune control of fully transformed leukemic cells. As the subsequently described work will illustrate, I demonstrated that the absence of type 1 NKT cells accelerates leukemia onset, and that the *in vivo* activation of iNKT cells using α-GalCer during the pre-malignant stage of disease in Eμ-ret mice induces IFN-γ-dependent depletion of LICs. Additionally, my results indicate that the neoantigen-driven protective immune response achieved via epitope spreading is limited in the absence of NKT cells. Importantly and rather unexpected, I found that type 1 NKT cell deficiency leads to the rejection of adoptively transferred primary leukemia cells.

5.2   NKT cells delay leukemia onset

The size of pre-leukemic cell population has been reported to inversely correlate with age at diagnosis [383,384]. To determine whether NKT cells influence disease progression in the Eμ-ret mouse, I first investigated if the absence of NKT cells affects the survival and expansion of LICs during the early stage of pre-leukemia. The splenic LIC burden in two-week old wild-type, NKT cell-deficient (CD1d$^{-/-}$), and type 1 NKT cell-deficient (Jα18$^{-/-}$) Eμ-ret mice were compared. The absolute number of LICs was the highest in CD1d$^{-/-}$ Eμ-ret mice, significantly exceeding that of both wild-type and Jα18$^{-/-}$ Eμ-ret mice (Figure 5.1). No difference in the early LIC burden was observed between Jα18$^{-/-}$ Eμ-ret mice and their wild-type counterparts. As prolonged survival and evolution of pre-leukemic cell population are critical for the development of overt leukemia, disease-free survival of the three mouse strains was compared to determine the impact of this NKT cell-mediated influence on early LIC burden on subsequent disease progression. Despite their differences in early splenic LIC burden, CD1d$^{-/-}$ and Jα18$^{-/-}$ Eμ-ret mice had no difference

in disease-free survival and both had significantly reduced disease latency compared to their

wild-type counterparts (Median survival 113 days versus 113 days versus 151 days) (Figure 5.2).
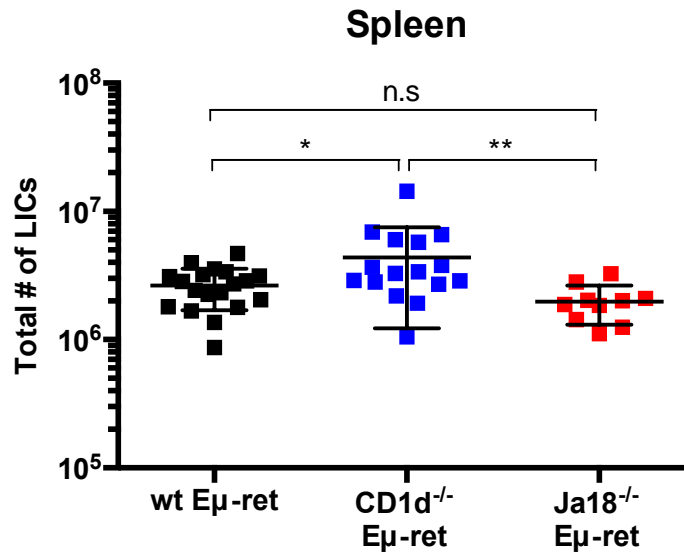
Figure 5.1. LIC burden is significantly higher in CD1d$^{-/-}$ Eμ-ret mice than wild-type and Jα18$^{-/-}$ Eμ-ret mice

LIC burden in spleen of two-week old wild-type (n=19), CD1d$^{-/-}$ (n=16), and Jα18$^{-/-}$ Eμ-ret (n=10) were compared using flow cytometry based on the characteristic B220^{int}/BP-1^{hi} phenotype of Eμ-ret LICs. Mann-Whitney test, bars represent mean±S.D.; *$p<0.05$, **$p<0.01$. n.s = not significant
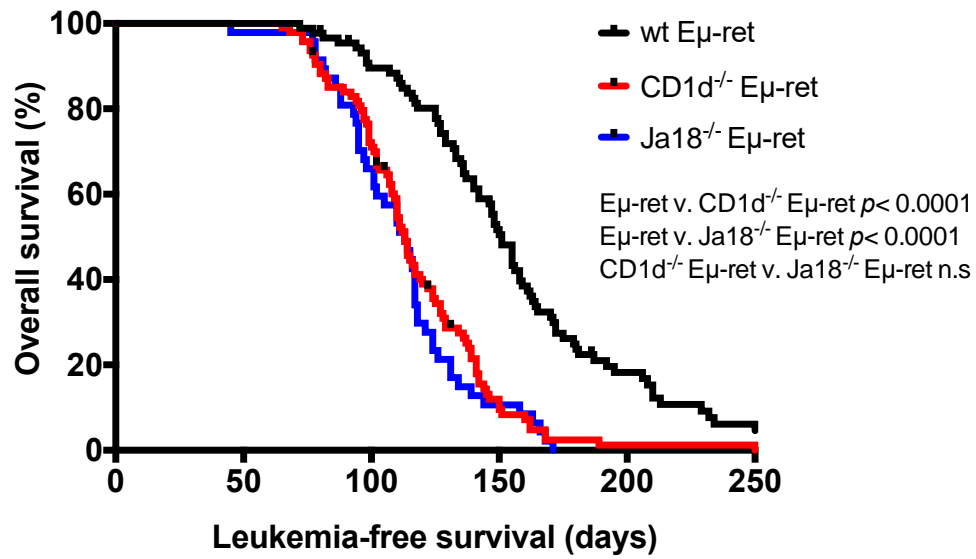
Figure 5.2. NKT cell deficiencies accelerates disease onset in the Eµ-ret mouse

Disease-free survival compared in wild-type (n=89), CD1d^{-/-} (n=94), and Jα18^{-/-} Eµ-ret (n=47). Median survival = 151 days (wild-type), 113 days (CD1d^{-/-}), and 113 days (Jα18^{-/-}). Log-rank test

5.3 α-GalCer administration induces significant depletion of LICs in an age- and IFN-γ-dependent manner

Following the observation that a lack of iNKT cells accelerates disease progression in the Eμ-ret mouse model, I next examined the therapeutic potential for *in vivo* activation of iNKT cells in Eμ-ret mice. A single dose of α-GalCer (4 μg) was administrated to wild-type, IFN-γ$^{-/-}$, and CD1d$^{-/-}$ Eμ-ret mice to determine whether any potential α-GalCer-induced NKT cell-mediated effect on LICs during early stage of disease is IFN-γ-dependent. Mice were treated with α-GalCer at either 2 or 3 weeks of age and then the splenic LIC burden of treated and untreated were assessed and compared 7 days later. Administration of α-GalCer at 3 weeks, but not 2 weeks of age, induced a significant depletion of LICs in spleens of wild-type Eμ-ret mice (

Figure **5.3**). Unlike their wild-type counterparts, splenic LIC burden remained unaffected in IFN-γ$^{-/-}$ and CD1d$^{-/-}$ Eμ-ret mice treated at either age. The absolute number of iNKT cells was also assessed on 7 days following α-GalCer treatment to confirm that α-GalCer had indeed induced an NKT response. Although no depletion of LICs was observed, a significant increase in the number of iNKT cells was detected in IFN-γ$^{-/-}$ Eμ-ret mice at day 7 following α-GalCer treatment at either age (Figure 5.4). This suggests that the primary response of activated iNKT cells is regulated by an IFN-γ-dependent negative feedback mechanism.
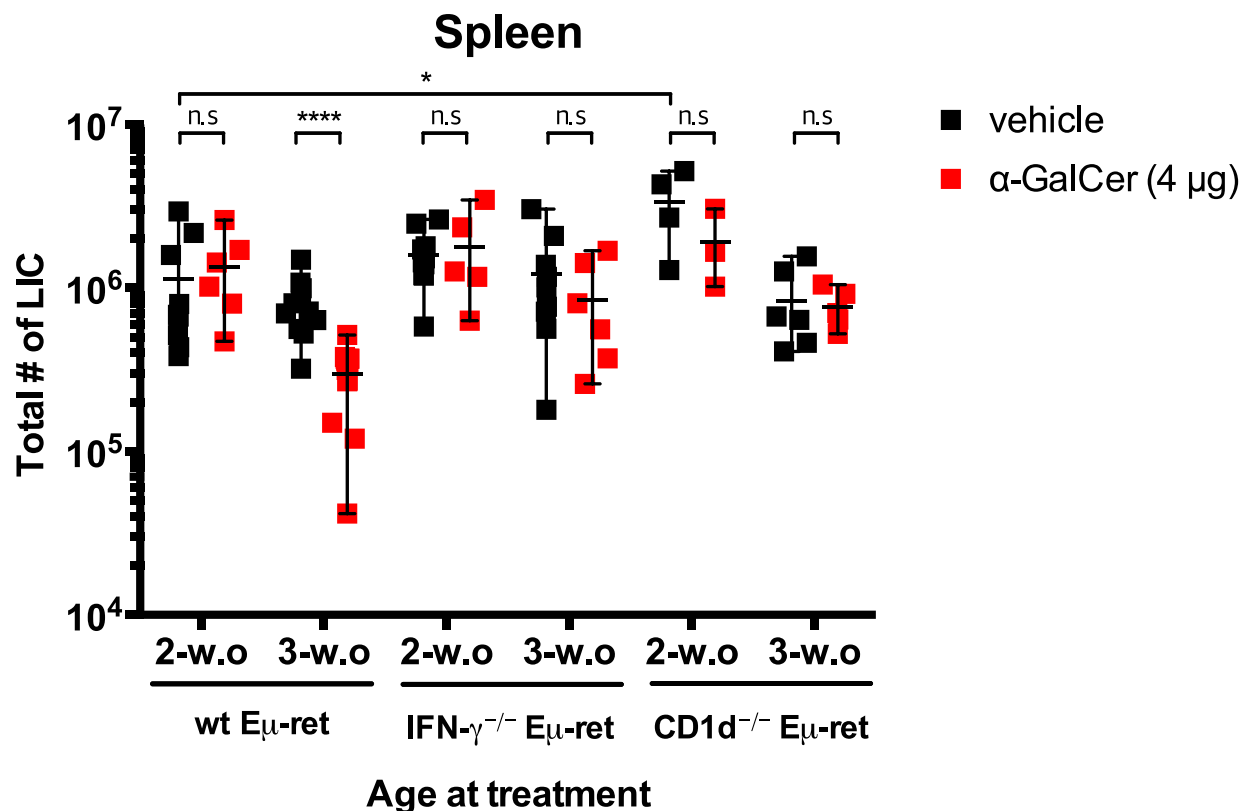
Figure 5.3. α-GalCer administration at 3 weeks but not 2 weeks of age induces significant depletion of LICs in the Eµ-ret mice in an IFN-γ-dependent manner

Wild-type, IFN-γ$^{-/-}$, and CD1d$^{-/-}$ Eµ-ret mice were injected intraperitoneally with a single dose of α-GalCer (4 µg) or vehicle (untreated) at either 2 or 3 weeks of age. On day 7, splenic LICs burden of treated and untreated mice was compared using flow cytometry based on the characteristic B220^{int}/BP-1^{hi} phenotype of Eµ-ret LICs. N=87; 2-week-old wild-type Eµ-ret + vehicle, n=9; 2-week-old wild-type Eµ-ret + α-GalCer, n=6; 3-week-old wild-type Eµ-ret + vehicle, n=11; 3-week-old wild-type Eµ-ret + α-GalCer, n=13; 2-week-old IFN-γ$^{-/-}$ Eµ-ret + vehicle, n=10; 2-week-old IFN-γ$^{-/-}$ Eµ-ret + α-GalCer, n=5; 3-week-old IFN-γ$^{-/-}$ Eµ-ret + vehicle, n=9; 3-week-old IFN-γ$^{-/-}$ Eµ-ret + α-GalCer, n=6; 2-week-old CD1d$^{-/-}$ Eµ-ret + vehicle, n=4; 2-week-old CD1d$^{-/-}$ Eµ-ret + α-GalCer, n=3; 3-week-old CD1d$^{-/-}$ Eµ-ret + vehicle, n=6; 3-week-old CD1d$^{-/-}$ Eµ-ret + α-GalCer, n=5. Results shown are pooled from three independent experiments. Mann-Whitney test, bars represent mean±S.D.; *$p<0.05$, ****$p<0.0001$. n.s = not significant
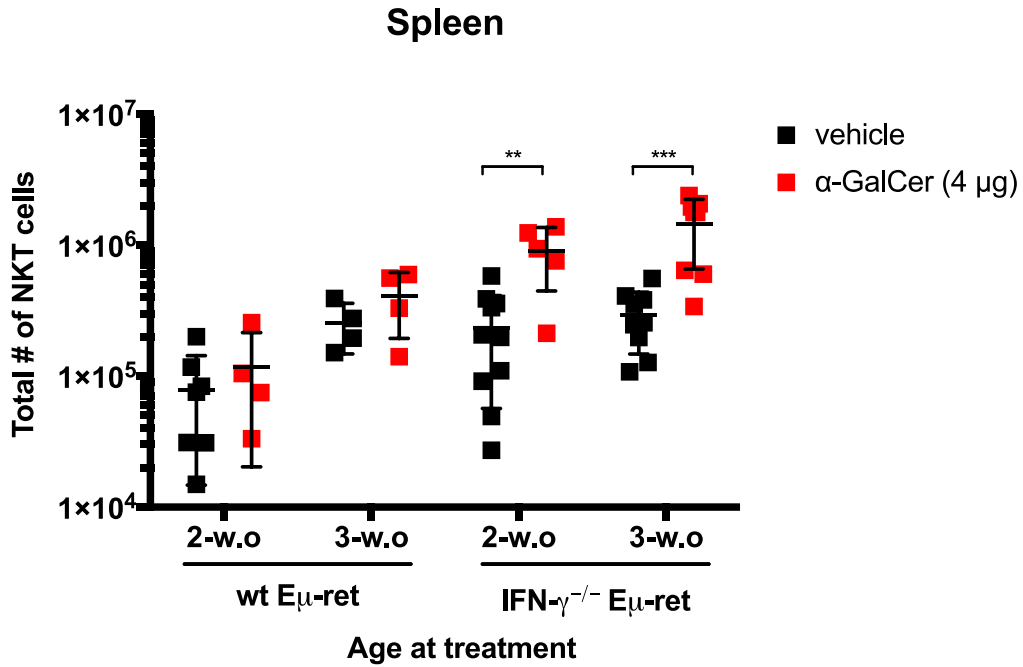
Figure 5.4. The absolute number of splenic iNKT cells increased significantly in IFN-$\gamma^{-/-}$ Eμ-ret mice following α-GalCer treatment

Total number of NKT cells (CD1d tetramer$^+$TCRβ^+B220$^-$) in spleen of wild-type and IFN-$\gamma^{-/-}$ Eμ-ret mice were compared using flow cytometry on day 7 following a single intraperitoneal injection of α-GalCer (4 μg) or vehicle (untreated) at either 2 or 3 weeks of age. N=51; 2-week-old wild-type Eμ-ret + vehicle, n=7; 2-week-old wild-type Eμ-ret + α-GalCer, n=4; 3-week-old wild-type Eμ-ret + vehicle, n=4; 3-week-old wild-type Eμ-ret + α-GalCer, n=4; 2-week-old IFN-$\gamma^{-/-}$ Eμ-ret + vehicle, n=10; 2-week-old IFN-$\gamma^{-/-}$ Eμ-ret + α-GalCer, n=5; 3-week-old IFN-$\gamma^{-/-}$ Eμ-ret + vehicle, n=9; 3-week-old IFN-$\gamma^{-/-}$ Eμ-ret + α-GalCer, n=8. Results shown are pooled from three independent experiments. Mann-Whitney test, bars represent mean±S.D.; **p<0.01, ***p<0.001.

5.4   Investigating immunoediting in the Eµ-ret mouse model in the absence of NKT cells

The critical role of the immune system in shaping the immunogenicity of tumors is well-established [385,386]. The concept of cancer immunoediting hypothesizes that the immune system not only protects the host against tumor formation, but also functions to promote tumor variants with reduced immunogenicity [230,387]. This extrinsic tumor suppressor mechanism of the immune system leads to the selection of tumor cells that are more fit to survive in an immunocompetent host [230,331]. Collectively, such the dynamic process of cancer immunoediting encompasses three distinct phases namely Elimination, Equilibrium, and Escape [387]. While the evidence of loss or downregulation of HLA expression as a mechanism of immune escape has been reported in adults with hematological cancers [388,389], the exact mechanism of immunoediting in the pediatric setting is less clear. As NKT cells help shape the strength and type of immune response that develops following activation by serving as early source of cytokines, including IFN-γ [333–336], I assessed whether NKT cells contribute to sculpting immunogenicity of ALL cells via exerting selective pressure. The immunogenicity of established B-ALL cells from CD1d$^{-/-}$ (CD1d-negative ALL), Jα18$^{-/-}$ (CD1d-positive ALL), and wild-type (CD1d-positive ALL) Eµ-ret mice were compared using an adoptive transfer approach in secondary wild-type hosts, where survival time is an indirect measure of immunogenicity. Wild-type BALB/c recipients of ALL derived from Jα18$^{-/-}$ Eµ-ret mice succumb to disease significantly earlier than the recipients of leukemia cells from wild-type or CD1d$^{-/-}$ Eµ-ret mice, but with median survival of 21 days compared to 28 days and 24 days, respectively (Figure 5.5). No difference in disease kinetics was observed between recipients of ALL cells derived from wild-type or CD1d$^{-/-}$ Eµ-ret mice.
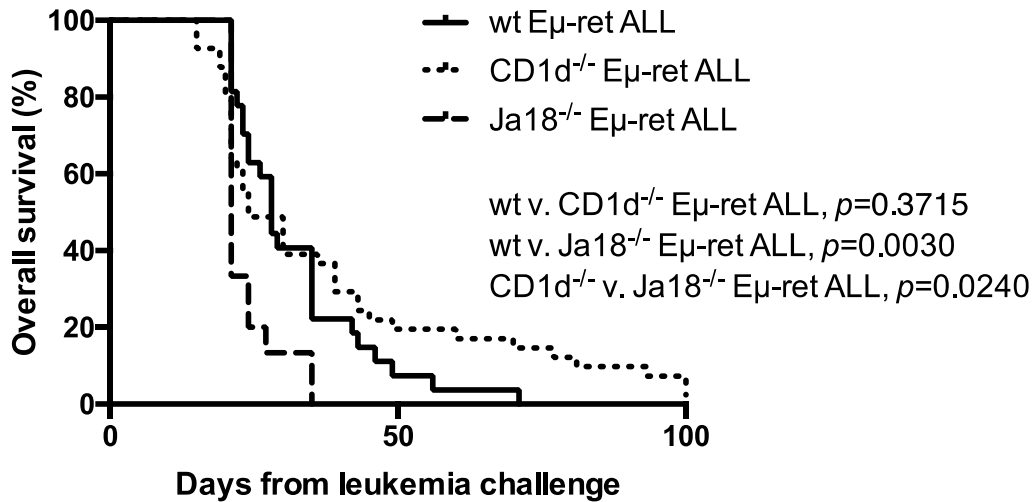
Figure 5.5. Disease progression was significantly accelerated in immunocompetent recipients of primary ALL cells derived from Jα18$^{-/-}$ Eμ-ret

Wild-type BALB/c mice were adoptively transferred with syngeneic primary B-ALL derived from wild-type, CD1d$^{-/-}$, or Jα18$^{-/-}$ Eμ-ret mice and then followed for disease-free survival. Disease progression was monitored via flow cytometric assessment of peripheral blood blast burden. Overt disease was defined by the presence of palpable/enlarged lymph nodes or white blood cell count of >15,000/μL in peripheral blood. Primary B-ALL cells derived from 5 individual wild-type, 6 individual CD1d$^{-/-}$, and 2 individual Jα18$^{-/-}$ Eμ-ret mice. N=82; Wild-type leukemia challenged, n=27; CD1d$^{-/-}$ leukemia challenged, n=40; Jα18$^{-/-}$ leukemia challenged, n=15. Median survival = 28 (Wild-type Eμ-ret ALL), 24 days (CD1d$^{-/-}$ Eμ-ret), and 21 days (Jα18$^{-/-}$ Eμ-ret). Log-rank test

5.5 NKT cell deficiency limits the generation of durable protection via epitope spreading in neoantigen-driven remission model

Cross-presentation and recognition of tumor-derived glycolipids in a CD1d-dependent manner is critical for the induction of NKT cell-mediated immunosurveillance [346,350–352,390–392]. Previously in Chapter 3, I have demonstrated that the generation of an immune response against a diverse range of leukemia-associated antigens is required to achieve durable control of ALL. Given the significantly reduced disease-free survival observed in Eμ-ret mice in the absence of iNKT cells, I extended this finding to determine the potential role for the leukemia-derived glycolipids–NKT cell interaction during epitope spreading in our neoantigen-driven remission model. To test this, wild-type and CD1d$^{-/-}$ BALB/c mice were initially challenged with GFP/luc-modified ALL as described in Chapter 3 (Figure 3.4) to induce neoantigen-directed primary response. Consistent with their ability to mount an immune response against CD1d as a neoantigen (Figure 3.8), all CD1d$^{-/-}$ recipients of GFP/luc-modified ALL went into spontaneous remission like their wild-type counterparts (data not shown). Then, these wild-type and CD1d$^{-/-}$ BALB/c mice in spontaneous remission and leukemia-naïve counterparts were challenged with CD1d-negative syngeneic primary B-ALL derived from CD1d$^{-/-}$ Eμ-ret mice. At day 21, the disease status of recipients, indicated as either positive or negative, was assessed based on the presence or absence of blasts in peripheral blood. Eight out of eleven (73%) and two out of five (40%) of rechallenged CD1d$^{-/-}$ recipients and their wild-type counterparts, respectively, were positive for disease, while leukemia-naïve wild-type (6/6) and CD1d$^{-/-}$ (9/9) recipients were all leukemia-positive, as expected (Figure 5.6a). This observation was confirmed by comparing disease progression in these recipients. Showing no significant difference in disease kinetics, leukemia-naïve wild-type and CD1d$^{-/-}$ recipients both quickly succumb to disease (Figure 5.6b).

Consistent with their peripheral blood disease burden at day 21, rechallenged CD1d$^{-/-}$ recipients had a limited survival advantage compared to their wild-type counterparts as the majority of mice failed to achieve remission and eventually succumbed to disease.
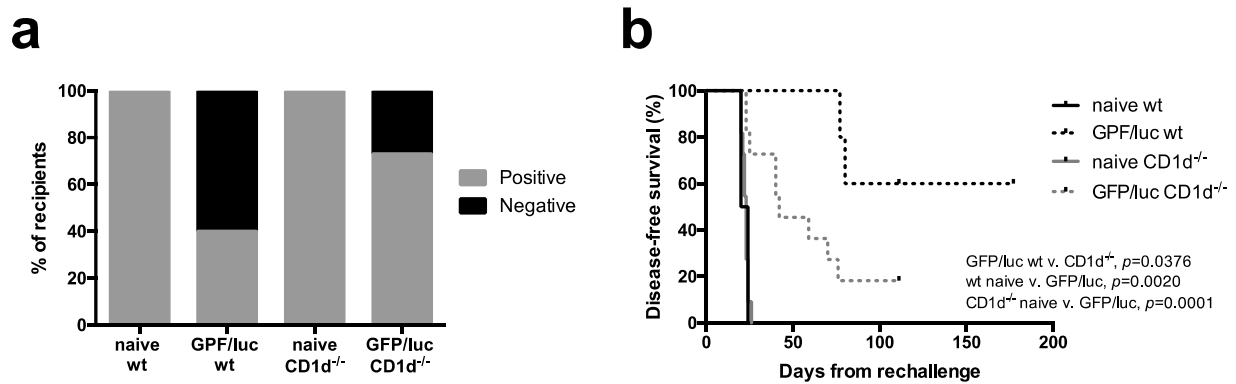
Figure 5.6. Neoantigen-driven protective immune response is limited in the absence of NKT cells

Wild-type and CD1d$^{-/-}$ BALB/c mice, either leukemia-naïve (naïve) or in spontaneous remission following adoptive transfer of GFP/luc-modified ALL (GFP/luc), were challenged with CD1d-negative syngeneic primary B-ALL. Mice were followed for disease-free survival. Disease status at day 21 based on peripheral blood burden was evaluated (a) and disease progression (b) were monitored via flow cytometric assessment of peripheral blood blast counts. N=31; Naïve wild-type, n=6; GFP/luc-modified ALL-cleared wild-type, n=5; Naïve CD1d$^{-/-}$, n=9; GFP/luc-modified ALL-cleared CD1d$^{-/-}$, n=11. Median survival = 22 (Naïve wild-type), 23 days (Naïve CD1d$^{-/-}$), and 42 days (GFP/luc CD1d$^{-/-}$). Mann-Whitney test, bars represent mean; **$p<0.01$ (a). Log-rank test (b)

5.6 Type 1 NKT cell deficiency leads to the rejection of adoptively transferred primary leukemia cells

To build on the observation that the duration of remission is reduced in the absence of NKT cells in our neoantigen-driven remission model, I repeated the experiment described as in Figure 5.6 using $J\alpha18^{-/-}$ BALB/c mice. In contrast to the situation in $CD1d^{-/-}$ syngeneic mice, CD1d on wild-type Eμ-ret mice-derived ALL cells is not a neoantigen in $J\alpha18^{-/-}$ mice as CD1d is also expressed on B-ALL cells derived from $J\alpha18^{-/-}$ Eμ-ret mice. Despite the absence of neoantigen, the majority of naïve $J\alpha18^{-/-}$ recipients rejected the primary B-ALL cells derived from wild-type Eμ-ret mice and maintained durable disease-free status, unlike their wild-type counterparts which all succumbed to disease, as expected, with median survival of 25 days (Figure 5.7a). Although disease-free survival was significantly improved compared to their wild-type counterparts, with median survival of 57 days, the percentage of $CD1d^{-/-}$ recipients of wild-type ALL cells that achieved long-term disease-free status was significantly lower than that of $J\alpha18^{-/-}$ recipients, in spite of the presence of neoantigen. To confirm these observations in the absence of neoantigen-specific immune responses, I repeated the experiment using primary B-ALL cells derived from $CD1d^{-/-}$ Eμ-ret mice. Again, the majority of naïve $J\alpha18^{-/-}$ recipients were protected from $CD1d^{-/-}$ B-ALL challenge, while naïve $CD1d^{-/-}$ and wild-type recipients both rapidly succumbed to disease with median survival of 21 days and 22.5 days, respectively (Figure 5.7b).
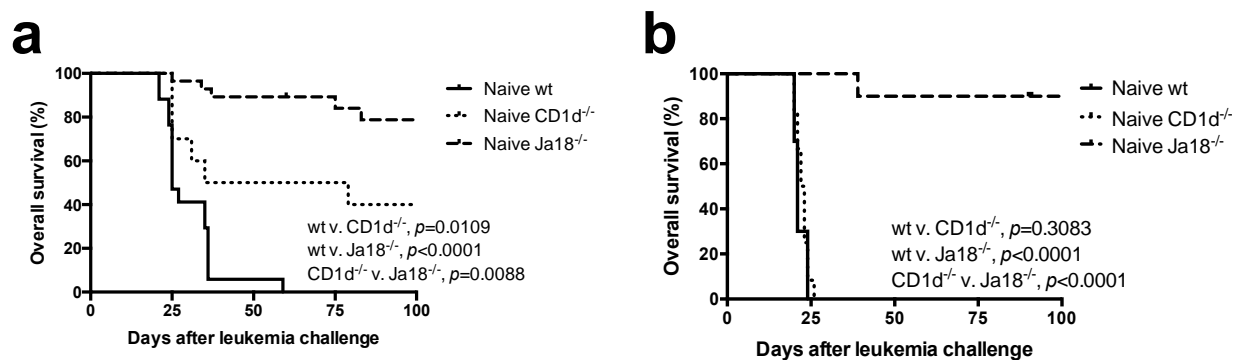
Figure 5.7. Jα18$^{-/-}$ mice rejected adoptively transferred primary B-ALL cells derived from wild-type and CD1d$^{-/-}$ Eµ-ret mice

Syngeneic primary B-ALL cells derived from wild-type (a) and CD1d$^{-/-}$ (b) Eµ-ret mice were adoptively transferred to naïve wild-type, CD1d$^{-/-}$, and Jα18$^{-/-}$ BALB/c mice and then followed for disease-free survival. Disease progression was monitored via flow cytometric assessment of peripheral blood blast burden. Overt disease was defined by the presence of palpable/enlarged lymph nodes or white blood cell count of >15,000/µL in peripheral blood. Primary B-ALL cells derived from 5 individual and 3 individual wild-type and CD1d$^{-/-}$ Eµ-ret, respectively. N=87; wild-type (n=17), CD1d$^{-/-}$ (n=10), Jα18$^{-/-}$ (n=28) recipients of wild-type Eµ-ret-derived ALL; wild-type (n=10), CD1d$^{-/-}$ (n=10), Jα18$^{-/-}$ (n=12) recipients of CD1d$^{-/-}$ Eµ-ret-derived ALL. Median survival = 25 days (wild-type + wild-type Eµ-ret ALL), 57 days (CD1d$^{-/-}$ + wild-type Eµ-ret-ALL), 21 days (wild-type + CD1d$^{-/-}$ Eµ-ret ALL), 22.5 days (CD1d$^{-/-}$ + CD1d$^{-/-}$ Eµ-ret ALL). Log-rank test

5.7 Discussion

The physiological roles of iNKT cells, in the absence of exogenous stimulation, were first demonstrated in immunosurveillance of methylcholanthrene (MCA)-induced spontaneous sarcomas [393,394]. Subsequent study indicated the potential role for endogenous ligands as effective activators of iNKT cell-mediated immunosurveillance [391], further supporting an ability of iNKT cells to suppress the development of a broad range of spontaneous tumors. While the important contribution of NKT cells to immune surveillance against hematological cancers, such as B and T cell lymphomas, CLL, and MM is well-established [369,379–382], the potential role for the CD1d–NKT cell axis during ALL progression remains poorly understood.

The results outlined in this chapter uncover the protective role of NKT cells, as a population, against leukemia progression in Eμ-ret mice as evident from the significantly reduced disease-free survival in both CD1d$^{-/-}$ and Jα18$^{-/-}$ Eμ-ret mice. While my results do not define functional differences between type 1 and type 2 NKT cells during spontaneous leukemia development, I demonstrated that type 1 NKT cells singly act as barrier against leukemia progression in the Eμ-ret mouse model. The reduced disease latency observed in Jα18$^{-/-}$ Eμ-ret mice, despite no differences in the early-life burden of LICs compared to their wild-type counterparts, suggests that type 1 NKT cells are likely to act during later stages of disease progression to restrict the survival and/or proliferation of maturation-arrested LICs or overt leukemic cells. While the absence of the entire NKT cell population resulted in greater numbers of LICs early in life, no differences in the overall disease progression between CD1d$^{-/-}$ and Jα18$^{-/-}$ Eμ-ret mice were observed. In mice, the first 4 weeks of life is a critical period for postnatal development of the immune system [395–397]. The number of immune cells in spleen remains relatively constant from 1 to 2 weeks after birth following a rapid increase in the number of

splenocytes during the first 7 days of life [396,397]. As the number of spleen cells significantly increase during week 3 after birth, primarily due to the increase in the B cell population, the relatively higher LIC burden at 2 weeks of age observed in all three mouse strains may be as a result of LICs filling the unoccupied lymphocyte niche prior to the establishment of niche-specific cell populations until after 4 weeks of age. Similarly, the size of NKT cell populations and the proportion of phenotypically mature subsets steadily increase for about 3 weeks once these cells begin to appear in the thymus at day 5 after birth in mice [398]. In line with this notion, the elevated splenic LIC burden observed in CD1d$^{-/-}$ Eμ-ret mice at 2 weeks of age may represent LICs occupying the extra niche size available as a result of the absence of the NKT cell population, especially during the time of extensive NKT cell expansion. Along with IL-15, IL-7 is an important survival factor for the development and homeostasis of NKT cells, although its functional role differs for specific NKT cell subsets [399–403]. The availability of IL-7 for IL-7-driven proliferation of LICs in the Eμ-ret model [292] may increase in the absence of NKT cells. This may explain the elevation of LIC burden in CD1d$^{-/-}$ Eμ-ret mice very early in life, which have no differences in overall disease progression in comparison with that of Jα18$^{-/-}$ Eμ-ret mice.

The importance of iNKT cells for initiating potent anti-tumor responses via Th1 immunity was first discovered with a broad range of experimental tumor lines following the administration of the exogenous immunotherapeutic stimulators, IL-12 [404–406] and α-GalCer [407–409]. Soon, the anti-tumor effects of α-GalCer were further demonstrated against spontaneous, carcinogen- or oncogene-induced murine tumor models [410]. The IL-12-dependent production of IFN-γ by iNKT cells is critical for the potent α-GalCer-induced anti-tumor response [411–413]. These studies suggest that rather than eliminating tumor cells by direct cytolytic activities, α-GalCer-stimulated iNKT cells primarily contribute to tumor protection by recruiting and

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activating a perforin-dependent cytolytic function of downstream NK cells in an IFN-γ-dependent manner. Likewise, my results demonstrate the ability of α-GalCer-stimulated iNKT cells to deplete LICs in Eµ-ret mice. My results reveal that α-GalCer treatment given at 3 weeks of age, but not 2 weeks, induce IFN-γ-dependent depletion of LICs in the Eµ-ret mouse model. The primary response of activated iNKT cells is strictly regulated by an IFN-γ-dependent negative feedback mechanism and hence is of limited duration [414]. Consequently, antigen-primed iNKT cells, especially those α-GalCer-primed, are refractory to secondary stimulation due to IFN-γ-mediated downregulation of iNKT cell activity via inhibitory NK cell receptors (CD94/NKG2A) [414]. Therefore, the significantly increased number of splenic iNKT cells observed in IFN-γ$^{-/-}$ Eµ-ret mice, but not their wild-type counterparts, regardless of age at treatment may indicate the loss of IFN-γ-mediated negative feedback regulation of homeostatic proliferation of iNKT cells following α-GalCer stimulation.

While numerical and functional defects in iNKT cells have both been reported in patients with hematological cancers [363,364,368,369,371,373,415], abnormal expression of CD1d by malignant cells is commonly implicated in progressive impairment of iNKT cell functions [369,379,416–421]. Immunophenotyping analysis of CD1d expression in B-ALL, however, is limited to a single report from the Grossi group, which investigated a cohort of 80 pediatric patients [422]. CD1d surface expression was mostly absent in both normal BCPs and B-ALL cells, where its expression was detected in leukemic blasts from only a small minority of the patients (n=12) with some degree of variability in the intensity of surface expression. More highly represented in high-risk subgroups of B-ALL, CD1d-positive cases were significantly associated with adverse prognosis. Importantly, this study reported that CD1d-positive, but not CD1d-negative blasts are

able to present α-GalCer via CD1d to healthy donor iNKT cells, which induced NKT cell-mediated apoptosis of ALL cells.

My results do not address the functional consequences of positive CD1d expression on B-ALL cells nor the functional integrity of autologous iNKT cells over the course of disease in the Eμ-ret model. In adoptive transfer settings, however, immune response to ALL cells are not influenced by the expression of CD1d by malignant ALL cells in immunocompetent hosts. A significantly reduced disease latency observed in Jα18^{-/-} Eμ-ret mice suggests that type 1 NKT cells are likely to exert selective pressure sculpting leukemia cells, if they do play a role in immunoediting, for their protective role during disease progression. Leukemia cells derived from Eμ-ret mice deficient in type 1 NKT cells, in this case, are likely to display an increased immunogenicity. The poorer survival rate observed in immunocompetent hosts bearing B-ALL cells derived from Jα18^{-/-} Eμ-ret mice compared to those challenged with B-ALL derived from either wt or CD1d^{-/-} Eμ-ret mice may indicate that B-ALL derived from Jα18^{-/-} Eμ-ret mice display a reduced immunogenicity, but it is insufficient to demonstrate the role of NKT in tumor sculping in the Eμ-ret mouse model. The two types of NKT cells often have opposing functions, but counteract each other in tumor immunity [357,382,392]. While my results do not functionally distinguish type 2 from type 1 NKT cells, it appears that the absence of type 1 NKT cells leads to the disruption of possible interactions between two types of NKT cells, conferring protection against adoptively transferred B-ALL cells.

Furthermore, my results indicate that while NKT cells are not required for eliciting immune response against neoantigens, their responses are critical for generating durable protection against B-ALL. The results presented in Chapter 3 illustrate the ability of a primary immune response to diversify to previously non-immunogenic leukemia-associated antigens. The

results presented here suggest that these additional leukemia-associated antigens include leukemia-derived glycolipids that are recognizable by NKT cells in a CD1d-dependent manner. These results are relevant to my finding that endogenous iNKT cells, in the absence of exogenous stimulation, are capable of influencing the disease progression in the Eµ-ret mouse, as leukemia-derived glycolipids may be important activators of iNKT cells in the Eµ-ret mouse model.

The two similar, but not equivalent mouse models of deficiency in type 1 NKT cells have served as important tools to facilitate our understanding of functional roles of NKT cells in various experimental settings [424,425]. The first model makes use of mice deficient in CD1d molecules, which prevents the development of any CD1d-reactive NKT cell development, therefore, is expected to lack both type 1 and type 2 NKT cells [426]. The other model is the originally established Jα18-deficient mice, described by the Taniguchi group in 1997, which directly targets Jα18 (*Traj18*) that is specifically required for type 1 NKT-TCR formation [427]. There is, however, an important caveat to this Jα18-deficient mice; an up to 60% of reduction in the diversity of TCRα chain repertoire due to a defective transcription of *Traj* gene segments upstream of *Traj18* has been confirmed [428]. To circumvent such limitation, four new *Traj18*^{-/-} lines of iNKT cell-deficient mice have been generated between 2015 and 2017 [424,429–431]. Apart from the absence of *Traj18*, and hence a lack of iNKT cells, an undisturbed TCRα chain repertoire has been confirmed in all four models [424,429–431]. While the use of the original Jα18-deficient mice is the critical limitation of my work presented in this chapter, the findings from this work reveal a unique subpopulation(s) of T cells that provide protection against B-ALL.

Chapter 6: General discussion and perspectives

6.1 The current immunotherapeutic approach for B-ALL

As has been extensively described in previous chapters, remarkable clinical successes have been achieved with targeted immunotherapies directed at surface antigens of leukemia blasts in patients with relapsed or refractory B-ALL. This single antigen-targeted approach, however, is highly prone to immune escape of tumors. The outgrowth of target-negative escape variants, which eventually leads to the development of resistance to therapy, remains a major drawback of CD19-directed therapies in B-ALL. The efficacy of strategies that direct T cell-mediated cytotoxicity towards leukemic cells bearing non-immunogenic antigens is limited, with a lack of evidence that these interventions generate long-term immune memory [215]. Hence, the goals of my thesis work were to better understand the limitations of current single antigen-targeted immunotherapies and to identify immune responses required for the establishment and importantly, maintenance of remission in childhood B-ALL.

The general hypothesis of this thesis work was that the generation of a protective T cell-mediated immune response with a broad specificity for range of leukemia-associated antigens is required for durable control of B-ALL. In this thesis, I have demonstrated the ability of target-directed therapy to generate long-term protection against B-ALL by inducing a secondary immune response against additional non-targeted leukemia-associated antigens. Nonetheless, these results also suggest that immune tolerance established towards leukemia-associated antigens precludes the efficacy of such protective immune responses for sustaining durable control of B-ALL. As a potential complement to MRD-directed contemporary chemotherapeutic regimen, I have also shown the ability of innate immune-mediated non-target specific immune modulation to induce a broad and durable protection against B-ALL. Overall, my findings have

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implications for further advances in the development of immunotherapy for B-ALL and suggest that overcoming immune tolerance established against leukemia-associated antigens may be critical for maximizing the therapeutic benefits of immunotherapies in general.

6.2 Epitope spreading allows unmasking of previously non-immunogenic leukemia-associated antigens for immune recognition

Epitope spreading has been increasingly proposed as a secondary mechanism underlying immune-mediated durable control of cancer following a single antigen-targeted and checkpoint blockade-mediated interventions. While the contribution of this immune diversification phenomenon to durable remission has been reported in the patients with solid malignancies [284,285], it has yet to be demonstrated in the context of B-ALL following single antigen-targeted therapies. Of the two common forms of relapse that have been observed following CD19-directed therapies against B-ALL, target-positive and target-negative relapses, the incidence of latter was independent of poor expansion and persistence of the engineered T cells [155,223]. Therefore, the incidence of target-negative relapses is unlikely to be reduced by enhancement of the persistence of engineered T cells [214,215]. The work detailed in Chapter 3, therefore, evaluated the hypothesis that epitope spreading after a target antigen-specific response enables the generation of a diversified immune response that contributes to durable control of ALL, thereby minimizing the risk for therapy-driven mechanisms of immune escape by blasts.

The results presented in Chapter 3 highlight the importance of a multi-antigen-directed immune response for the maintenance of remission in B-ALL following a target-directed therapy. These results represent the first demonstration of the ability of target-directed immunotherapies to prime secondary immune responses from existing host immune system

against additional non-targeted, previously non-immunogenic, antigens present on blasts via epitope spreading in the setting of B-ALL. The work detailed in Chapter 3, therefore, unveiled another mechanism of action necessary for sustaining long-term remission and/or leukemia eradication following antigen-directed immunotherapy, in addition to the direct killing of cells expressing the target antigen by target-specific T cells. Importantly, the findings uncovered in the Eμ-ret mouse model that the therapeutic efficacy of epitope spreading is limited in an immunological setting where immune tolerance to leukemia-associated antigens has likely been established early in the course of leukemia progression, which is commonly the case in the clinical setting, therefore, may have a relevance to patients. Together, these results may provide possible explanation for the reported cases of durable long-term remissions in a subgroup of B-ALL patients [294], as well as identify an obstruction (tolerance) to achieving long-term sustained remissions via desired epitope spreading following CD19-directed CAR T cell therapy. Identification of the underlying mechanism of epitope spreading may provide insights into the development of strategies to selectively induce the necessary secondary immune responses required for the maintenance of remission.

6.3   The impact of therapeutic innate immune modulation on B-ALL

Increase in the availability of leukemia antigens as a result of chemotherapy-mediated cell death can contribute to the elicitation of therapeutically relevant leukemia antigen-specific immune responses associated with immunological memory provided appropriate immunostimulatory signals [306–308]. Limited neoantigenicity [124,187] and weak immunogenicity [312–314] render ALL blasts a challenging target for immunotherapy. The poor antigen presentation capacity of ALL blasts [312,315] simultaneous with a rapid induction of T cell dysfunction by

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progressive leukemia [293], therefore, may contribute to the failure of immunotherapy in many ALL patients. Nonetheless, appropriate immune stimulation, previously demonstrated with TLR9 agonist CpG ODN in particular, has been shown to be sufficient to induce depletion of both mouse and human ALL blasts [272,316]. Combining the beneficial effects of chemotherapy with leukemia antigen-specific immune response, therefore, may lead to synergistic clinical activity against MRD-positive disease and further improve survival rates. The recently demonstrated divergent effects of endosomal TLR stimulation on pre-leukemic cells and human leukemic cells [314,316–318], however, suggest that there are significant variables that contribute to the outcome of TLR stimulation in the context of BCP cell malignancy. Having demonstrated the capability of the host immune system to mount protective immune response against previously non-immunogenic leukemia-associated antigens upon proper immune priming (Chapter 3), I speculated that TLR agonists have differential capacities for inducing innate immune responses required for the productive activation of leukemia antigen-specific T cells.

Extending on the previously reported ability of CpG ODN to induce T cell-mediated long-term protection against outgrowth of transplanted syngeneic ALL cell lines [272], the results presented in Chapter 4 represent the first comparison of the organ-specific anti-leukemia immune activity induced by endosomal TLR agonists, and identification of immune components associated with the most durable immune-mediated control of B-ALL in syngeneic transplantable leukemia model using primary B-ALL cells. The results in Chapter 4 demonstrate that while systemic administration of endosomal TLR agonists is sufficient to prolong disease-free survival, CpG ODN, which induced early control of disease in the bone marrow with high consistency, conferred the most significant durable control of B-ALL. Additionally, I also validated the sensitivity of human blasts to endosomal TLR-mediated killing using mouse

xenografts. The observed association between rapid treatment response in the bone marrow, but not spleen nor peripheral blood, and sustained TLR-induced remission may have therapeutic significance. It is in line with the prognostic significance of early reduction of leukemic blasts in bone marrow during remission-induction therapy as reported in a number of pediatric ALL trials [296,432]. Furthermore, assessment of the bone marrow is more sensitive than evaluation of the peripheral blood for early response [296] and unlike children with T-ALL, MRD levels in bone marrow and peripheral blood are not comparable in those with B-ALL [433].

The clinical significance of an early bone marrow response corresponds with the fact that B-ALL relapse often originates from this location. Therefore, the ability of CpG ODN to induce a potent anti-leukemia immune activity in bone marrow has valuable therapeutic perspectives. The direct intratumoral administration of TLR agonists is now gaining momentum as a preferable route of delivery in oncological settings [434]. Despite the accumulating preclinical and clinical evidence of the superior effectiveness of intratumoral immune modulation in generation of a therapeutic systemic anti-tumor immune response [236,270,271,434], intratumoral treatment approaches are not applicable to a systemic disease, such as B-ALL. Systemic administration of CpG ODN in the context of B-ALL may feasibly achieve eradication of MRD, a relatively low-level disease in bone marrow, and thus reduce the risk of relapse in MRD-positive patients. Optimal timing, dose, and duration of treatment will be critical for achieving maximal therapeutic benefits of such approach.

6.4   Influence of NKT cells on B-ALL progression

Epidemiological studies have revealed that a number of polymorphisms in cytokine genes is associated with increased risk or reduced latency of of B-ALL [325–328]. Although the mechanism for these associations remains unclear, polymorphisms in cytokine genes may represent immune defects underlying the development of early-life immune environment that is pro-leukemic [292,435]. NKT cells are capable of rapidly producing a large array of cytokines in copious amounts following stimulation [333–336]. For their functional properties, NKT cells have been implicated in the modulation of immune responses, and are attributed a role in immunosurveillance [436]. Unlike for other types of hematological cancers, the understanding of a role for the CD1d–NKT cell axis in the control of B-ALL is limited. The work detailed in Chapter 5, therefore, investigated the hypothesis that NKT cells inhibit B-ALL progression by acting as key early players constraining the survival of pre-leukemic cells.

While the work described in Chapter 5 is exploratory, the results highlight NKT cells as an important population of immune cells playing a role in leukemia progression in the context of B-ALL. Although the mechanisms by which NKT cells exert inhibitory effects on the progression of B-ALL remain to be examined in future studies, the deficiency of type 1 NKT cells leads to accelerated leukemia progression, but with no evidence of NKT cell-driven immunoediting. In line with the direct proliferation-inhibitory effect of basal IFN-γ exclusively during the pre-leukemic phase of disease, the therapeutic activation of type 1 NKT cells using α-GalCer during early stage of disease in Eμ-ret mice achieves IFN-γ-dependent depletion of LICs. The absence of type 1 NKT cells, however, shows a minimal impact on the early-life proliferation of LICs, at least during the very early stages of disease progression. As an extension of the work described in Chapter 3, the work detailed in Chapter 5 also uncovered the

requirement of NKT cells to epitope spreading, which enables the generation of a diversified immune response that contributes to durable B-ALL control. This finding indicates that immune recognition of leukemia-derived glycolipids, as well as diverse range of leukemia-associated antigens, is critical for durable control of B-ALL.

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