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Redirecting T Cells With Chimeric Antigen Receptors To Target Cd123+ Leukemia

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A

DISSERTATION

Presented to the Faculty of
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DOCTOR OF PHILOSOPHY

by

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Houston, Texas

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DEDICATION

JESUS ALMIGHTY
MY BELOVED PARENTS

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This work could not have been accomplished without the help of many people. I would like to thank my mentor Dr. Laurence Cooper with utmost gratitude for the opportunity to work in his laboratory. His guidance and supervision throughout my graduate studies have been instrumental for my development as an independent researcher. My sincere thanks to Dr. Lee for being my mentor for the last few months and his valuable guidance and support for my dissertation, when Dr. Cooper moved to Ziopharm as C.E.O. I would also like to extend my thankfulness to my advisory and examination committee members Dr. Richard Eric Davis, Dr. Dat Tran, Dr. Kenneth Tsai, Dr. Elizabeth Shpall, Dr. Francois Claret for their important contribution, guidance and support for my PhD training.

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ABSTRACT

REDIRECTING T CELLS WITH CHIMERIC ANTIGEN RECEPTORS TO TARGET CD123^{+} LEUKEMIA

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CD123 or interleukin receptor alpha (IL-3Rα) is expressed on hematological malignancies such as acute myeloid leukemia (AML) and some acute lymphoblastic leukemia (ALL). Significantly, CD123 is over-expressed on leukemic stem cells (LSCs) compared to normal hematopoietic stem cells and thus targeting this tumor-associated antigen (TAA) provides the potential to prevent relapse. The prototypical chimeric antigen receptor (CAR) is fashioned by combining the variable light (V_L) and heavy (V_H) as a scFv derived from a single monoclonal antibody (mAb) specific for the TAA. We describe a new approach for generating CD123-specific CARs generating a chimeric scFv that is made up of the V_L and V_H harvested from two mAbs that are each specific for CD123. The hypothesis is V_L and V_H from different antibodies to the same TAA can be recombined to form unique binding domains that retain antigen specificity but may have altered binding characteristics. This non-homologous recombination of antibody binding domain may be used to select CAR for optimal anti-tumor characteristics, such as increasing the therapeutic index. The chimeric scFvs were derived by fusing the V_L and V_H chains derived from mAbs 26292, 32701, 32703, 32716 specific to

CD123. *Sleeping Beauty* (SB) was employed as a non-viral gene transfer system to stably express 2nd generation CARs in T cells derived from peripheral blood mononuclear cells (PBMC). The CARs were co-expressed with inducible Caspase 9 (iCaspase9) for conditional ablation of T cells in case of off-target toxicities. The SB plasmids coding for two CARs (transposons) activated T cells via chimeric CD28 with CD3-zeta and CD137 with CD3-zeta were electroporated into PBMC. Following electrotransfer of the SB system the genetically modified T cells were preferentially propagated on activating and propagating cells (AaPC) designated as Clone 1-CD123. The AaPC were derived from K562 cells genetically modified to co-express co-stimulatory molecules (CD86 and CD137L), a membrane bound cytokine (IL-15 fused to IL-15Rα), and the TAAs CD123 and CD19. CAR^+ T cells specifically produced IFN-γ and lysed $CD123^+$ leukemic cell lines and primary AML patient samples, but did not lyse $D123^{neg}$ tumor cells. The addition of a chemical dimerizer to activate iCaspase9 resulted in destruction of genetically modified T cells. Both populations of CAR^+ T cells produced and eliminated leukemic tumors *in vivo*. We observed no difference in the anti-tumor effects whether the CARs triggered T cells via CD28 or CD137. These studies suggest that CD123 can be targeted by CAR^+ T cells and that the hybrid arrangement of V_L and V_H maintained specificity for CD123.

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ABBREVIATIONS

aAPC: Artificial antigen presenting cell

AaPC: Activating and propagating Cells

APC: Antigen Presenting Cell

Ab: Antibody

Ag: Antigen

ALL: Acute Lymphoblastic Leukemia

AML: Acute Myeloid Leukemia

ATCC: American Type Culture Collection

BLI: Bioluminescence Imaging

CAR: Chimeric Antigen Receptor

CCL: CC Chemokine ligands

CCR: CC Chemokine Receptors

CD: Cluster of Differentiation

CDR: Complementarity Determining Regions

cGMP: Current Good Manufacturing Practices

CLL: Chronic Lymphocytic Leukemia

CML: Chronic Myeloid Leukemia

CMV: Cytomegalovirus

CRA: Chromium Release Assay

DC: Dendritic Cell

eGFP: enhanced Green Fluorescent Protein

EGFR: Epidermal Growth Factor Receptor

FACS: Fluorescence Activated Cell sorting

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

ffLuc: Firefly Luciferase

GvHD: Graft-versus-Host Disease

HLA: Human Leukocyte Antigen

HIV: Human Immunodeficiency Virus

HSC: Hematopoietic Stem Cell

ICOS: Inducible T-cell Co-Stimulator

ICS: Intracellular Cytokine Staining

IFNγ: Interferon-γ

Ig: Immunoglobulin

IL: Interleukin

IRB: Institutional Review Board

LCA: Lymphocyte Code-set Array

mAb: monoclonal Antibody

MDACC: MD Anderson Cancer Center

MHC: Major Histocompatibility Complex

MRD: Minimal Residual Disease

NIH: National Institutes of Health

NKT cells: Natural Killer T cells

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

PD1: Programmed Death-1

PI3K: Phosphoinositide 3-Kinase

PKC: Protein Kinase C

polyA: polyadenylation tail for mRNA transcripts

pSBSO: *Sleeping Beauty* transposon plasmid

ROR1: Receptor tyrosine kinase-like Orphan Receptor-1

RPMI: Roswell Park Memorial Institute medium

SB: *Sleeping Beauty*

scFv: single-chain variable fragment

SCID: Severe Combined Immunodeficiency

STAT: Signal Transducer and Activator of Transcription

T_{CM}: Central memory T cell

T_{EFF}: Effector T cell

T_{EM}: Effector memory T cell

T_{EMRA}: Effector memory RA T cell

T_{M}: Memory T cell

T_{N}: Naïve T cell

TAA: Tumor-associated antigen

TCR: T-cell Receptor

UCB: Umbilical Cord Blood

UPenn: University of Pennsylvania

WBC: White Blood Cell

CHAPTER-I

INTRODUCTION

I.1. Hematological malignancies

Hematological malignancies affects blood, bone marrow (BM) and lymphatic system. They originate from BM or the cells of immune system and are the fifth most commonly occurring cancers and the second leading cause of cancer death. Based on the type of white blood cells affected hematological malignancies are broadly classified as **i) Lymphoma**: affects the lymphatic system, produces uncontrolled growth of white blood cells (WBCs) in lymph nodes. Lymphoma can be further classified as Hodgkin's lymphoma (HL) and Non-Hodgkin's lymphoma (NHL) **ii) Myeloma**: also known as plasma cell myeloma, myelomatosis, or Kahler's disease a type of cancer affecting plasma cells that produces antibodies. It begins in the BM by accumulation of abnormal plasma cells **iii) Leukemia**: leukemia is the most common type of cancer in children younger than 15 years and adults older than 55 years. Leukemia begins with the abnormal accumulation of lymphocytes or myeloid cells in the BM. The four major types of leukemia are acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML) acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL). Approximately 75% of leukemias affecting children are ALL, whereas AML and CLL are the most common among adults followed by ALL and CML **(1-3).** Immunotherapies targeting tumor associated antigens (TAAs) e.g CD19 by adoptive transfer of genetically engineered T cells resulted in drastic regression of tumors and complete remission in CLL patients in clinicalsetting **(4-8).** The focus of this dissertation is on developing adoptive immunotherapies by targeting surface proteins expressed on for B-ALL and AML through genetic modification of T cells.

I.1.A. B-cell Acute Lymphoblastic Leukemia

ALL originates from B or T lymphocytes in the BM. B-cell acute lymphoblastic leukemia (B-ALL) is clonal accumulation of B cell blasts resulting in suppression of normal hematopoiesis. More than 80% of ALLs in children and 70% ALLs in adults belong to B-ALL lymphoid group **(9, 10).** Key tools to diagnose B-ALL include cytogenetic studies to identify genetic alterations in B cell blasts, molecular studies to detect translocations, genome-wide associations to detect genetic changes where routine techniques are unavailable, flow cytometry to analyze surface phenotype and monitoring minimal residual disease (MRD) **(11).** Improved chemotherapeutic approaches and radiation followed by allogeneic hematopoietic stem cell transplantation (HSCT) with cord-blood and haplo-identical approaches over the past decade enhanced the long-term survival in 90% of children.

Although transplant related mortality (TRM) has decreased markedly over the past 15 years, relapse remains a concern in high risk group children. Several groups reported that presence of MRD pre and post HSCT is a predictable tool to detect relapse. Rate of relapse can be decreased by monitoring MRD and occurrence of Graft versus Host Disease (GvHD) in First 2 months after the transplant. Employing novel agents and immunotherapies before and after HSCT will lower MRD and improve Graft versus Leukemic effect (GvL) and survival in children and adults **(12-16).**

I.1.B. Acute Myelogenous Leukemia

AML is the most common form of leukemia mostly affecting adults over 55 years. AML is a clonal proliferation of malignant myeloid blast cells in the BM with impaired normal hematopoiesis. Despite many advances in treatments AML still remains a lethal disease. Standard chemotherapy and radiation regimens ensure long-term remission only in 30 to 50% of patients with a low survival probability resulting in resistance and relapse **(17-19).** The relapse in AML is due to MRD caused by small population of Leukemic stem cells (LSCs) resistant to drugs and radiation. Initial treatment strategy for AML patients include induction chemotherapy to eliminate blast cells, followed by consolidation therapy to target the leukemic stem cells. Because of abundant availability of AML samples, relative simplicity of acquiring them from BM, recent advances in the understanding of molecular aspects such as role of

Chromosomal translocations, easy to analyze AML subsets by flow cytometry enable to progress the studies on AML. Introducing advanced treatment options beyond or in addition to current standard treatments will radically change the survival rates of people diagnosed with AML. Antigen specific based adoptive immunotherapy will play a complimentary role in eradicating MRD by targeting leukemia associated antigens expressed on LSCs and leukemic cells **(20-22).**

I.2. T lymphocytes and adaptive immune system

Immune system protects organisms from infection and disease and broadly classified as innate immune system and adaptive immune system. The innate immune system serves as first line of defence in case of infection and has broad range of specificity for different pathogens. The blood cell types that mediate innate immune system include i.e macrophages, natural killer (NK) cells. In contrast adaptive immune system is specific to part of pathogen (tumor associated antigens and peptides) resulting in long-lasting response through formation of immunological memory **(78-82).** T lymphocytes are a type of white blood cells that plays a major role in adaptive immune system by cell-mediated-response. Based on TCR structure, T cells can be classified into two types, **i) alpha/beta (αβ) T cells**: TCR is a heterodimer composed of an alpha and beta chains. Each chain has a variable (V) region and a constant

(C) region. The V regions each contain 3 hyper variable regions that make up the antigen-binding site. αβ T cells comprises up to 95-99% of circulating T cells **ii) gamma/delta (γδ) T cells**: TCR is a heterodimer composed of gamma and delta chains. The TCR of αβ T cells binds to a bimolecular complex consisting of peptide of antigen lying within the groove of MHC displayed at the surface of antigen presenting cell (APC) i.e. dendritic cells (DCs), B-cells, macrophages **(83).** Aβ T cells are distinguished from other lymphocytes such as NK cells and B cells by the presence of T cell receptor (TCR) on their surface and recognize its antigens in the context of major histocompatibility complex **(MHC).** Most of T cells in the body belong to sub sets CD4 or CD8. $CD8^+$ T cells bind to epitopes that are part of major MHC class I and $CD4^+$ T cells bind to epitopes that are part of MHC class II molecules. All most all the cells in the body express MHC-class I and professional antigen APCs DCs, B cells and macrophages express MHC-class II molecules. The best understood $CD8^+$ T cells cytotoxic lymphocytes (CTLs) whose main function is to destroy infected or a tumor cell by binding to its specific peptide or antigen. $CD4^+$ T cells are essential for both cell mediated and antibody mediated (Humoral) immunity. In cell mediated immunity $CD4^+$ T cells binds to antigen presented by APCs by releasing lymphokines that attract other immune cells to the area resulting in inflammation. Humoral immunity is mediated by B cells primarily through production of antibodies. $CD4^+$ cells, called helper T cells binds to antigen presented by B cells resulting development of clones of plasma cells secreting antibodies **(84-85).**

I.3. Chimeric Antigen Receptors

The concept of redirecting T-cells to TAAs by genetic modification was first developed by Prof. Zelig Eshhar and colleagues at the Weizmann Institute of Science in Rehovot, Israel in 1980s. By 1989, the same group had created the first functional CAR T cells **(25)**. Chimeric antigen receptors (CARs) are recombinant receptors derived by fusing single chain fragment variable (scFv) region of a monoclonal antibodies (mAbs) specific to TAAs to T cell signaling domains (i.e. CD3ζ, CD28) via a transmembrane domain

CD8α, CD28) and a hinge (i.e IgG4, CD8α, CD28) **(Figure 1).** Generally the scFvs used in making CARs are derived from well characterized murine mAbs or fully humanized mAbs (hmAbs). CARs recognize targeted antigen in its native form independent of major MHC compatability. The moieties used to recognize antigens by CARs can be broadly fall into three categories

i) scFv derived from mAbs specific to targeted antigen **ii)** fragment antigen-binding (Fab) selected from libraries **iii)** nature ligands that binds to their cognate receptors. The "generation" in the CAR refer to the intracellular signaling domains. First generation CARs include only CD3ζ as signaling domain and showed limited T cell activation and short term T cell expansion but enabled cytotoxicity. Second generation CARs include one co- stimulatory domain such as CD28 or 41BB exhibited improved T cell expansion, cytokine production and T cell persistence. Third generation CARs include three intracellular endo-domains the most common combination has been CD28, CD137 (4-1BB), and CD3ζ **(26-29) (Figure 2).** The efficacy of CAR T cells targeting its TAAs depends on various factors such as **i)** position and distance of epitope from cell surface and formation of optimal T cell synapse **ii)** length and flexibility of hinge region between scFv and transmembrane domain **iii)** antigen density on tumor cells **iv)** Activation of endo-domains **(30-33).**

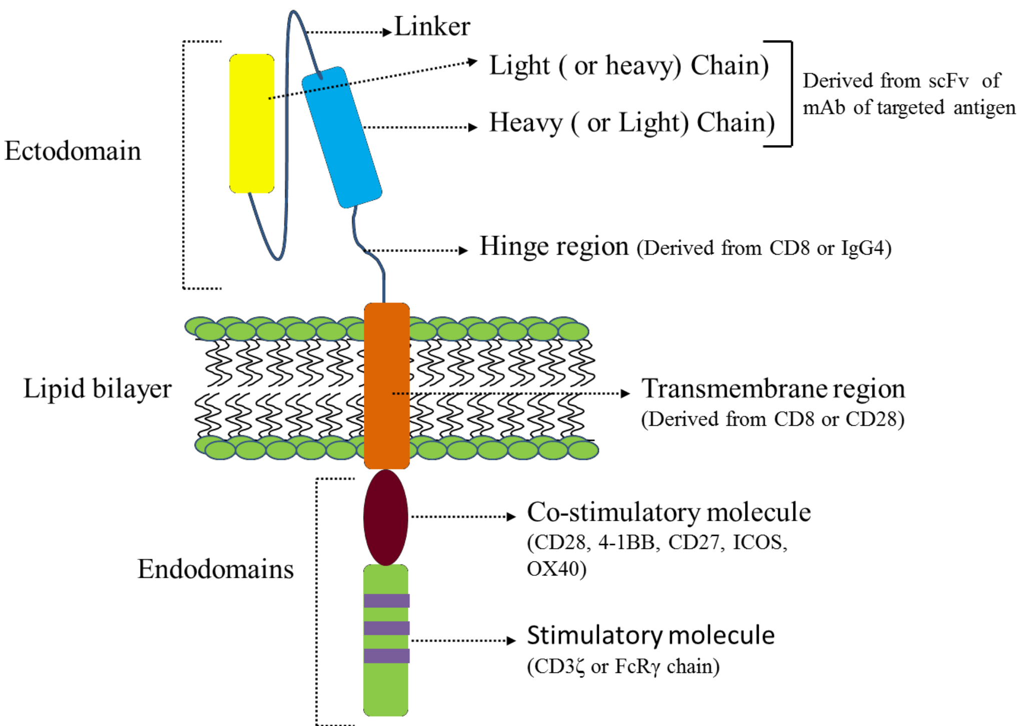

Figure 1. Schematic representation of 2nd generation chimeric antigen receptor. Single chain fragment variable (scFv) region of a monoclonal antibodies (mAbs) specific to TAA fused to T cell signaling domains (i.e. CD3ζ, CD28) via a transmembrane domain (i.e CD8α, CD28) and a hinge (i.e IgG4, CD8α, CD28).

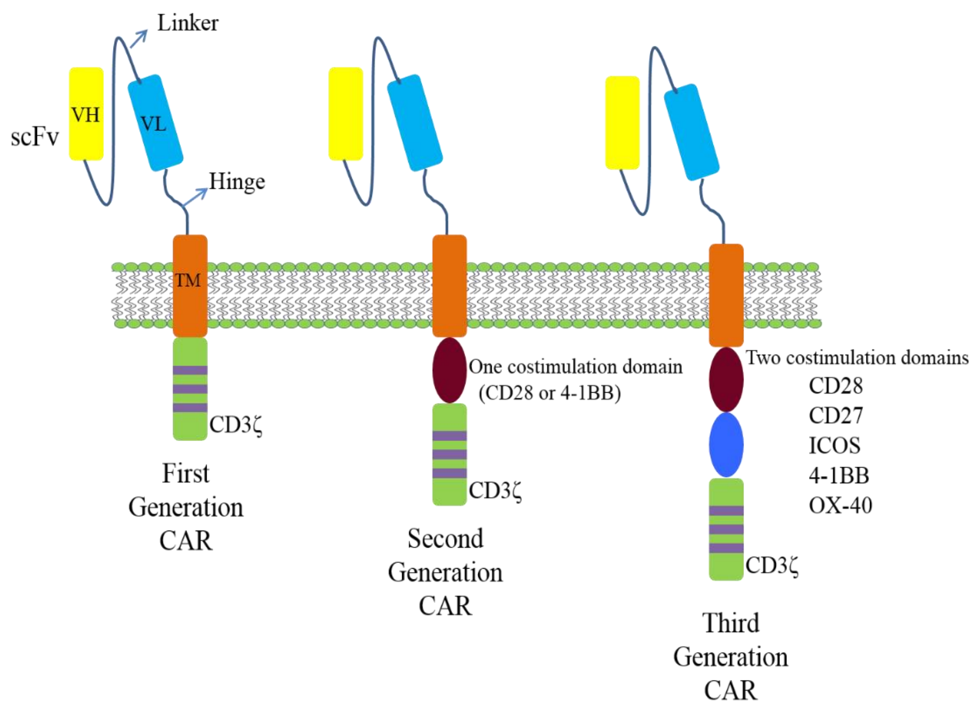

Figure 2. Schematic of three generations of CARs. The first-generation CARs consisted of the single-chain variable fragment of monoclonal antibodies specific for tumor associated antigen fused IgG4 constant region followed by CD28 transmembrane alpha helix and CD3ζ signaling endodomain. Second generation CARs were generated to incorporate the intracellular domains of one or more costimulatory molecules such as CD28 or CD137 within the endodomain. Third-generation CARs include a combination of costimulatory endodomains e.g CD28 and CD137.

I.4. Clinical trials and CAR T cells

CD19 was the first antigen targeted by CAR engineered T cells since it is expressed by most of B-cell leukemias and lymphomas but not on tissue other than normal B lineage cells **(34, 35).** Successful eradication of tumors with different CD19 directed CARs resulted in multiple clinical studies targeting large number of surface molecules expressing on hematological malignancies as well as solid tumors such as HER2, GD2, prostate-specific membrane antigen (PSMA) and mesothelin **(36).** To date the most promising clinical outcome including complete remission have been reported with second generation CARs targeting CD19 expressed by B-cell leukemia and lymphoma **(37-39)**. In July, 2014, CD19-specific CAR T cell therapy (CTL019) developed at University of Pennsylvania (UPenn) was granted "breakthrough therapy" status by Food and Drug Administration (FDA) **(40).** Second generation CARs with CD3ζ and CD137 signaling domains out- performed the ones signaling through CD28 and CD3ζ in terms of therapeutic efficacy though the preclinical models have not shown any difference between them **(41).** The reasons for better efficacy of CD137 CARs over CD28 CARs not known at present, chapter III of this dissertation will describe the comparative evaluation of efficacy CD123-specific CARs with CD28 and CD137 co-stimulatory domains.

I.5. *Sleeping beauty* transposition

Stable integration of transgenes can be accomplished by viral and non-viral methods. Most of the clinical trials currently use retroviral or lentiviral vectors for CAR transgene transfer **(42).** Viral vectors are efficient in gene transfer but often associated with genotoxic effects and immunological complications **(43-44).** DNA transposons have been developed as an alternative method for gene transfer. *Sleeping beauty* (SB) transposon system is a molecular reconstruction from evolutionarily decayed sequences in salmonid genomes **(45).** Unlike lentiviral and retroviral vectors, SB gene transfer requires less production cost for manufacturing clinical grade T cells and does not integrate at sites of active transcription. It has been shown SB transposons do not activate oncogenes though the mode of integration into genome by random method. The SB system has a two DNA plasmids a transposon with the gene of interest (e.g CAR) flanked by Inverted repeats/Direct repeats (IR/DR) and a transposase that catalyzes excision and integration of gene of interest into TA dinucleotide site of recipient genome **(46).** TA nucleotides are randomly distributed in the genome enabling random integration of transgenes through SB system and has been shown to be safe in preclinical studies **(47- 49).** Electro-transfer of two transposons into peripheral blood mononuclear cells (PBMC) results in transient expression of SB transposase and stable expression and integration of CAR transgene into the genome. The major safety concern for CAR T cells is genotoxicity and the risk of insertional mutagenesis associated with introduced genetic material. The risk of insertional mutagenesis can be alleviated by transiently expressing CAR by mRNA electroporation. This would require multiple infusions of CAR T cells to generate effective anti-tumor effect but it may reduce the cytotoxicity to normal tissues **(50)**.

I.6. Activating and Propagating Cells

Activating and Propagating Cells **(**AaPCs) are a group of immune cells that mediate immune response by presenting antigens complexed with MHC to certain lymphocytes such as T cells. Classical APCs include dendritic cells (DCs), macrophages and B cells among which DCs are the most efficient and equipped with MHC I and MHC II molecules on their surface **(51)**. Adoptive transfer of mature DCs augment T-cell responses in humans, hence DC immunization is considerably important in immunotherapy of cancer **(52).** However development of DCs as T cell expanding platform is expensive and laborious and sometimes dysfunctional in cancer patients **(53, 54).** Since CARs activate T cells independent of MHC and TCR specificity, a method of propagation avoiding TCR/MHC interactions is also needed for ex vivo propagation. Different platforms do exist to achieve this most popular are CD3/CD28 coated beads and artificial antigen presenting cells (aAPC) (2). This dissertation uses an approach that focuses on expanding S*leeping beauty* modified T cells on Activating and Propagating Cells (AaPCs) a K562 is a CML

Cell line genetically modified co-expressing co-stimulatory molecules and cytokines for T cell expansion **(Figure 3)**. The advantages of K562 as APC over DCs are they **i)** do not express MHC class I and II molecules except limited expression of MHC-class C which therefore prevents allogenic T cell responses **ii)** can be easily manipulated genetically by viral or non-viral methods **iii)** express adhesion molecules required for aAPC-T cell interactions **iv)** do not skew endogenous TCR response to particular antigens **(55).** Patients treated with T cells expanded on K562-AaPC did not show toxicity, suggesting this is a safe approach for manufacturing clinical grade T cells. So far K562-derived aAPC have been used in 4 clinical trials at MD Anderson cancer center (NCT01497184, NCT01653717, NCT01619761, NCT00968760). Various versions of K562 based aAPC had been created by University of Pennsylvania (UPenn) to expand CD19-specific CAR T cells in autologous as well as allogeneic setting. K562-clone 4 was developed by enforced expression of CD19, CD32, CD64, CD86, CD137L, and enhanced green fluorescence protein (eGFP) tagged Fc- IL15 fusion protein. This dissertation used K562-Clone1 which is similar to clone 4 except it has enforced expression of IL-15 fused to IL-15Rα replacing (eGFP) tagged Fc- IL15 fusion protein, ROR1 and CD123 in addition to CD19, CD64, CD86, CD137L **(Figure 3).**

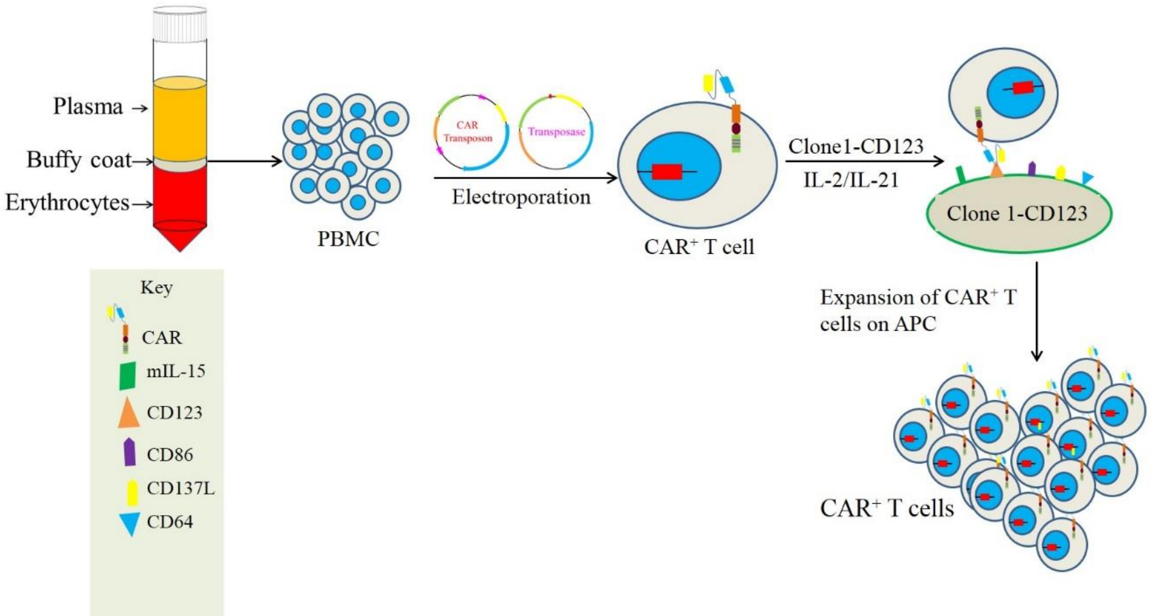

Figure 3. Schematic of CAR T cells Expansion on AaPC. PBMC are isolated from whole blood by density gradient centrifugation using Ficoll-Hypaque and are electroporated with plasmids encoding either **(i)** *Sleeping Beauty* transposase or **(ii)** *Sleeping Beauty* transposon containing CAR. Transfected cells were phenotyped for CAR expression next day and cells stimulated with γ-irradiated K562-derived AaPC every 7 days supplementing with IL2 and IL-21. Following 3-4 weeks of co-culture, CAR T cells expanded to clinically relevant numbers are ready for cryopreservation and then infusion into cancer patients. Figure includes K562- Clone1-CD123 that express CD19, ROR1, CD64, CD86, CD137L, mIL15 (1IL15 fused to IL15Rα) and CD123.

I.7. Interleukin-3 Receptor α (CD123)

CD123 is the α subunit of Interleukin-3 cytokine receptor (IL-3Rα) which forms high-affinity functional hetero-dimeric receptor along with its β subunit CD131. Binding of IL-3 to IL-3R activates the receptor leading to cell survival and proliferation **(56, 57).** IL-3 stimulated activation of spontaneous signal transducer and activator of transcription 5 (STAT5) is correlated with over expression of CD123 on AML cells resulting in proliferation of tumor cells **(58-60).** CD123 has been reported to be overexpressed on up to 95% of leukemic blasts and leukemic stem cells (LSCs) in AML, majority of B- ALL blasts, but not on normal hematopoietic stem cells (HSC) and no cells outside hematopoietic lineage **(61-66).** Clinically, high CD123 expression in AML patients at diagnosis is associated with higher blast counts and a lower complete remissions resulting in reduced survival **(58-60).** Collectively, these findings point to the significance of CD123 expression in leukemia cell stimulation and AML patient outcome. Phase1 clinical trials targeting CD123 in AML using neutralizing mAbs and cytotoxic protein fused to IL-3 cytokine showed limited therapeutic efficacy pressing the need for more novel efficacious treatments **(67-68).** Thus CD123 is a viable target in AML through chimeric antigen receptors in AML give n its wide expression on leukemic blasts, progenitors, LSCs and weak or no detectable expression on hematopoietic stem cells. The main goal of this dissertation is, to redirect T –cell specificity to CD123 through chimeric antigen receptors (CARs) to target AML and to generate preclinical data in support of an adoptive immunotherapy trial.

I.8. Leukemic Stem cells and minimal residual disease

The majority of treated AML patients deemed to be in complete remission by chemo and radiation therapies resulting in relapse. The relapse is due to MRD attributed to LSCs **(74).** LSCs are pre-leukemic clonal population of HSC by genetic and molecular alterations capable of self- renewal, able to initiate leukemia when transplanted in SCID mice by generating rapidly proliferating progenitors and leukemic blasts **(69-71).** HSCs and LSCs have common features such as basic phenotype (Lineage^{neg}CD34^{+}CD38^{neg}), slow division, self-renewal capacity **(69, 72).** To our knowledge most of the antigens that are expressed in AML are also present on hematopoietic stem cells and progenitors. However certain AML markers are over expressed on LSC while there is weak or no detectable expression on normal HSCs. CD123 is highly expressed on the CD34^{+}CD38^{neg} fraction, leukemic blasts and bulk of AML cells when compared to normal hematopoietic cells **(73).** CD123 and C- type lectin-like molecule1 (CLL-1) are robust markers for MRD and highly expressed on LSC **(75, 76).** Employing CAR T cells specific to CD123 after hematopoietic transplantation eradicate MRD which contain residual leukemic stem cells. Moreover some of the B-ALL patients treated with CD19 CAR T resulted in relapse with the residual population of leukemic cells negative for CD19 and positive for $CD123^{+}$ **(77).** These patients can be treated using CAR T cells specific to CD123. The increased expression of CD123 on LSCs compared with weak or no detectable expression on HSC presents an opportunity for selectively targeting LSCs on AML with CD123-specific CAR^{+} T cells.

I.9. Dissertation specific aims

This dissertation focuses on three specific aims described as follows

I.9.A. Specific aim #1. To determine if scFvs of chimeric antigen receptors derived by "Mix-and-Matching" V_L and V_H domains from two monoclonal antibodies can redirect specificity to $CD123^{+}$ leukemias. The V_L and V_H of scFvs of CARs usually derived from single monoclonal antibody specific to targeted antigen. However this dissertation describe a new approach for generating CD123-specific CARs generating a chimeric scFv that is made up of the V_L and V_H harvested from two mAbs that are each specific for CD123. The major **hypothesis** for this specific aim is that CARs generated by combining V_L and V_H chains from two different mAbs for CD123 will retain specificity for CD123. We hypothesize that the CARs can be selected for targeting CD123 overexpressing leukemia while sparing normal hematopoietic cells expressing CD123 at low levels for improved therapeutics. Rationale for this specific aim is i) TAAs are not specific to tumors but also may be expressed at low levels on normal cells, potentially resulting in on-target, off-tumor toxicities ii) CD123 is expressed on hematopoietic progenitors and weakly expressed on monocytes, neutrophils, basophils and megakaryocytes iii) The affinity of the scFv for TAA also affects the density of TAA required for efficient killing. iv) CAR T cells preferably target tumors with high antigen density, while cells with lower density are more resistant to CAR T cells (97, 98). A panel of CARs have been generated by mix-and-matching VL and VH of four mAbs specific to CD123 and tested their cytolytic efficacy in B-ALL and normal BM cells.

I.9.B.Specific Aim#2: Comparative evaluation of CD123-specific chimeric CARs containing CD28 or CD137 endo-domains for enhanced survival and anti-tumor efficacy in AML. The hypothesis of this aim is that CAR T cells containing CD137 endo-domain will be superior to those signaling through CD28 in therapeutic efficacy. The *rationale* is i) Optimal CAR design enhances the persistence of CAR T cells ii) studies showed that CARs that incorporates CD137 has enhanced survival and anti- tumor efficacy compared to CARs with CD28 endo-domain iii) the clinical outcome of complete remission of CAR T cells correlated with long-term persistence of CAR T cells iv) CD123, the IL-3 receptor α- subunit has been reported to be overexpressed in AML. Two second-generation CD123- specific CARs were generated from chimeric scFv by fusing V_H and V_L from two mAbs specific to CD123 to CD3ζ and CD28 signaling domains (designated CD123-CD28 CAR) and by fusing the same scFv to CD3ζ and CD137 signaling domains (designated CD123-CD137 CAR). Each CAR connect the scFv region to the endodomains via a modified hinge and Fc region from IgG4. The Sleeping Beaut y (SB) system was used for non- viral gene transfer to stably express CARs into T cells derived from peripheral blood mononuclear cells (PBMC). Two SB plasmids coding for transposons (CARs co-expressed with iCaspase9) and transposase (SB11) were electroporated into PBMC and numerically expanded on designer AaPCs (designated Clone 1-CD123) a genetically modified K562 cells co-expressing co-stimulatory molecules (CD86 and CD137L), a membrane bound cytokine mIL15 (IL-15 fused to IL-15R) and the TAAs ROR1 CD19 and CD123 supplemented with cytokines IL-2 and IL-21. Expanded T cells were monitored for CAR expression, counted to determine expansion kinetic s over a period of 4 to 5 weeks. At the end of 4 weeks of co-culture the surface and memory phenotype were determined. The effector function of CAR^{+} T cells were determined by assessing *in vitro* lysis of $CD123^{+}$leukemic cell lines and primary AML patient samples. To evaluate in vivo tumor clearance $CD123^{+}$ leukemia xenografts were established in NSG mice and treated with CAR T cells.

I.9.C **Specific Aim#3:** *in vitro* targeting of AML leukemic stem cells by CAR T cells specific to CD123. The hypothesis for this specific aim is to determine if CD123-specific CAR T cells can eliminate leukemic stem cells in AML. The *rationale* is **i)** The relapse in AML is due to minimal residual disease caused by small population of LSCs resistant to drugs and radiation **ii)** high expression of CD123 on LSCs compared with weak or no detectable expression on HSCs presents an opportunity for selectively targeting AML-LSCs **iii)** Antigen specific based adoptive immunotherapy will play a complimentary role in eradicating MRD by targeting TAAs expressed on LSCs. CD123 expression levels were determined in AML primary samples and phenotypically defined LSCs. The efficacy of CAR cells in elimination of LSCs and HSCs were determined *in vitro* by co-culture killing assays.

CHAPTER-II

Redirecting specificity of T cells To Target CD123^{+} B-ALL Tumors

II.1. Introduction

CARs can empower T cells with an antibody-like specificity and is able to transmit signals leading to T cell activation, proliferation and its effector functions upon binding its specific antigen. The binding chemistry of CAR's scFv with its cognate antigen is not well studied at present. Eshhar et.al demonstrated that the antigen binding site and idiotope for anti-2, 4, 6- trinitrophenyl (TNP) antibody (SP6) reside exclusively in VH region. In general, T cells expressing chimeric antigen receptors (CARs) are generated by combining the variable light (V_L) and heavy (V_H) chains of scFv derived from single mAb specific to targeted antigen **(86).** Examination of the contribution of V_H and V_L chains of scFvs specific to targeted antigen may help us to better understand the functionality of CARs and to derive CARs with different affinities to targeted antigen. One of the limiting factors in CAR T cell therapy is TAAs are not tumor "specific" but also expressed at low levels on normal cells and often associated with off tumor toxicities. Recent preclinical studies targeting EGFR and erbB2 with affinity lowered CAR T cells have demonstrated potent antitumor effect on tumors with high antigen density while sparing normal cells **(87, 88).** The present chapter describes a new approach for generating CD123-specific CARs derived from a chimeric scFv that is made up of the V_L and V_H harvested from two mAbs that are each specific for CD123. The major hypothesis for this specific aim is that CARs generated by combining V_L and V_H chains from two different mAbs for CD123 will retain specificity for CD123. We hypothesize that the CARs can be selected for targeting CD123 overexpressing leukemia while sparing normal hematopoietic cells expressing CD123 at low levels for improved therapeutics. To test this hypothesis we have generated six CARs with chimeric scFvs by mix and matching V_H and V_L of four mAbs specific to CD123. CARs derived from V_H and V_L of original mAbs without mix and matching were used as control. We have chosen the one with least killing and effector functions in normal hematopoietic cells carried forward to target B-ALL (described in present chapter) and AML (described in chapter III).

II.2.Results

II.2.A. Generation of CD123$^+$ Activating and Propagating Cells (AaPC)

Activating and Propagating cells (AaPC) has been successfully shown to expand antigen specific CAR T cells *ex vivo* **(45-49).** Binding of T cells to its cognate antigen on APC cell surface results in CAR$^+$ T cell clustering, phosphorylation of immune-receptor tyrosine-based activation motifs (ITAMs) there by activating T cells **(89).** K562 based AaPC-Clone 1 was previously made to expand CAR T cells co-express TAAs (CD19 and ROR1) co-stimulatory molecules (CD86 and CD137L), Fc receptors (endogenous CD32 and transfected CD64) for loading of agonistic anti-CD3 antibody OKT3 and IL-15 fusion protein (IL-15 fused to IL-15Rα) **(Figure 4).** However AaPC-Clone 1 do not express CD123. Therefore a new AaPC has been derived to expand CD123-specific CAR T cells by enforced expression of CD123 on AaPC-Clone 1 (designated as Clone1-CD123). The CD123 DNA sequence was synthesized and codon optimized by Gene Art (Regensburg, Germany) fused to hygromycin resistance gene through F2A peptide and sub cloned into a SB transposon plasmid **(Figure 5A).** AaPC-Clone 1 cells were co-electroporated with CD123 transposon and transposase SB11 and CD123$^+$ positive cells were selected by hygromycin selection. Within 9 days after electroporation more than 98% of cells express CD123 (Figure 5B)

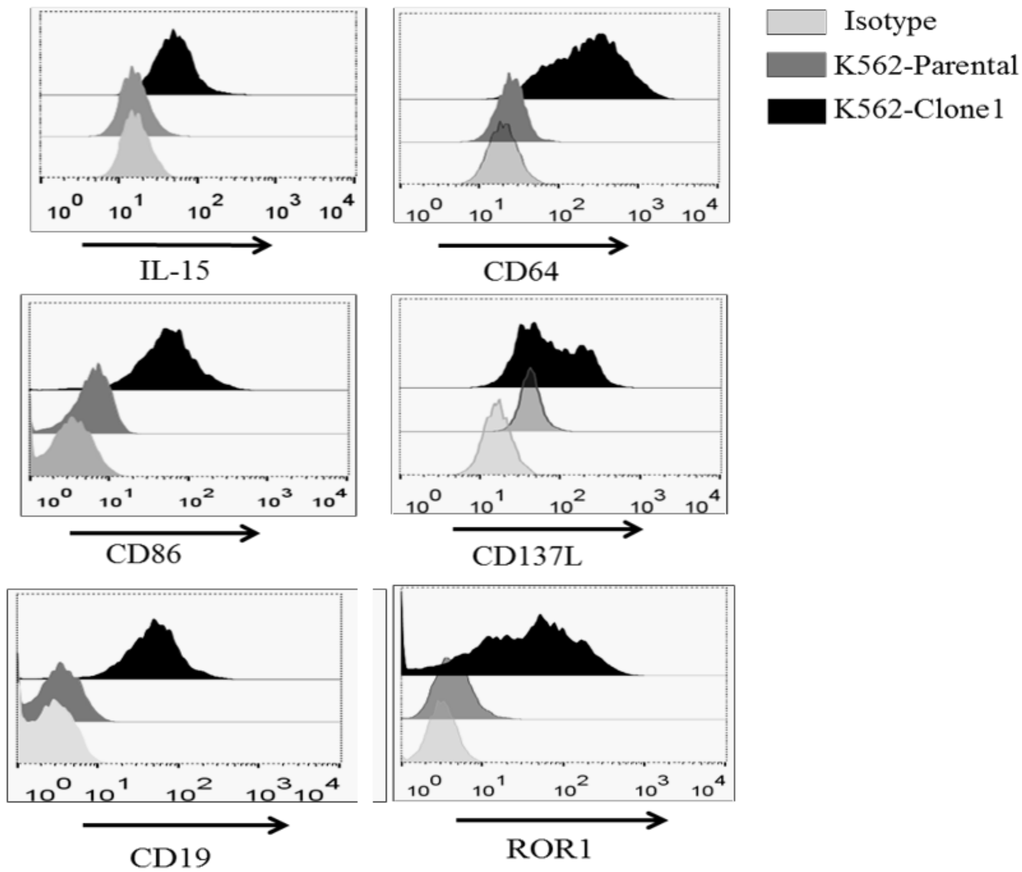

Figure 4. Surface phenotype of AaPC-Clone1. Surface expression of IL-15, CD64, CD86, CD137L, CD19 and ROR1 were analyzed by flow cytometry on parental K562 (deep grey histogram) and Clone1 (black histograms) and appropriate isotypes controls (light grey).

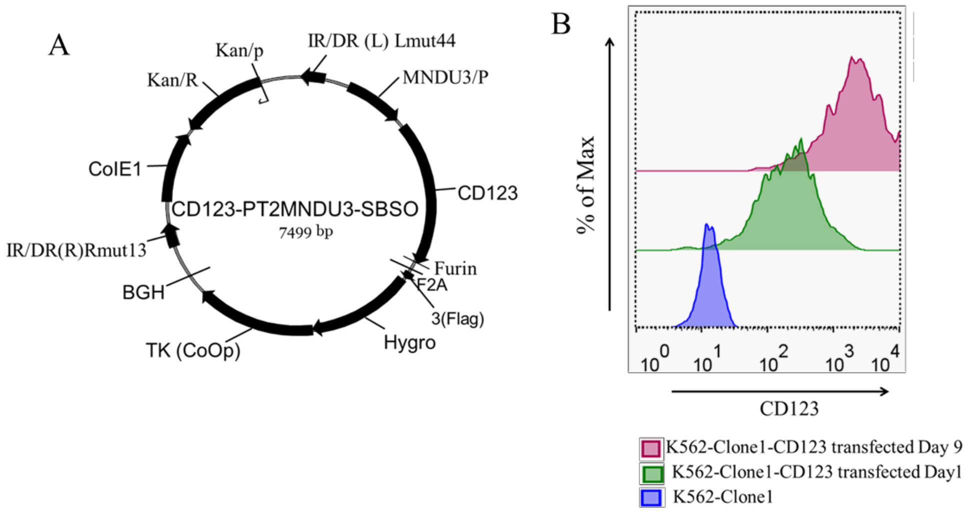

Figure 5. Generation of Clone 1-CD123.

A) *Sleeping Beauty* transposons expressing CD123 antigen. DNA plasmid vector maps for CD123 antigen IR/DR: *Sleeping beauty* Inverted Repeats/Direct Repeats, MNDU3/P: modified myeloproliferative sarcoma virus long terminal repeat enhancer–promoter (MNDU3) CD123: Human codon optimized CD123 antigen fused to hygromycin resistance gene through flag and F2A peptide. TK- codon optimized thimidine kinase gene BGH polyA; Bovine growth hormone polyadenylation sequence, ColE1: A minimal *E.coli*origin of replication, Kanamycin (Kan/R): Bacterial selection gene encoding Kanamycin resistance, Kanamycin promoter (Kan/p); Prokaryotic promoter **(B)** Histograms showing CD123 expression after electroporation of CD123 transposon and SB11 transposase into AaPC-Clone1 transfected with nucleofector solution without plasmids (blue) with plasmids on day1 (green) with plasmids day 9 (pink)

II.2.B. Chimeric CARs numerically expand on AaPC and stably express CAR

5 second generation CARs with chimeric scFvs were generated by mix and matching V_L and V_H chains of mAbs 26292, 32701, 32703 and 32716 specific to CD123 (CARs 5 to 9 **Figure 6A right**). All the scFvs except CAR-10 were fused in frame to CD3ζ and CD28 endo domains via CD8α hinge and CD8 transmembrane domain (TM) whereas IgG4 hinge and CD28 TM were used for CAR-10. For simplicity these CARs are designated as "chimeric CARs" and CARs derived from regular scFvs of mAbs were used as positive control and called "Regular CARs" **(**CARs 1 to 4 **Figure 6A left)**. These mAbs recognize different epitopes on CD123 with different binding affinities **(96)**. All CAR constructs were custom synthesized and cloned into *Sleeping Beauty* system.

CAR plasmids **(**typical representation of CAR plasmid is given in **Figure 6B left)** along with transposase SB11 **(Figure 6B right)** were electroporated into $CD56^+$ NK cell depleted PBMC and expanded on Clone1- CD123 at 2:1 AaPC:T cell ratio in presence of recombinant cytokines IL2 and IL-21. T cell cultures were stimmed with AaPC and surface phenotyped every 7 days

starting from dayone. CAR expression was detected with CD123 recombinant protein fused to Fc followed by serial staining with antibodies specific to Fc and CD3. Within 21 days chimeric contain more than 90% CD3 and CAR double positive cells like regular CAR cultures **(Figure 7A).** Cultures were devoid of NK cells though a small proportion of T cells express CD56, they do not express CD3 (data not shown)**.** Chimeric CARs expanded at similar rates as regular CARs in sufficient amounts for clinic **(Figure 7B and 7C).**

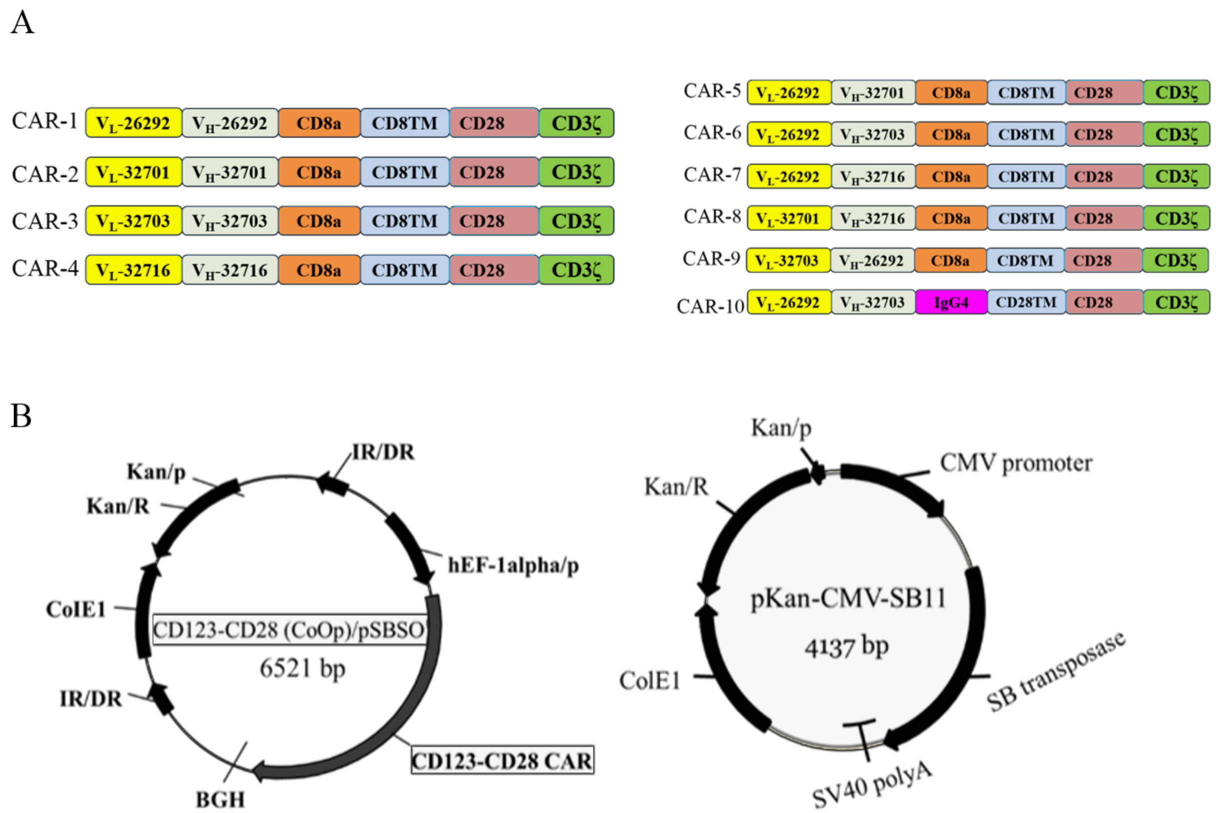

Figure 6. CD123-specific CARs with chimeric scFvs: (A) Left**.** Regular CARs (CARs 1 to 4) derived by fusing V_H and V_L chains of mAbs specific to CD123. Right**.** Chimeric CARs (CARs 5 to 10) derived from chimeric scFvs of mAbs by mix and matching V_H and V_L chains **(B)** Left. Typical representation of S*leeping Beauty* transposon plasmid containing CD123-specific CAR with CD28 co-stimulatory domain. IR/DR: *Sleeping Beauty* Inverted Repeat/Direct repeats, ColE1: A minimal E.coli origin of replication, Kanamycin (Kan/R): Bacterial selection gene encoding Kanamycin resistance, Kanamycin promoter (Kan/p); Prokaryotic promoter. hEF-1alpha/p: human Elongation Factor-1 alpha region hybrid promoter; CD123-CD28 CAR:

Human codon optimized CD123-specific CAR with CD28 co-stimulatory domain; BGH polyA; Bovine growth hormone poly adenylation sequence, (right) SB11 transposase; CMV promoter (Cytomegalovirus promoter) SV40 PolyA (Simian Virus 40 PolyA).

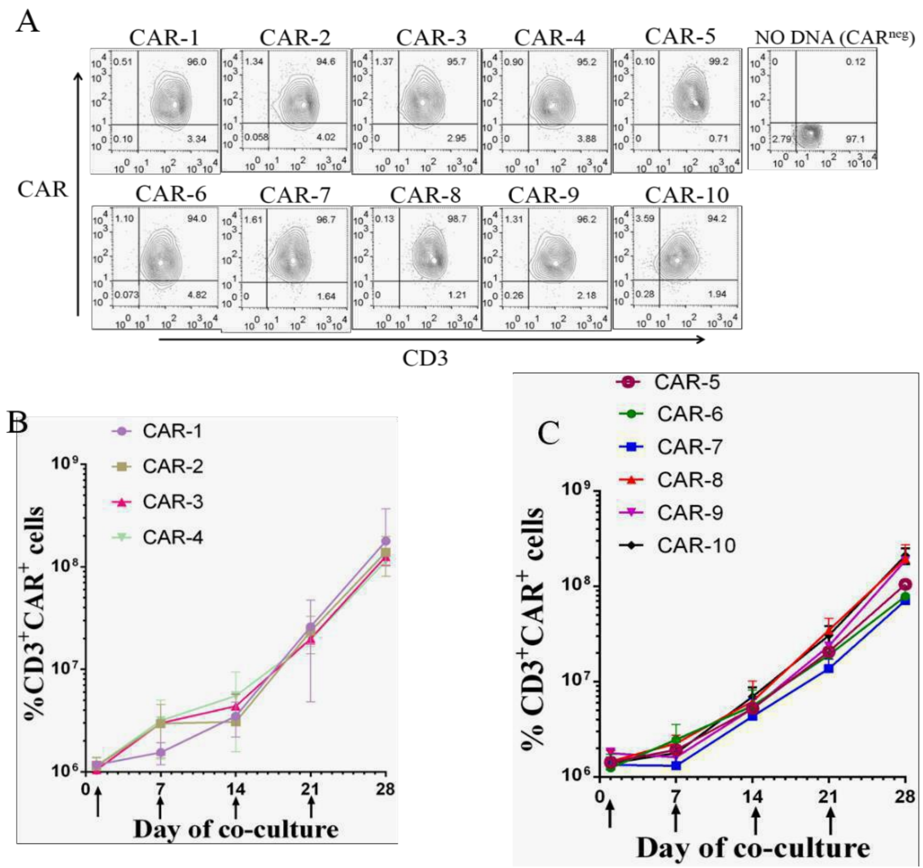

Figure 7. Expression and expansion kinetics of chimeric CARs

CAR expression and expansion kinetics following electroporation and xpansion on Clone 1-CD123 in presence of IL-2 and IL-2 (A)CAR expression on Day21 after electroporation detected byCD123 recombinant protein fused to Fc followed byserial staining with Fc and CD3 antibodies. PBMC electroporated with nucleofector solution without CAR plasmids (CAR^{neg}) used as negative control **(B)** Expansion kinetics of CARs 1 to 4 **(C)** CARs 5-10 over a period of 28 days and data pooled from 3 donors mean $\pm$ SD

II.2.C. Chimeric CARs maintain specificity to CD123

Before testing *in vitro* efficacy of chimeric CARs several leukemic cell lines including pre-B-ALL cell line Nalm6 and AML cell line TF1 and human embryonic kidney cell line 293T **(Figure 8).** To test chimeric CAR T cells demonstrate effective specific lysis of $CD123^{+}$ tumor cells *in vitro*, a chromium-51 labeled target cell lines were co-cultured with CAR T cells in a standard 4 hour chromium release assay effector: target (E:T) ratio 20:1 . $CD123^{+}$ pre B-ALL cell line Nalm6, and AML cell line TF1 were used as positive controls and 293T human embryonic kidney cell line used as negative control. CAR T cells able to lyse $CD123^{+}$ B-ALL tumor cell lines **(Figure 9A)** but not $CD123^{neg}$ cell line 293T **(Figure 9B).** To further verify killing by CAR T cells we co-cultured CAR^{neg} with target cell lines in 20:1 they fail to kill $CD123^{+}$ ALL cell lines. To test antigen-specific lysis 293T cells CAR T cells were co-cultured with 293T cells CAR T cells and 293T cells transfected with CD123. CAR T cells lysed transfected cells but not $CD123^{neg}$ 293T **(Figure 9A).** This data suggests that chimeric CARs recognize the CD123 antigen and execute antigen specific killing.

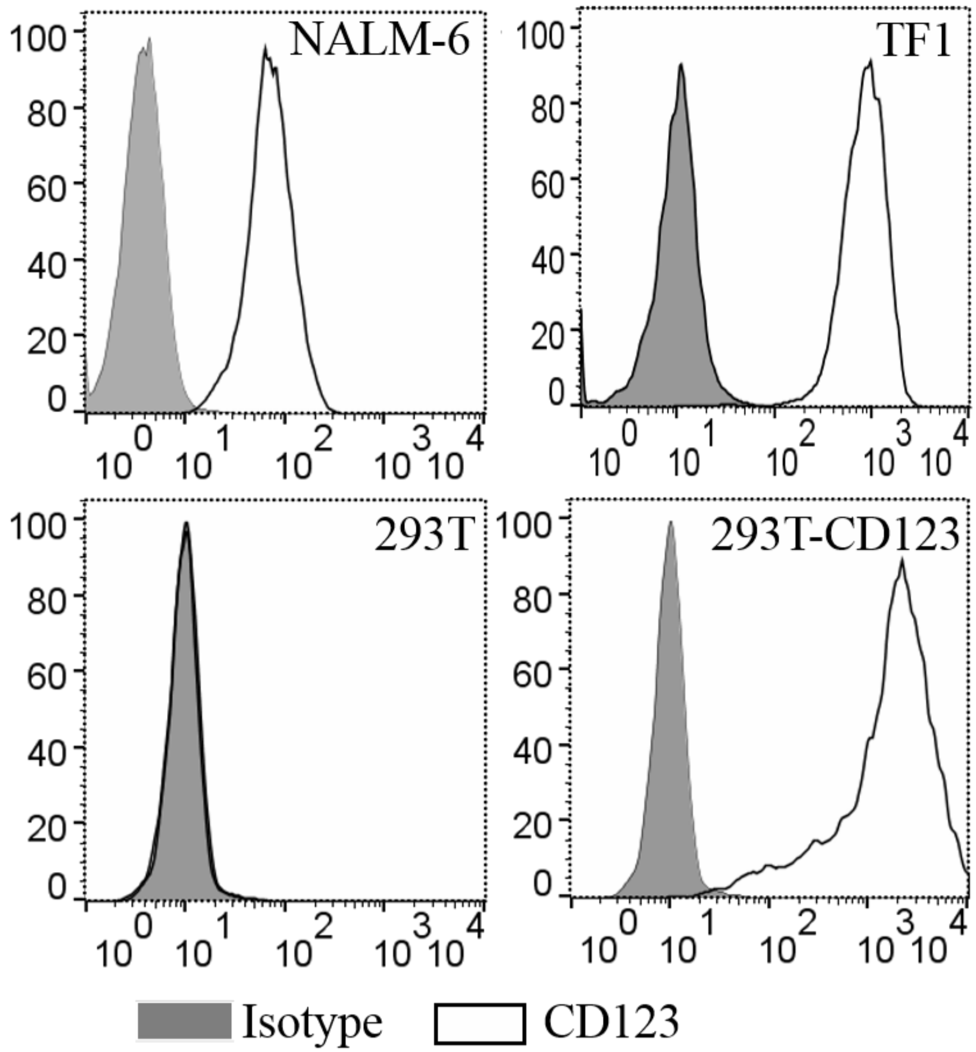

Figure 8. CD123 expression on leukemic cell lines and 293T cells CARs. CD123 expression assessed by flowcytometry in CD123^{+} Leukemic cell lines NALM6, TF1, CD123^{neg} human embryonic kidney cell line, and 293T transfected with CD123.

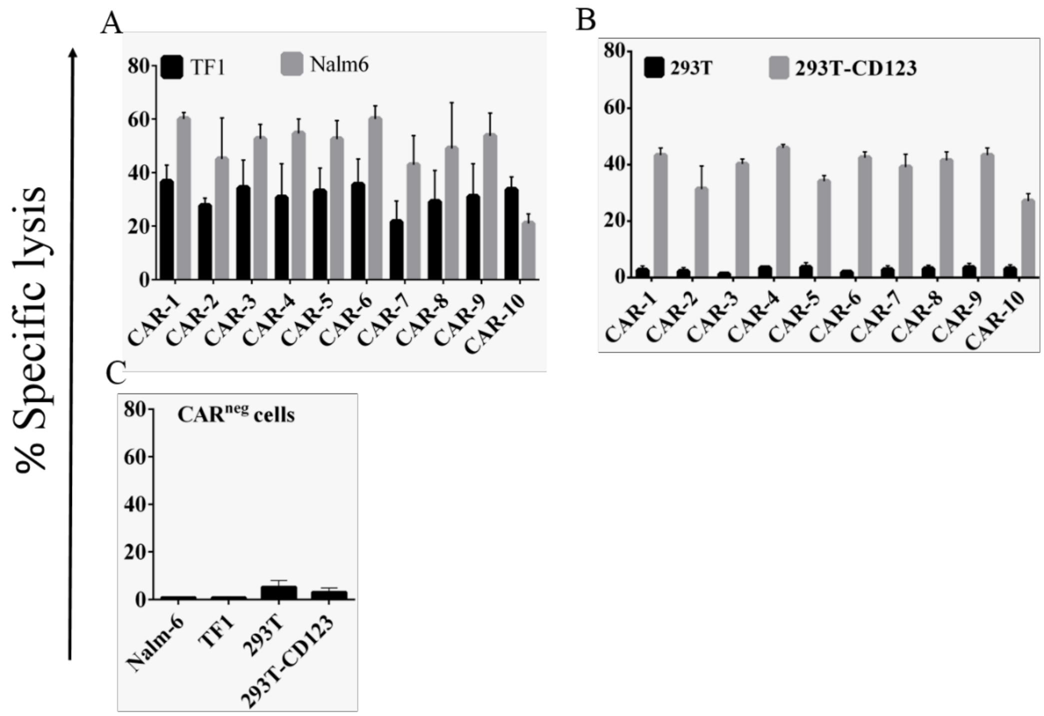

Figure 9. Specific cytolysis of chimeric CAR T cells (A) Left. *in vitro* efficacy of CAR T cells in established CD123^{+} pre B-ALL cell line Nalm6 and CD123^{+} AML cell line TF1 with E:T ratio 20:1 **(B)** Right. Antigen specific cytolysis in CD123^{neg} human embryonic kidney cell line 293T and 293T cells stably transfected with CD123 antigen E:T ratio 20:1 **(C)** Cytolysis by CAR^{neg} T cells in NALM6, TF1, 293T and 293T-CD123, E:T ratio 20:1. All data are mean ± SD of triplicate measurements in CRA.

II.2.D. IFN-γ production by chimeric CARs in response to CD123 antigen

In order to assess antigen-specific effector function of chimeric CARs IFN-γ production was assessed in $CD123^+$ Nalm6 cells. 293T cells used as negative control. T cells on Day 21 after electroporation were incubated with Nalm6 and 293T cells in E:T ratio 2:1 for 48 hours. T cells without targets used to see the difference with and without targets. Nalm6 stimulated chimeric CAR T cells produced IFN-γ in significant amounts compared to CAR T cells treated with 293T and T cells alone **(Figure 10).** These data established the effector function and functionality of chimeric CARs in response to antigen.

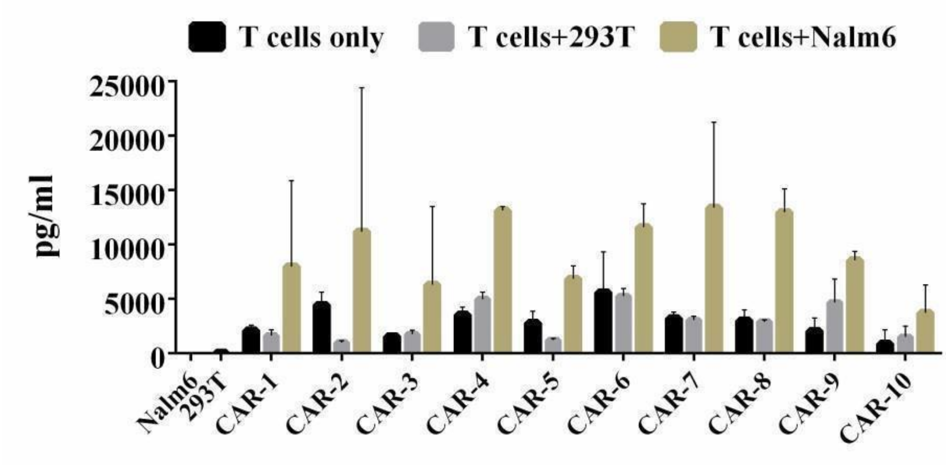

Figure 10. IFN-γ production by chimeric CARs in response to CD123 antigen. T cells on Day 21 after electroporation were incubated with Nalm-6 and 293T cells in E:T ratio 2:1 for 48 hours. IFN-γ production was assessed by cytokine capture beads by LEGEND plex ™ Human Th1 panel kit (Biolegend). Samples were run in iQue Screener Systems (intellicyt) and analyzed by LEGEND plex™ software provided with the kit.

II.2.E. *in vitro* toxicity of chimeric CAR T cells in normal hematopoietic cells

Many studies explored the expression of CD123 indicate that part of hematopoietic progenitors from human cord blood, bone marrow, peripheral blood and fetal liver express CD123 while primitive population of HSCs express at low levels or absent **(157).** Though the antibody based CD123-targeting therapies in AML reported to be well tolerated sparing normal hematopoietic cells, recent pre-clinical studies employing CD123-specific CAR T cells resulted in eradication of normal human myelopoiesis **(161).**

To test the *in vitro* toxicity of chimeric CARs for normal hematopoietic cells, we isolated lineage^{+} and HSCs enriched lineage^{neg} fractions from normal BM cells and labeled with PKH-26. CAR T cells co-cultured with PKH-26 labeled cells for 48 hours with E:T ratio 2:1.CD19 CAR T cells used as control. Cells were stained with 7AAD and live/dead cells were enumerated by 7AAD exclusion. CAR T cells are apparently lysed both lineage^{+} and lineage^{neg} hematopoietic cells (**Figure 11A**). CD19 is expressed on differentiated cells but not expressed on HSCs. This is apparent by minimal lysis in lineage^{neg} population than lineage^{+} population. These data raises concern that CD123- specific CAR therapy can be detrimental to normal hematopoiesis. However IgG4 hinge based CAR-10 showed less cytotoxicity to normal hematopoietic cells when compared to its counterparts with CD8α hinge (CARs 5-9) (Figure 11B).

We have chosen CAR-10 (referred as CD123- IgG4 CAR rest of the chapter) to take forward to generate preclinical data in support of clinical trials in B-ALL (Chapter-II) and AML (Chapter-III). Before moving to forward for testing the *in vivo* efficacy of CD123-IgG4 CAR T cells in NSG mice in B-ALL, we reconfirmed *in vitro* efficacy in additional cell lines. CD123 expression was assessed in $CD123^{+}$ B-ALL tumor cell lines RCH-ACV, kasumi-2 and $CD123^{neg}$ cell lines OCI-Ly19and EL4 **(Figure 11C).** CAR T cells were co-cultured with 51 chromium labeled target cells in different ratios in 4 hour chromium release assay. CAR T cells able to lyse $CD123^{+}$ B-ALL tumor cell lines, but not OCI-Ly19. Antigen specific killing was determined by using EL4 and EL4 transfected with CD123 where CAR T cells able to lyse EL4-CD123 but not EL4-parental **(Figure 11D)**.

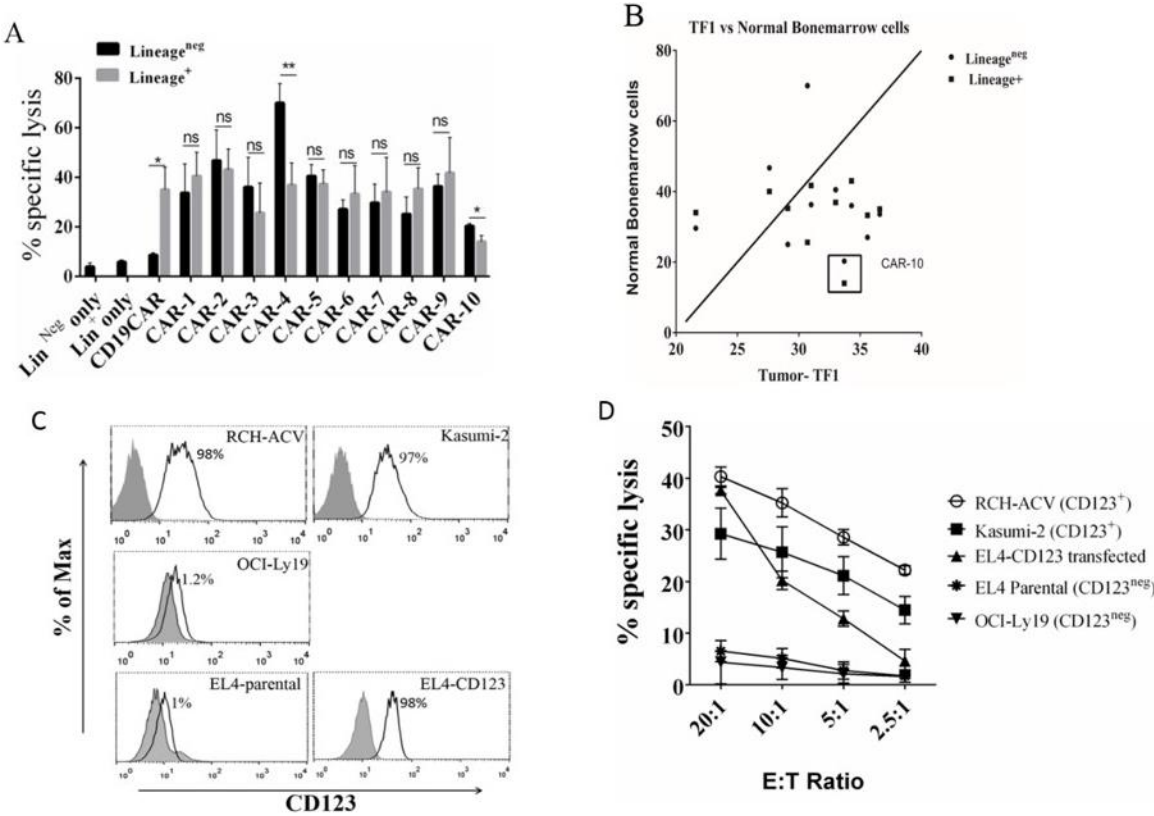

Figure 11. Anti-tumor efficacy of chimeric CARs (CAR-10)

A) *in vitro* lysis of normal hematopoietic cells by chimeric CARs. **(A)** Mononuclear cells isolated from normal BM samples and separated into lineage^{+} and lineage^{neg} cells labeled with PKH-26 and co-cultured with CAR T cells at E:T ratio 1:1 for 48 hours. 7-AAD added to distinguish live and dead cells to assess killing.

B) In vitro lysis of TF1 tumor cells vs Normal BM cells by chimeric CARs Reduced cytolytic activity of CAR-10 compared to CARs 1-9 shown inbox

C**)** Flow analysis of CD123 expression on B-ALL cell lines RCH-ACV, KASUMI-2, Nalm6 and B-cell lymphoma OCI-Ly19. D**)** *in vitro* efficacy of CD123-chimeric CAR (CAR-10) specific CAR^{+} T cells in B-ALL cell lines in a standard 4 hour chromium release assay. CD123^{neg} mouse T cell lymphoma cell line EL4 was transfected with CD123 antigen to determine antigen specific killing. Data was reported as mean ± SD

II.2.F. *in vivo* clearance of B-ALL tumors by CD123-specific T cells

In order to test *in vivo* efficacy of CAR T cells, B-ALL cell line RCH-ACV was transduced with lentiviral vector pLVU3G effluc T_2A mKateS158A **(Figure 12A)** and $mKate^+$ cells were Fluorescence-activated cell (FACS) sorted and the clones from single cells were developed for uniform mKate expression for bioluminescent imaging (BLI). RCH-ACV cells expressed luciferase confirmed by standard luciferase assay (****p<0.0001) **(Figure 12B).** On Day 0 and day1 mice were intravenously treated with tumor cells and CAR T cells respectively. 3 more infusions of CAR T cells were given on day 7, 14 and 21 followed by intraperitoneal treatment of IL2 (60000 units/mice). Untreated group did not receive CAR T cells **(Figure 13A).** CAR treated group showed reduced tumor burden quantified by BLI **(Figure 13B)** and flux activity (****p<0.0001 **(Figure 13C)** and significant improvement in survival (**p<0.01 **(Figure 13D)** compared to control mice. These data suggests that CD123 provides additional approach to treat B-ALL through chimeric antigen receptors in addition to targeting CD19.

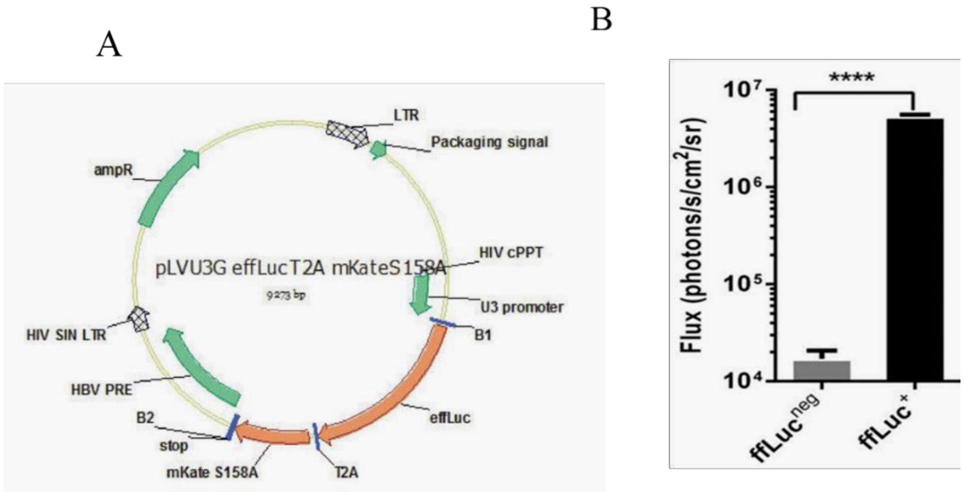

Figure 12. Expressing firefly luciferase on RCH-ACV

(A) Lentiviral vector pLVU3G effluc T2A mKateS158A transduced to genetically modify RCH-ACV to express mKate red fluorescence protein and *firefly luciferase* (*ffLuc*; bioluminescence reporter) for non-invasive bioluminescence imaging (BLI) of tumor burden *in vivo* **(B)** Flux activity in B-ALL cell line RCH-ACV transduced with lenti-viral vector expressing firefly luciferase compared to effluc^{neg} control (****p<0.0001 unpaired t-test)

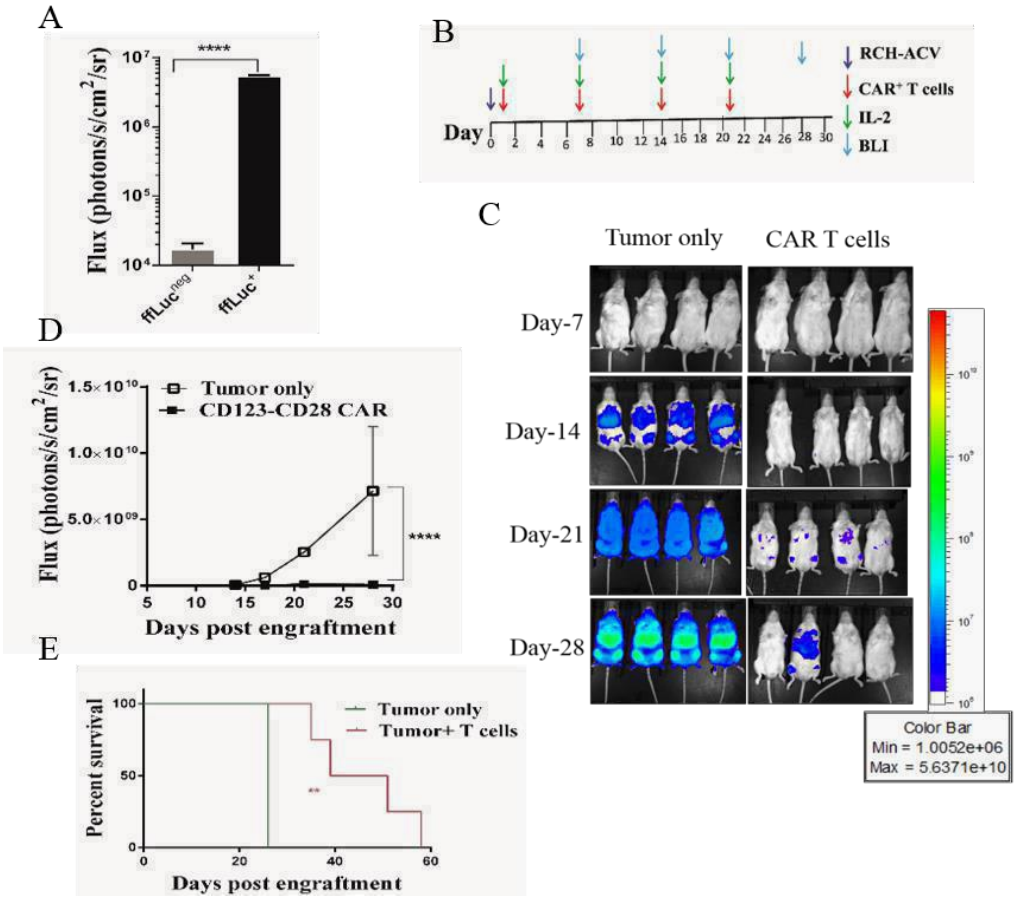

Figure 13. Anti-leukemic effects of CD123-IgG4 CAR T cells in B-ALL. **(A)** Experimental plan. RCH-ACV a pre B-ALL cells were infused intravenously on day 0 followed by 4 infusions of CD123-specific CAR T cells along with IL-2. **(B)** Graphic images of BLI of CAR treated and Control group on day 7, 14,21and 28 (C**)** Flux activity measured by BLI in CAR treated group in comparison to untreated group. Statistical analysis by two way ANOVA (****p<0.0001) (D**)** CAR treated mice (grey) showed significant survival in comparison to untreated group (black). Log-rank (Mantel-Cox) test was used for statistical analysis. **p<0.01

II.3. Discusssion

Cell-based immunotherapies have demonstrated efficacious results in cancer treatment modalities. This dissertation aimed to develop pre-clinical data to support a clinical trial of CD123-specific CAR T cell treatments for $CD123^{+}$ B-ALL and AML malignancies. We used existing platforms, S*leeping Beauty* system non-viral gene transfer and AaPC for expanding genetically modified T cells with CARs **(45-49, 55).**

Traditional CARs have been generated using single-chain variable fragments (scFv), often derived from a single mAb. Here we described a novel approach for making CARs using chimeric scFvs deriving by assembling V_H and V_L chains from two mAbs specific to CD123. Six CARs were generated by mix and matching of V_H and V_L chains mAbs 26292, 32701, 32703 and 32716 specific to CD123. The CARs with chimeric scFvs were expressed, expanded and mediated target cell lysis *in vitro* in similar fashion as CARs derived from regular scFvs of mAbs. This approach may allow us to design affinity tuned CARs with chimeric scFvs by mix and matching of V_L and V_H chains of mAbs of various affinities. Clinical outcome of CAR T cells attributes to several factors including CAR design, affinity of scFv to targeted antigen, density of targeted molecule on tumor cells age and strength of immune system of blood donor used for manufacturing T cells.

CD123 is over expressed in more than 95% of B-ALL patients while it is absent in normal early B-cell precursors and weakly expressed on intermediate and mature normal B cells. CD123 expression is correlated with hyper diploid genotype a frequent genetic abnormality in childhood ALLs. In contrast B-ALLs associated with other genetic abnormalities such as chromosomal translocations or normal karyotype do not express CD123 **(99, 100).** The overexpression of CD123 expression on B-ALL compared to normal B cells and correlated expression in hyper diploid B-ALL, provide s an opportunity to therapeutically target B-ALL through chimeric antigen receptors.

Relapse is the main reason for treatment failure in ALL patients, minimal residual disease (MRD) has significant prognostic value in pediatric and adult ALL **(101-107).** Leukemic stem cells are well documented in AML, their existence and relevance in ALL is less clear. However, several reports suggested that, a majority of leukemic populations with primitive stem-like phenotype can propagate leukemia in the appropriate experimental setting and their hierarchial organization is less strict like LSCs in AML **(158)**. As reported by several groups TEL/AML1-positive $CD34^+$ cells that carried no lineage markers specific to lymphoid differentiation (CD19 or CD10) were capable of engraftment and propagating leukemia and even engraft secondary recipients **(159-160).** These findings corroborate recent clinical findings by June et.al while targeting B-ALL by CAR T cells specific to CD19. In their studies though CD19 CAR^+ T cells have been shown to induce potent anti-tumor activity against B-ALL tumors, some of the CD19 CAR treated B-ALL relapsed patients exhibited phenotype that were negative for CD19 but expressed D123 **(108).** It appears that, employing CD123-specific CAR T cells for relapsed patients after CD19 CAR therapy feasible strategy to prevent relapse and improved survival.

One of the limiting factors in CAR T cell therapy is TAAs are not tumor "specific" but also expressed at low levels on normal cells often associated with off tumor toxicities. This is a considerable concern since CD123^{+}B-ALL antigen targeted therapies results in elimination of HSCs along with leukemic cells. Though the effect of antigen density for CAR therapy is not well defined yet it appears that CAR T cells preferably target tumors with high antigen density while the ones with lower density are resistant to therapy **(97, 98).** Recent preclinical studies with CAR T cells with lowered affinity targeting EGFR and erbB2 showed potent antitumor effect on tumors with high antigen density while sparing normal cells **(87, 88)**.

Though CARs typically are identified by their endo-domains and scFv, the other components of CARs, including the hinge/spacer region, also play a crucial role in their function and clinical efficacy. The constant region of IgG4 and CD8α re frequently used extracellular (stalk) hinge regions, though the Fc region has been reported to engage Fc receptors and activate innate immune cells (137). To avoid off target activation of CARs and unwanted immune responses we have generated a CD123 specific CAR construct by introducing L235E and N297Q mutations in the CH2 region of IgG4-Fc spacer.

We showed that CAR constructs using CD8α-derived hinge provide highly effective cytolysis in our CD123-specific constructs. Interestingly, the choice of spacer had a much greater impact on target cytolysis than expected, with a CAR utilizing a CD8-derived spacer achieving much better cytolysis than the same scFv using an IgG4-derived spacer. Importantly CAR-10 with IgG4 hinge showed minimal lysis in normal BM cells compared to CAR-6 with same scFv with CD8α hinge (Figure 6A). This observation requires further investigation in future models. By choosing different sources of V_H and V_L chains and perhaps different hinge regions, we may be able to tune the activation threshold for CAR T cells further, especially if a wider range of antibody affinities is used than was chosen for these studies. This finding may allow us to generate CARs with low affinity to selectively target high antigen density tumors while sparing normal hematopoiesis.

CHPTER-III

Comparative evaluation of co-stimulatory signals in targeting AML with CD123-specific T cells

III.1. Introduction

Acute myeloid leukemia (AML) is a clonal proliferation of malignant myeloid blast cells in the BM with impaired normal hematopoiesis. Despite many advances AML remains a lethal disease. Standard treatment regimens chemotherapy and radiation ensure long-term remission only in 30 to 50% of patients with a low survival probability resulting in resistance and relapse **(109-111).** CARs have demonstrated clinical efficacy in treating leukemia in preclinical models and are being tested in several clinical trials and emerging as powerful tools for adoptive immunotherapy **(115).** CD123, the IL-3 receptor α- Sub unit has been reported to be overexpressed on up to 95% of leukemic in AML with weak on normal HSCs and absent on cells outside hematopoietic lineage **(120-124).** Phage1 clinical trials targeting CD123 in AML using neutralizing mAbs and cytotoxic protein fused to IL-3 cytokine showed limited therapeutic efficacy pressing the need for more novel efficacious treatments **(125, 126).** Several pre-clinical and animal models have demonstrated that CAR T cells including CD28 or CD137 co-stimulatory domains as a built in source of signal 2 have improved persistence compared with those containing the CD3ζ signaling domain alone (119,196,197). However, the anti-tumor efficacy of one over the other costimulatory domain has not been investigated in depth.

An additional challenge in developing CAR T cells for immunotherapy is the management of toxicities, especially those related to excess activation of infused cells or targeting of TAA expressed on normal tissues (195). To address these questions, we engineered constructs in which the CAR10 CD123-specific second generation CAR was fused to either the CD28 (designated as CD123-CD28 CAR) or CD137 (designated as CD123- CD137 CAR) co-stimulatory domains. To reduce off-target toxicities, the utility of the inducible suicide switch iCaspase9 has been evaluated in this context. The main goal of this study is comparative functional evaluation of two CD123- specific CARs with CD28 or CD137 co-stimulatory domains. The *hypothesis* of this aim is that T cells expressing CD123-specific CARs will re-direct the specificity of T cells to target $CD123^{+}$ AML and CARs containing CD137 endo-domain will be superior to those signaling through CD28 in therapeutic efficacy. The *rationale* is i) Optimal CAR design enhances the persistence of CAR T cells ii) studies showed that CARs that incorporates CD137 has enhanced survival and anti-tumor efficacy compared to CARs with CD28 endo-domain iii) the clinical outcome of complete remission of CAR T cells correlated with long-term persistence of CAR T iv) CD1 23, the IL-3 receptor α -subunit has has reported to be overexpressed in AML **v)** evidence that complete remissions (CR) were observed in B-ALL and CLL patients treated with CD19 CAR T cells.

III.2. RESULTS

III.2.A. **Construction of CD123-specific CAR SB plasmids**

Two codon-optimized *Sleeping Beauty* transposons encoding CD123-specific second generation CARs fused to suicide gene iCaspase9 with CD28 **(Figure 14A)** or CD137 **(Figure 14B)** co-stimulatory domains were swapped into previously made iCaspase 9 co-expressing CD19 CARs in SB system. Swapping replaces CD19-specific scFv sequence with CD123-specific scFv keeping rest of the plasmid intact. The CAR plasmids were constructed in the following order: human elongation factor-α (hEF-α) promoter was used to drive expression of CARs. Following promoter, 5' to3' CAR open reading frame (ORF) consisting of signal peptide, scFv, whitlow linker, modified IgG4 hinge, CD28 transmembrane domain, CD28 or C D 137 endo-domain and CD3ζ signaling domain. The scFv is derived from V_L of mAb 26292 and V_H of mAb 32703 specific to CD123 (**Figure 6A, CAR- 10 Chapter II**). To distinguish CARs with CD28 and CD137 endo-domains by PCR in cells isolated from in vivo studies a unique oligonucleotides SIM for CD123- CD28 CAR and FRA for CD123-CD137 CAR were interspersed between stop codon of CAR and BGH polyA tail. Upon electroporation the indirect repeats (IR) of SB system flanking 5' end of hEF-α promoter and 3' end of Poly A tail is cut by SB11 transposase and integrates within the TA repeats in human T cell genome. Kanamycin resistance gene will allow to amplify the SB plasmids in large numbers in bacteria.

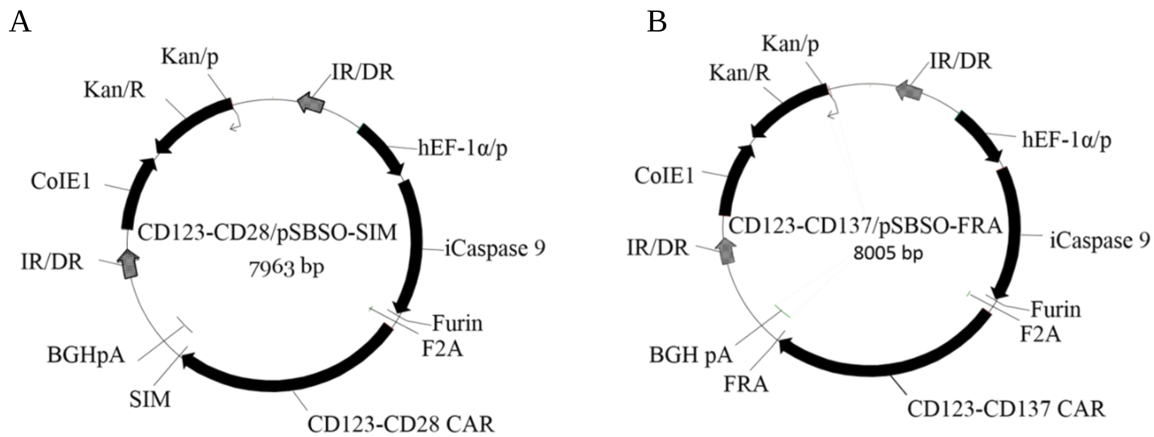

Figure 14: **CD123-specific CAR plasmids.** DNA plasmid vector maps for (**a**) CD123-CD28 CAR and (**b**) CD123-CD137 CAR. Abbreviations are as follows, IR/DR: *Sleeping Beauty* Direct repeats/Inverted Repeat, ColE1: A minimal E.coli origin of replication, Kanamycin (Kan/R): Bacterial selection gene encoding Kanamycin resistance, Kanamycin promoter (Kan/p); prokaryotic promoter. hEF-1alpha/p: human elongation factor-1 alpha region hybrid promoter iCaspase 9; induced caspase 9 suicide gene. CD123-CD28 CAR: human codon optimized CD123-specific scFv fused to Fc, CD28 endo-domain and CD3 zeta chimeric antigen receptor, CD123-CD137CAR: human codon optimized CD123-specific scFv fused to Fc, CD137 endo- domain and CD3 zeta chimeric antigen receptor SIM: “SIM” PCR tracking oligonucleotides, FRA: “FRA” PCR tracking oligonucleotides, BGH polyA; Bovine growth hormone poly adenylation sequence,

III.2.B. SB modified T cells stably co-express CD123-specific CAR and iCaspase9

PBMC from normal donors were co-electroporated with CD123-CD28 or CD123-CD137 transposon and SB11 transposase co-cultured with CD123^{+} AaPC (designated as clone1-CD123) for 4 to 5 weeks. PBMC electroporated with nucleofector solution without CAR plasmids used as negative control ("NO DNA" CAR^{neg} T cells) were expanded on OKT3 loaded Clone1- CD123. By da y 35 more than 95% of T cells expressed CAR **(Figure 15A)** and CD3 **(**Both CARs expanded at similar rates as noted by total number of cells counted at the end of culture (p=0.14) Two-way ANOVA) **(Figure 16).** Genomic DNA from Day 35 CAR T cells amplified by using primers and probes specific to IgG4-Fc and CD28 transmembrane domains showed on an average integration of 1 copy of CAR transgene per cell. Jurkat clone1 of known copy number per cell used as positive control and NO DNA cells used as negative control **(Figure 15B).** Thus SB transposition of CAR into PBMC and selective propagation on AaPC, Clone 1-CD123 resulted in generation of CAR T cells to clinically sufficient numbers with high CAR expression.

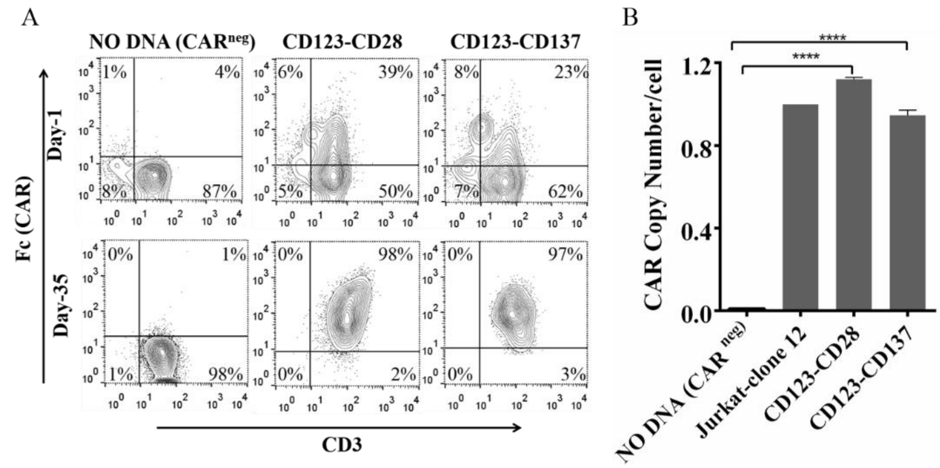

Figure 15. CAR Expression and copy number in CD123-specific CARs. (A) CAR Expression in CD123-CD28 (middle) and CD123-CD137 (right) T cells on day1and 35after electroporation and co- culture on AaPC Clone 1-CD123 where CAR^{neg} T cells (left) were used as negative controls. T cells were detected with CD3 antibody and CAR expression with Fc-specific antibody against IgG4 **(B)** CAR copy number was determined on day 28 using primers and probes specific for CD28 transmembrane and IgG4 hinge region. CAR^{neg} and Jurkat cells were used as negative and positive controls respectively.

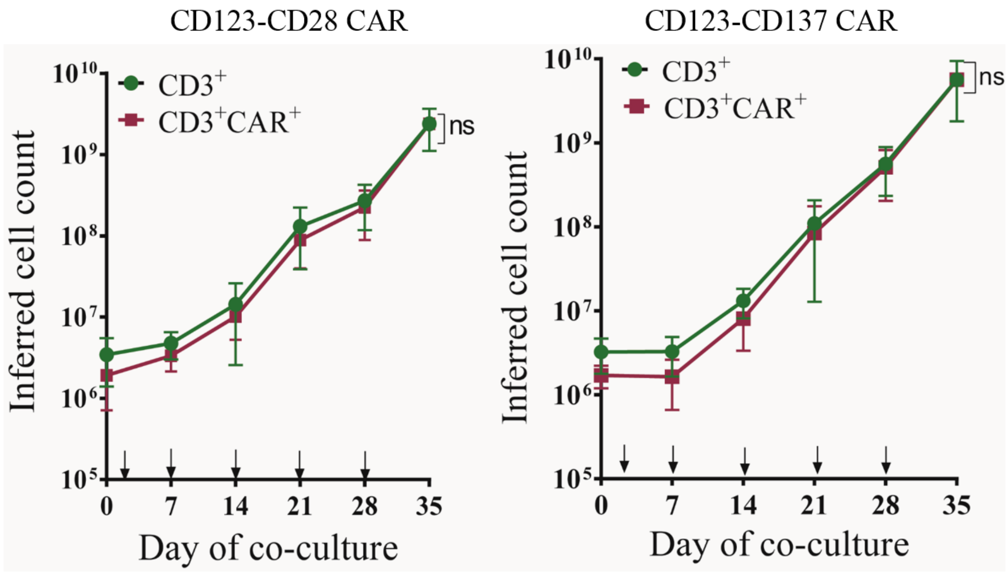

Figure 16. Expansion kinetics of CD123-specific CARs. Expansion of $CD3^+$ and $CD3^+CAR^+$ T cells over a period of 35 days after electroporation in CD123-CD28 and CD123-CD137 CAR T cells as noted by total number of cells counted at the end of culture (p=0.14) Two-way ANOVA ($CD3^+$ and $CD3^+$ CAR^+ T cells).

III.2.C. **Immuno-phenotype of CD123-specific CAR T cells**

The immunophenotypic analysis of CAR T cells by flow cytometry shows > 95% T cells co-expressing CD3 and CAR with a mixure of $CD8^+$ and $CD4^+$ T cells. **(Figure 17A right)**. Establishment of long term memory and survival is the key for improving anti-tumor efficacy of CAR T cells in clinical setting. Terminally differentiated effector memory (T_{EM}) T cells lose their capacity to expand and persist after adoptive transfer. On the contrary, less differentiated and minimally manipulated T cells with central memory phenotype (T_{CM}) can further expand, differentiate and self-renew with superior clinical response**.** To date, adoptively transferred CAR^+ T cells have demonstrated minimal *in vivo* expansion and antitumor efficacy in clinical trials **(130-132).** Though IL-2 is routinely used for T cell expansion recent reports suggests that other common gamma chain cytokines such as IL-15 and IL-21 more usefully suppress differentiation of naïve T cells into effector T cells **(133).**

SB transposition and expansion on $mIL15^+$AaPC in presence of IL-2 and IL-21 resulted in outgrowth of T cells with less differentiated phenotype and memory associated markers CD45RA, CD45RO, CD62L, CCR7, CD27, CD28, and no detectable expression of exhaustion markers CD57 and PD-1. Few cells express BM homing receptor CXCR4. **(Figure 17B).**

CAR^+ T cells belonged to less differentiated phenotype primarily composed of few naïve (T_N) defined by $CD45RA^+CD62L^+CD95^{neg}$ $CCR7^+$, T_{EMRA} ($CD45RA^+CD62L^{neg}CD95^{neg}$ $CCR7^{neg}$), T_{EM} ($CD45RA^{neg}CD62L^{neg}CD95^+$ $CCR7^{neg}$) and T_{CM} ($CD45RA^{neg}CD62L^+CD95^+$ $CCR7^+$) and co-express CD27 and CD28 to engage co-stimulatory ligands for long term survival (**Figure 17A and 17B**).

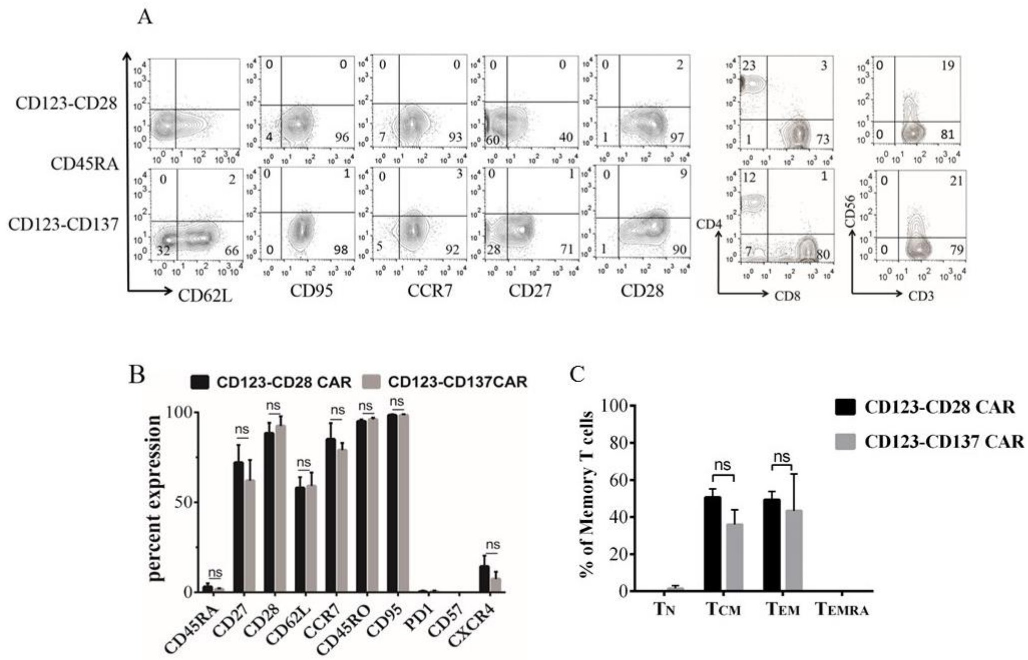

Figure 17. Immunophenotype of CD123-specific CAR T cells (A) Flow analysis of memory markers on $CD3^{+}Fc^{+}$ gated T cells. Representation of one donor of total 3 donors actually used in the experiment (left) and selective surface markers CD4, CD8, and CD56 (right) **(B)** memory and exhaustion markers CD57 and PD1 expressed (n=3) on CD123-CD28 and CD123-CD137 CAR^{+}T cells. Paired Student's two-tailed t- test was used *p<0.05 **(C)** T cell differentiation subsets gated on $CD3^{+}Fc^{+}$ population , histograms depicting cell percentage in each subset, T_{Naive} $CD45RA^{+}CD62L^{+}CD95^{neg}$ $CCR7^{+}$, T_{EMRA} ($CD45RA^{+}CD62L^{neg}CD95^{neg}CCR7^{neg}$), T_{EM} ($CD45RA^{neg}CD62L^{neg}CD95^{+}$ $CCR7^{neg}$) and T_{CM} ($CD45RA^{neg}CD62L^{+}CD95^{+}$ $CCR7^{+}$) in CD123-CD28 CAR^{+} T cells (Black bars) and CD123-CD137 CAR^{+} T cells (Grey bars) (n=3). Statistical analysis by Student's t test or nonparametric Mann–whitney Method.

III.2.D. **Transcriptional profile of CD123-specific CAR T cells**

Transcriptional profile of CAR T cells was assessed by nanostring digital multiplex array of mRNA showed expression of T cell activation markers CD69, CD44, TIM3, co-stimulatory molecules CD40L, CD27 CD28 and no expression of exhaustion and terminal differentiation markers above detectable levels B3GAT1 (Beta-1, 3-Glucuronyltransferase-1; CD57) and KLRG1 (KLRG1) by CAR T cells shows they are fully activated and has the potential for persistence after adoptive transfer **(Figure 18A).** Concurrent expression of transcription factors associated with less differentiate d phenotype i.e ID2 (Inhibitor of DNA Binding-2), KLF2 (Kruppel-like Factor-2), FOXO1 (Forkhead Box-O1), CTNNB1 (β-Catenin), BACH2 (BTB and CNC Homology-2), GFI1 (Growth Factor Independence-1), LEF1 (Lymphoid Enhancer Binding Factor-1) and later memory stages, i.e BCL6 (B-cell Lymohoma-6), PRDM1 (BLIMP-1), and TBX21 (T-bet), suggests that the expanded CAR^{+} T cells were heterogeneous in memory regulation **(Figure 18B).** Expression of cytokine receptors e.g., IL2RA (IL-2-Receptor- α; CD25), IL2RB (IL-2-Receptor-β; CD122), IL2RG (IL-2-Receptor-γ;CD132), IL7R (IL-7-Receptor-α; CD127), and IL15RA (IL-15-Receptor-α), suggests that CAR T cells has potential for continuous survival and persistence after adoptive transfer. CAR T cells express molecules associated with T cell effector (Granzyme A, Granzyme B, Perforin 1, Granulysin, IFN- γ and TNF) memory and trafficking (SELL (L-Selectin; CD62L), CD95, CCR7) predicts homing, persistence and therapeutic efficiency of CAR T cells **(Figure 18C).** In summary APC expanded, IL2/IL21 supplemented CAR T cells contain sub-populations with desirable phenotype and gene expression patterns predictive of therapeutic efficacy after adoptive transfer.

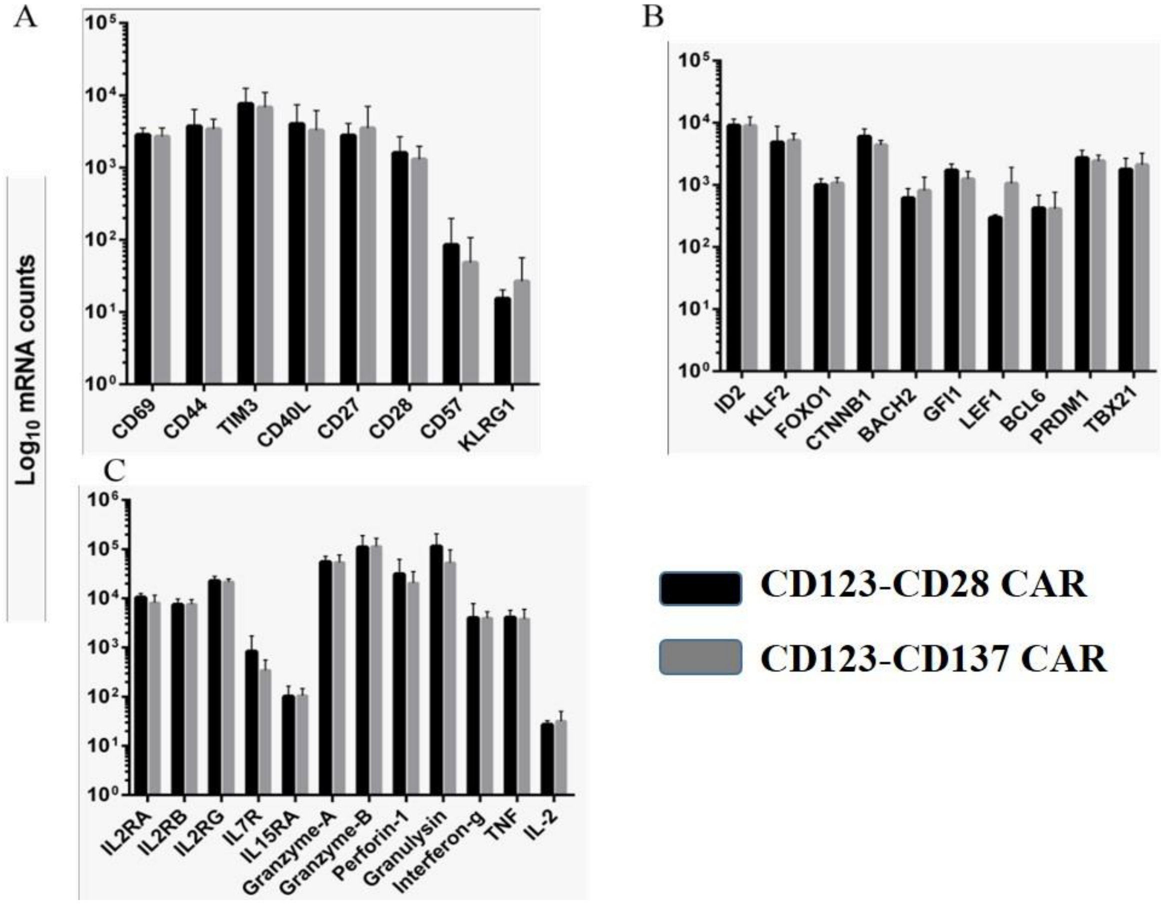

Figure 18. Transcriptional profile of CD123-specific CARs

mRNA transcripts of lymphocyte genes expressed on CAR T cells analyzed by non-enzymatic digital multiplex array of **(A)** Transcriptional profile of activation, co-stimulation and exhaustion **(B)** Transcription factors associated with less differentiated phenotype and late memory stages **(C)** Cytokine receptors for survival and markers associated with effector function

III.2.E. ***in vitro* functionality of CD123-specific CAR T cells**

Before testing functionality of CAR T cells, CD123 expression was evaluated on AML cell lines MV4-11, TF1, Molm-13, OCI-AML3 and mouse T cell lymphoma cell line CD123^{neg} EL4-parental (EL4-P) and EL4-P transfected with CD123 antigen. All the cell lines tested were positive for CD123 except EL4-P cells and OCI-Ly19 **(Figure 19A).** To evaluate functionality of CD123-specific CAR^{+} T cells *in vitro*, we used 4 hour chromium release assay for AML cell lines and flow-cytometry based killing assay for AML primary cells. CD123- specific T cells were able to lyse CD123^{+} AML cell lines but did not kill CD123^{neg} B-cell lymphoma cell line OCI-Ly19. To provide further evidence that CD123-specific CAR T cells specifically target CD123^{+} tumors we genetically modified EL4 parental cell line to enforce CD123 expression. CD123-specific T cells efficiently killed EL4-CD123 but not EL4 parental cells **(Figure 19B).**

In order to assess killing efficacy in primary patient samples, CD123 expression was analyzed on primary samples by flow cytometry **(Figure 20A).** All 4 primary samples do not express CD19 (data not shown). CAR T cells were co-cultured with PKH-26 labeled CD123^{+} primary AML cells in E:T ratio 2:1 for 72 hours and CD19 CAR T cells used as negative control. CD123-specific T cells recognized and killed CD123^{+} AML primary cells but not in CD19^{neg} AML primary cells co-cultured with CD19 CAR T cells

(Figure 20B). iCaspase 9 expression on CAR T cells was assessed by flow cytometry **(Figure 21A)** and *in vitro* functionality of iCasp9 was assessed by treating CAR T cells with 1 µM chemical inducer of dimerization (CID) a synthetic homo-dimerizer AP20187 for 24 hours. Untreated CAR T cells used as negative control. Within 24 hours the dimerizer drug rapidly eliminated CAR T cells in CID treated group **(Figure 21B)** compared to untreated control**.** In summary CD123-specific CAR T cells demonstrated anti-tumor efficacy in $CD123^{+}$ cell lines and primary tumors, and conditionally ablated CAR T cells.

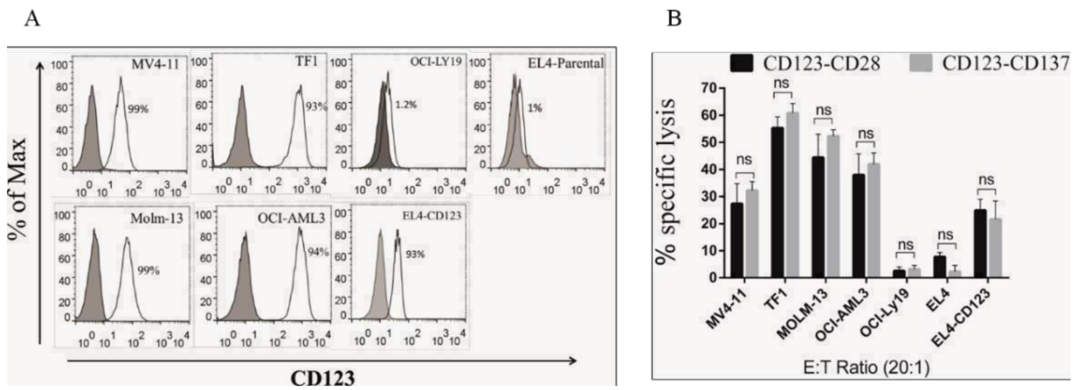

Figure 19. ***in vitro*** **lysis of CD123-specific CARs in AML**

A) Flow cytometric analysis of CD123 expression on AML cell lines MV4-11, Molm-13, TF1, OCI-AML3, EL4-Parental and EL4-Parental cells transfected with CD123. Percentage of CD123 positive cells (grey filled) over isotype controls (not filled) are indicated in each histogram **(B)** Specific lysis of CD123- CD28 and CD123-CD137 CAR^+ T cells against AML cell lines MV4-11, Molm- 13, TF1,OCI-AML3, $CD123^{neg}$ OCI-Ly19, EL4 and EL4 transfected with CD123 in a 4 hour chromium release assay, Data are mean ± SD n=3

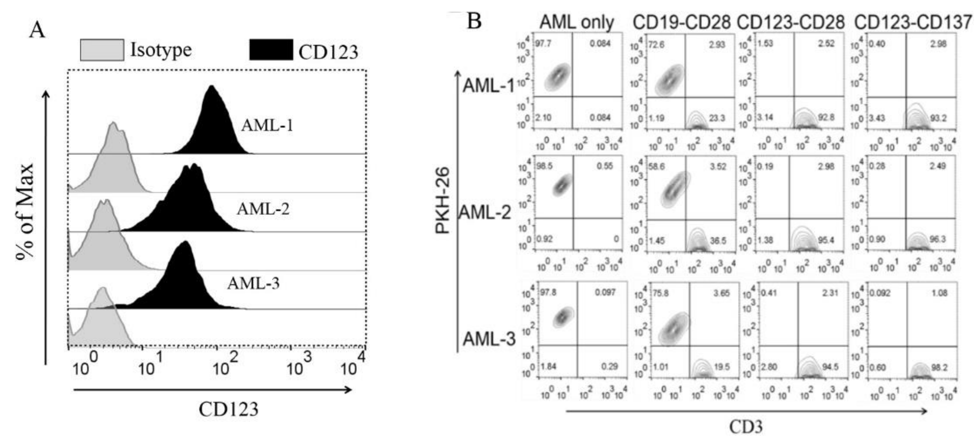

Figure 20. *in vitro* lysis of CD123-specific CAR T cells in AML primary samples (A) Flow cytometric analysis of CD123 expression on primary AML samples used in co-culture assay **(B)** PKH-26 labeled Primary AML primary cells were co-cultured with CD123-CD28 and CD123-CD137 CAR T cells at 1:1 ratio for 72 hours.CD19-CD28 was used as negative control. At the end of the culture, cells were stained using anti- CD3 to distinguish between T cells and PKH-26 labeled tumor cells.

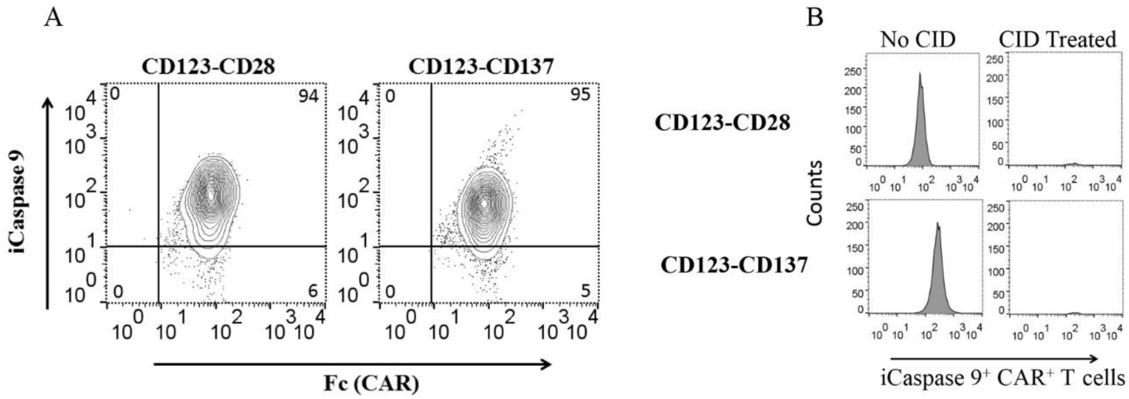

Figure 21. ***in vitro*** **functionality of iCaspase 9 in CD123-specific CARs**

(A) ICaspase 9 expression on CAR T cells, assessed by flow cytometry **(Figure 21A)** and *in vitro* functionality of iCasp9 was assessed by treating CAR T cells with 1µM chemical inducer of dimerization (CID) a synthetic homodimerizer AP20187 for 24 hours which rapidly eliminated T cells in CID treated group.

III.2.F. ***in vivo* efficacy of CD123-specific CAR T cells**

To evaluate antitumor activity of CAR T cells *in vivo* a xenograft model of AML was established in NSG mice transgenic for human interleukin-3 (IL-3), stem cell factor, and granulocyte macrophage colony-stimulating factor (NSGS). GMCSF dependent erythrocytic leukemia cell line TF1 was genetically modified with lentiviral particles to express mKate red fluorescent protein (RFP) and enhanced firefly luciferase (ffLuc) **(Figure 22A)** for allowing to track tumor burden by serial non-invasive bioluminescence imaging (BLI). On day 0 mice were injected with $2.5x10^6$ TF1-mKate-ffluc cells allowed to engraft for 5 days. On day 5 tumor engraftment confirmed by BLI and 10^7 CD123-CD28 or CD123-CD137 CAR^+ T cells/mice were infused along with intraperitoneal injection of IL-2 (60,000 units/mice). Untreated mice served as control. 2 more infusion of T cells were given on day 11 and 20 and mice were imaged for tumor burden on day 20 and 28 **(Figure 22B).** Untreated mice showed continuous tumor growth evidence d by increase in bioluminescence flux in comparison to CAR T cells treated group **(Figure 22C)**. Both CD123-CD28 and CD123-CD137 CAR T cells treated groups were able to reduce tumor burden compared to untreated group as measured by tumor BLI flux $p<0.01$ **(Figure 22D)**. Treatments with CD123-specific CAR T cells significantly prolonged survival of mice in both treated groups compared to control group **(Figure 22E)**. However we did not observe any statistically significant difference in survival between mice treated with CD123- CD28 and CD123-CD137 CAR T cells **(p value > 0.05).**

In summary, preclinical data thus generated so far will allow us to test CD123-specific T cells in clinical setting to treat $CD123^{+}$ malignancies in patients.

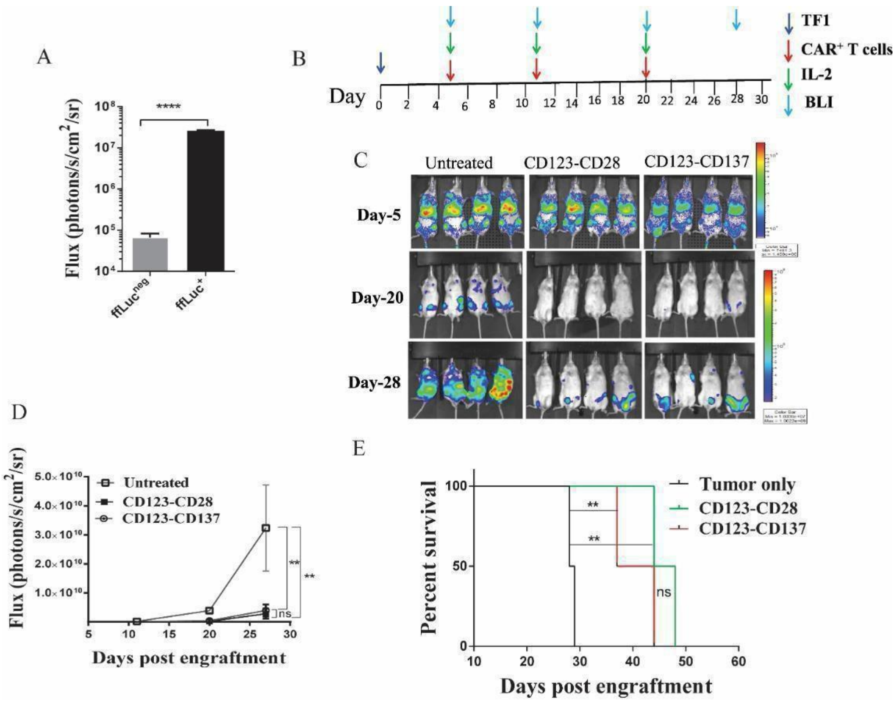

Figure 22. *in vivo* efficacy of CD123-specific CAR T cells in NSGS mice **(A)** AML cell line TF1 was genetically modified with lentivirus particles to express mKate red flourescent protein and enhanced firefly luciferase (effluc). Flux intensity in TF1 cell line compared to non-transduced TF1 cells, measured by firefly luciferase assay (****p<0.0001) **(B)** Schematic of TF1 xenograft model. 2.5x10e6 TF1-*effLuc*-mKate cells were intravenously injected into NSGS mice on day 0. On Day 5 tumor engraftment was quantified using Non- invasive bioluminescence imaging (BLI) and mice randomly divided into 3 groups and treated with 3 infusions of CD123-28 or CD123-CD137 CAR T cells and untreated group received no T cells followed by IL-2 treatment and BLI on day 5,11 and 20 **(C)** BLI images of mice showing tumor reduction in CD123-CD28 and CD123-CD137 CAR treated group compared to untreated group (**p<0.01 **(D)** Flux activity measured by BLI in CD123-CD28 or CD123- CD137 treated group in comparison to untreated group. Statistical analysis by two way ANOVA (**p<0.01)

(E) Survival of mice treated with CD123-CD28 CAR T cells compared to mice treated with CD123-CD137 CAR T cells. Log-rank (Mantel-Cox) test was used for statistical analysis. p>0.05 ns (not significant)

III.3. Discussion

CARs that activate through chimeric CD28 or CD137 endo-domains have anti-tumor activity and durable remissions in clinical trials with pros and cons for each design. However the improved efficacy over the other is unknown at present. Preclinical data that supports targeting CD123 on AML using CARs with CD28 and CD137 co-stimulatory domains have been reported by two groups respectively **(134, 135).** In this study we described the head to head comparison of CD123-specific CARs with co-stimulator y domains CD28 or CD137 and have been evaluated in the lines of CD19- specific CAR T cells currently in clinical trials (NCT00968760).

We have redirected the specificity of T cells using *Sleeping Beauty* system to stably express CARs and selectively propagated on Clone 1-CD123 AaPC, co-expressing CD123, co-stimulatory molecules CD86 and CD137L and a membrane bound IL-15. Trans-presentation of mIL-15/IL-15Rα fusion protein expressed on Clone 1-CD123 support enhanced proliferation and survival of CAR T cells without altering T-cell activation patterns and global T-cell receptor (TCR) repertoire **(136, 137).** Unlike lentiviral and retroviral vectors SB transposition is cost effective gene transfer system requires less production cost for manufacturing clinical grade T cells. SB system doesn't integrate at sites of active transcription, has been shown not to activate oncogenes **(127-129).** Establishment of long term memory and survival is the key for improving anti-tumor efficacy of CAR T cells in clinical setting.

Terminally differentiated effector memory (T_{EM}) T cells lose their capacity to expand and persist after adoptive transfer. On the contrary, less differentiated and minimally manipulated T cells with central memory phenotype (T_{CM}) can further expand differentiate and self-renew with superior clinical response**.** To date, adoptively transferred CAR T cells have demonstrated minimal *in vivo* expansion and antitumor efficacy in clinical trials **(130-132).** Though IL-2 is routinely used for T cell expansion recent reports suggests that other common gamma chain cytokines such as IL-15 and IL-21 suppress differentiation of naïve T cells into effector T cells **(133).**

SB transposition and expansion on $mIL15^{+}AaPC$ in presence of IL-2 and IL-21 resulted in outgrowth of T cells with less differentiated phenotype and memory associated markers CD45RA, CD45RO, CD62L, CCR7, CD27, CD28, with no detectable expression of exhaustion markers CD57 and PD1. Few cells express BM homing receptor CXCR4 **(Figure 17B)** which helps T cells to migrate to BM regions and clear leukemic cells. More than 95% of T cells express CAR and expanded to clinically relevant numbers with heterogeneous phenotype consistent with central memory T cells (T_{CM}) and effector memory (T_{EM}). Redirected specificity was established based on CAR-dependent T-cell effector function such as specific lysis of $CD123^{+}$ leukemic cell lines and primary AML samples, but not $CD123^{neg}$ targets. Most of the tumor associated myeloid antigens are also expressed on normal hematopoietic cells. Recent studies by Gill et al reported that CD123-specific CAR T cells treated mice showed marked reduction in myelo-ablation in a preclinical xenograft model suggests that new approaches needed to mitigate off-target toxicities **(134).** This raises concern for targeting CD123 in AML as it is expressed on normal hematopoietic cells. Our approach of using chimeric CARs with the combination of changing CAR hinge to IgG4 may enable us to generate low affinity CARs by minimal lysis of normal hematopoietic cells by CAR 10 **(Figure 11A Chapter II)**. Recent data have shown that mRNA modified T cells with transient CAR expression specific to CD19 and CD33 resulted in target specific killing **(139).** Identification of unique molecular abnormalities helps to develop targeted and personalized treatment options for AML patients. Flow cytometry and immuno-histochemical studies showed CD123-positive AML is most frequently encountered within the intermediate cytogenetic risk group and is associated with FLT3-ITD and NPM1 Mutations **(140).** These patients with FLT3-ITD and NPM1 Mutations can be benefited by CAR based therapy targeting CD123. Though CARs generated by viral vectors exhibit significant anti- tumor efficacy and *in vivo* persistence sometimes resulted in on-target and off-target cytotoxicities. Introduction of suicide genes such as iCapase 9 may mitigate the risks by conditional ablation of T cells off target toxicities evidenced by our *in vitro* data that addition of CID rapidly eliminated T cells in 24 hours at 1µM concentration. In summary, our data also shows that CARs activated through CD28 or CD137 showed similar efficacy *in vitro* and *in vivo*, and that inclusion of an iCasp9 domain in frame with a Furin/F2A domain does not impair CAR function and generates an effective suicide switch in CAR^+ T cells.

CHAPTER IV

Targeting leukemic stem cells by CD123-specific CAR T cells while sparing normal hematopoiesis

IV.1. Introduction

Leukemic stem cells (LSCs) are a rare population of cells resistant to conventional treatments and responsible for relapse and therapy failure. LSCs are pre-leukemic clonal population of HSCs arise by genetic and molecular alterations. This is evidenced by common features that LSCs share with HSCs including self- renewal, engraftment potential and are enriched in Lineage^{neg} (Lin^{neg}) fraction of blood cells with surface phenotype of CD34^{+}CD38^{neg}. LSCs are capable of self- renewal and able to initiate leukemia when transplanted in SCID mice. CD123 is overexpressed on AML blasts, hematopoietic progenitors and LSCs compared to normal hematopoietic stem cells and confers growth advantage in AML **(147-150).** Overexpression of CD123 is associated with higher blast counts, poor prognosis and reduced survival in AML patients **(147-150).** Phase-I clinical trials targeting CD123 by monoclonal antibodies had limited efficacy pressing the need for alternative and more potent treatments. **(151, 152).** However most of the tumor associated myeloid antigens that are expressed on LSCs are also expressed on normal hematopoietic stem cells and its progenitors. Tumor targeted immune therapies that damage normal hematopoietic stem cells are often associated with irreversible and reversible side effects. Clinical trials targeting CD33 antigen in AML using gemtuzumab ozogamicin (GO) antibody conjugated to a cytotoxic agent have been shown to have proplonged cytopenia though exhibited potent anti-tumor effect (139). Recent report by *Casucci et al* suggests though CARs targeting CD44v6^{+} AML effective in eliminating AML, but associated with reversible monocytopenia upon contraction of

T cells *in vivo* **(145).** Gill et.al reported off-target cytotoxicity to myeloid progenitors in CAR directed immunotherapy for $CD123^{+}$ AML **(146).** Hence targeting AML can be myelo-toxic, careful study of possible off-target cytotoxicity is an important concern while designing CAR therapies. In the present chapter we evaluated *in vitro* killing efficacy of CD123-specific chimeric CAR T cells targeting leukemic stem cells in AML and normal hematopoietic cells.

IV.2. Results

IV.2.A. CD123 is frequently expressed in AML and Leukemic stem cells

In order to decide whether CD123 is a suitable target for CAR therapy in AML we determined CD123 expression levels in 30 random primary AML patient samples. Peripheral blood samples of 30 patients were processed for mononuclear cells (MNCs) established protocols. Samples include treated non-treated and relapsed patients. FAB classification is not available for some of the patients. MNCs from each patient were stained with CD34, CD38 and CD123 antibodies. CD123 expression levels were assessed on LSC enriched fraction ($CD34^+CD38^{neg}$) fraction **(Figure 23A)** and blasts ($CD38^+$) population **(Figure 23B)**. In AML patients the percentages of total $CD34^+$ cells, $CD34^+$ $CD38^+$ cells, and $CD34^+$ $CD38^{neg}$ $CD34^{neg}CD38^+$ cells within the MNC fraction was highly heterogeneous. However Consistent with previous reports CD123 is frequently expressed on more than 95% of AML samples **(Figure 24).** List of patients samples used in the study and total % of CD123 on each sample listed in **table1.**

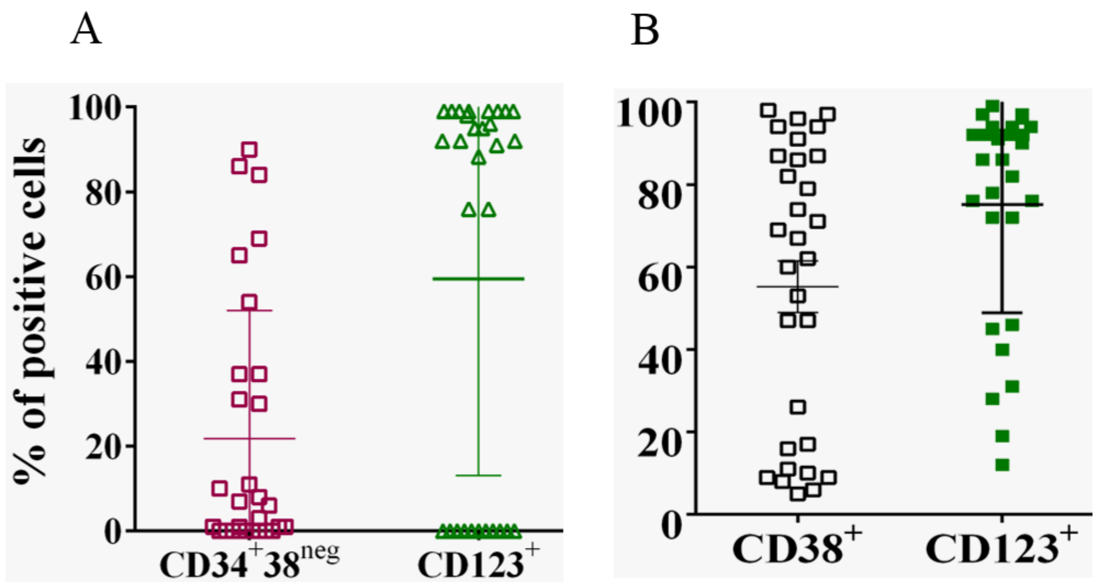

Figure 23. CD123 expression analysis on LSCs and blasts in AML

Mononuclear cells were isolated from peripheral blood from primary AML patients and stained with antibodies specific to CD123 CD34 and CD38.

(A) CD123 expression gated on LSCs (phenotypically defined $CD34^+$ and $CD38^{neg}$) and (B) on blasts (phenotypically defined $CD38^+$ fractions). Mean$\pm$SD N=30

Table.1. CD123 expression in primary AML assessed by flow cytometry

S.No	Patient	FAB	CD123⁺ (%)
1	5480	Relapsed	92
2	3469	Relapsed	72
3	2842	AML-TR	82
4	5586	AML-MRC	45
5	5812	AML-TR	97
6	6280	AML	76
7	6430	Relapsed	92
8	3162	M4	90
9	6542	AML	19
10	3206	MRC treated	99
11	3385	AML-TR	94
12	5402	AML	40
13	5595	M1	92
14	6059	AML-M5a	86
15	3515	N/A	91
16	5703	M5a	46
17	5757	AML-MRC	28
18	6037	M5a	12
19	3107	MRC	31
20	1983	M2	92
21	1929	M5	92
22	2004	M4	94
23	1592	N/A	78
24	6246	AML-treated	86
25	2842	relapsed	93
26	AML-1	N/A	94
27	AML-2	N/A	76
28	AML-3	N/A	96
29	AML-4	N/A	96
30	AML-5	N/A	93

IV.2.B. Leukemic stem cells express CD123

To determine whether CD123 is expressed on AML-LSCs we have isolated $CD34^{+}CD38^{neg}$ cells from lin^{neg} fraction of primary AML samples HTB numbers 5480, 6280, 6430,2842, 5586,5512. We have chosen 4 relapsed samples and 2 samples with high blast counts. Our analysis indicated that LSCs are enriched in relapsed patients (HTB2842, HTB5480, HTB6430 and HTB6280). Percentage of $CD34^{+}$ $CD38^{neg}$ cells are more in relapsed patients than the patients with higher blast count (HTB 5586 and HTB 5812). To isolate LSCs we isolated Lin^{neg} cells from MNCs of patient samples with CD34 diamond isolation kit (Miltenyi), next FACS sorted into $CD34^{+}CD38^{neg}$ population and stained with CD123 antibody with appropriate isotype controls. CD123 is expressed in all the samples tested **(Figure 24**). Contrary previous reports CD123 expression is no higher on phenotypically defined leukemic stem cells. These results suggests that CD123 is a therapeutic target in AML given its frequent expression on LSCs.

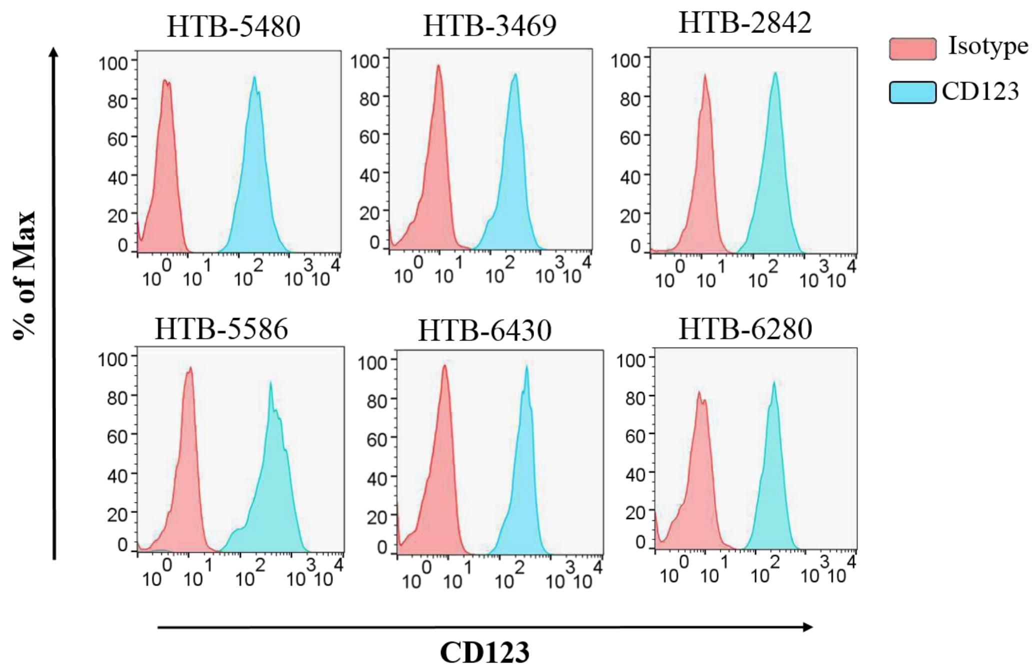

Figure 24. CD123 expression on AML isolated leukemic stem cells

lin^{neg} cells from MNCs of patient samples were isolated and FACS sorted into $CD34^{+}CD38^{neg}$ population and stained with CD123 antibody with appropriate isotype controls.

IV.2.C. *in vitro* cytotoxicity of chimeric CAR T cells against AML-LSCs and normal hematopoietic cells

Human HSCs express lineage associated genes during their differentiation into blood cells. However, HSCs are generally regarded as being devoid of lineage specific markers expressed by differentiated blood cells. Studies in mice indicate that well established myeloid lineage associated markers CD33, CD13, CD123 are expressed on long-te rm repopulating HSCs from cord blood and BM. This finding raises the concern that myeloid antigen targeted therapies has the potential of killing HSCs **(155).** To determine whether chimeric CARs target normal hematopoietic stem cells and progenitors we have isolated lineage positive and negative cells from normal BM samples, Lineage^{+} and HSCs (lin^{neg} CD34^{+}CD38^{neg}) from cord blood MNCs and co- cultured with chimeric CAR T cells in E:T ratio 1:1 for 48 hours. *in vitro* toxicity by CAR T cells was observed in lineage positive and lineage negative cells from BM **(Figure 25A**). However HSCs and lineage positive cells from cord blood showed minimal lysis by CAR T cells. **(Figure 25B).** Next we determined anti-tumor efficacy in freshly isolate d phenotypically defined lin^{neg} CD34+CD38neg AML-LSCs with similar co- culture conditions used for hematopoietic cells.

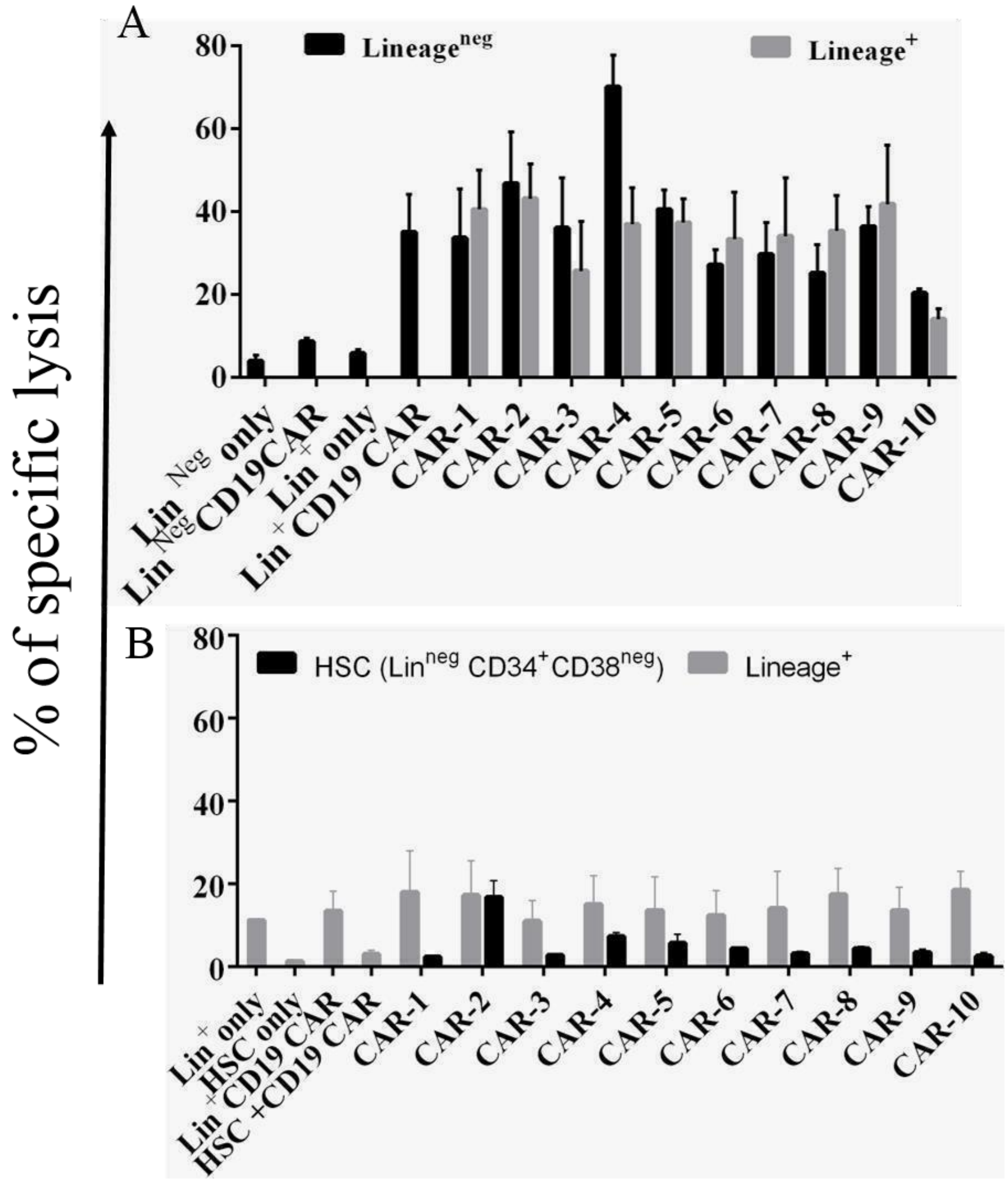

Figure 25. *in vitro* lysis of normal hematopoietic cells by chimeric CARs. Isolated lineage positive and negative cells from normal BM samples, Lineage+ and HSCs (lin^{neg} CD34^{+}CD38^{neg}) from cord blood MNCs, labeled with PKH-26 and co-cultured with chimeric CAR T cells in E:T ratio 1:1 for 48 hours.

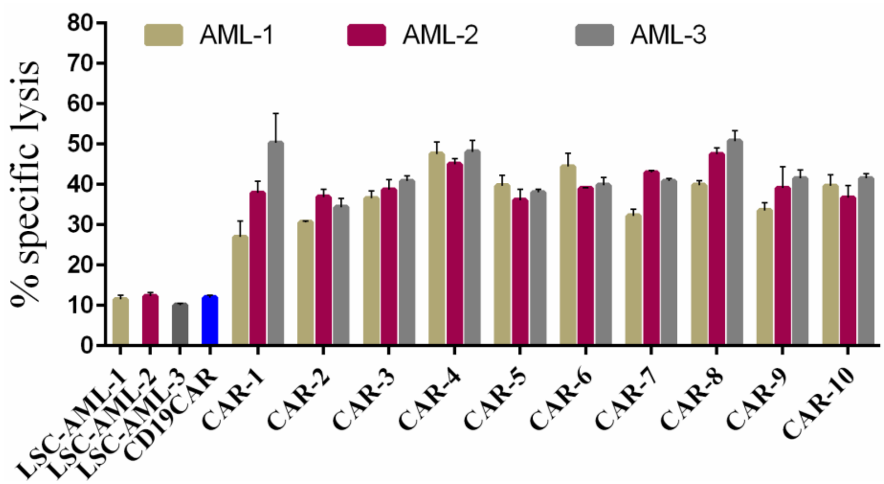

Figure 26. lin^{neg} CD34^{+}CD38^{neg}) from three AML patient samples labeled with PKH-26 and co-cultured with chimeric CAR T cells in E:T ratio 1:1 for 48 hours. CD19 CAR T cells used as negative control. Cells were stained with 7-AAD to distinguish dead and live cells to assess killing

IV.2.D. Expansion of LSC under hypoxia

Reports suggests that LSCs reside in hypoxic regions of bonemarrow micro environment in quiescent stage and resistant to conventional treatments. It has been demonstrated that intravenously injected AML-LSCs home to BM engraft and subsequently reside in endosteal regions. Therefore novel approached are needed to target LSCs in hypoxic regions of BM niche thereby preventing relapse and therapy failure **(167**). LSCs are rare and few in number in AML which limits the feasibility of cell-based assays. Current culture conditions do not prevent LSCs and HSCs from differentiation. It has been shown that Aryl hydrocarbon receptor (AHR) pathway is inactive *in vivo* and rapidly activated *in vitro* in HSCs and LSCs. Stem regenin1 (SR1) is an antagonist of the aryl hydrocarbon receptor that promotes the self- renewal of human HSCs and LSCs in culture supplemented by cytokines and prevents their differentiation **(168,169).**

To expand LSCs under hypoxic conditions we isolated lin^{neg} CD34^{+}CD38^{neg} fraction from relapsed AML patients cultured at 1% oxygen and 5% CO_2. Cells were cultured in serum free stemspan II media (stem cell technologies) in presence SR1 1µM/ml supplemented by cytokines stem cell factor (SCF), human FLT3 ligand, interleukin-3 for 7 days. SR1 non-treated cells used as control. All AML-LSCs treated with SR1 showed higher percentages of CD34^{+}CD38^{neg} fraction with relative CD123 expression after a 7-day culture period compared to SR1 non treated controls **(Figure 27).** On day 7, LSCs were labeled with PKH26 and co-cultured with CD123-specific chimeric CAR T cells in 1:1 ratio for 48 hours under hypoxic conditions. CD19 CAR T cells used as negative control. CD123-specific CAR T cells lysed LSCs expanded under hypoxia compared to CD19 CAR which exhibited minimal lysis **(Figure 28).**

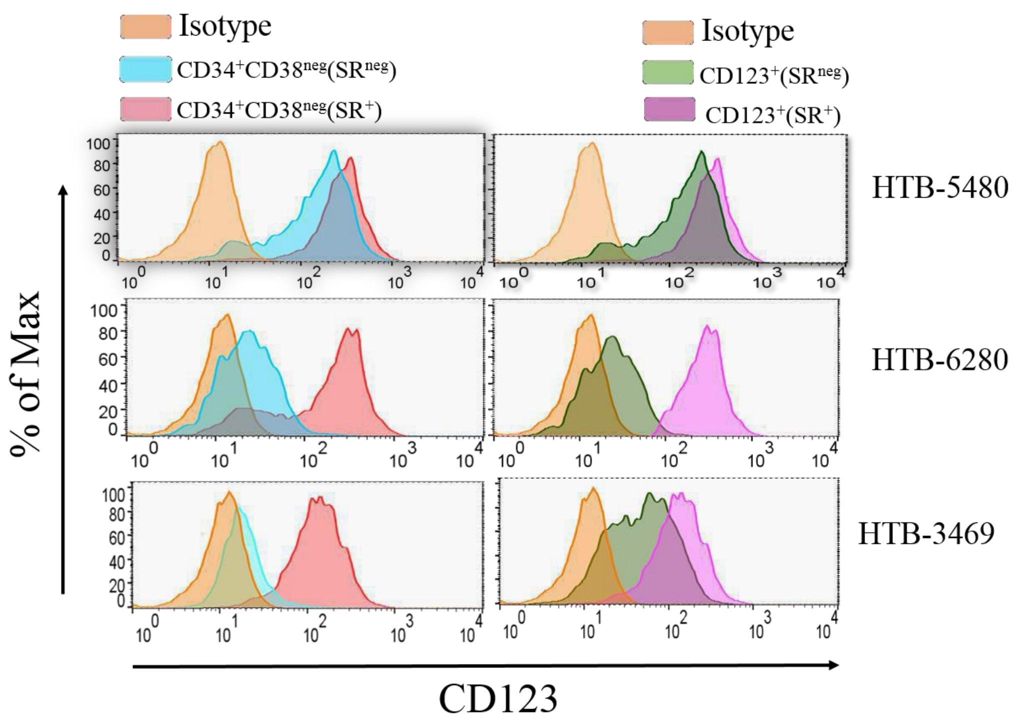

Figure 27. Expansion of AML-LSCs under hypoxic conditions. lin^{neg} CD34$^+$CD38^{neg} fraction was isolated from relapsed AML patients HTB- 5480, HTB-3469, HTB-6280 and cultured at 1% oxygen and 5% CO_2. Cells were cultured in serum free stemspan II media (stem cell technologies) in presence SR1 1µM/ml supplemented by cytokines stem cell factor (SCF), human FLT3 ligand, interleukin-3 for 7 days. SR1 non treated cells used as control.

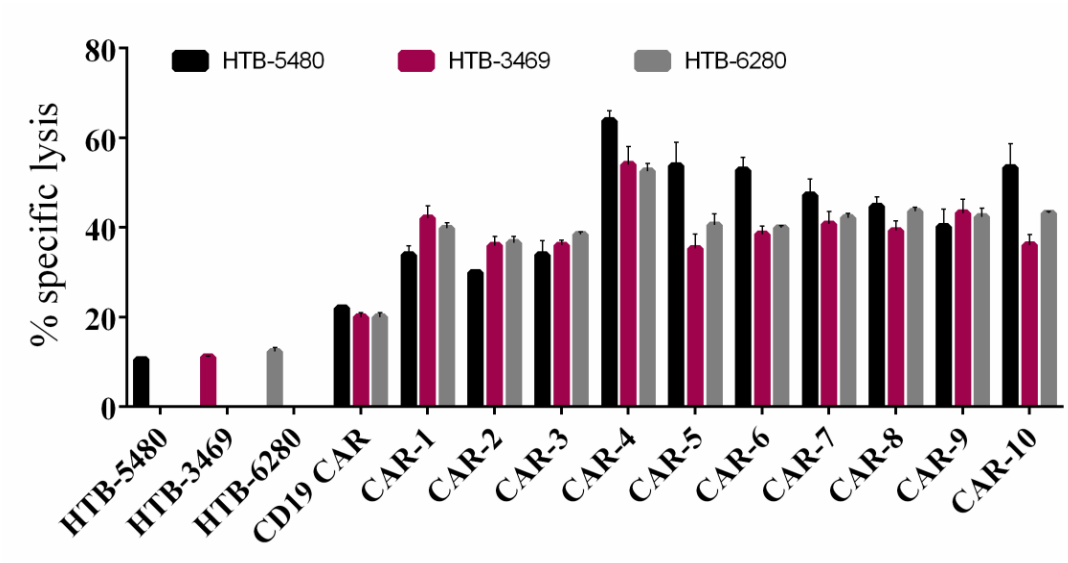

Figure 28. ***in vitro* lysis of LSCs by chimeric CAR T cells under hypoxia.** lin^{neg} $CD34^{+}CD38^{neg}$ fraction was isolated from relapsed AML patients cultured at 1% oxygen and 5% CO_2 in presence SR1 supplemented by cytokines stem cell factor (SCF), human FLT3 ligand, interleukin-3 for 7 days. SR1 non treated cells used as control. On day 7, LSCs were labeled with PKH26 and co-cultured with CD123-specific chimeric CAR T cells in 1:1 ratio for 48 hours under hypoxic conditions. CD19 CAR T cells used as negative control.

IV.3. Discussion

LSCs has many features in common with HSCs including self- renewal and engraftment potential and are enriched in lin^{neg} fraction of blood cells. Initial studies suggests that LSC activity was restricted to $Lin^{neg}CD34^{+}CD38^{neg}$ fraction in NOD/SCID mice models **(171).** Later studies using refined mice models have demonstrated LSC activity is confined to more than one compartment and even more mature $lin^{neg}CD34^{+}CD38^{+}$ progenitor population of some of AML patients able to initiate leukemia **(172).** LSCs were well documented in AML though they are reported in other leuekemic groups. Studies in ALL suggests that, greater degree of plasticity is observed in LSC compartment with phenotype $CD34^{neg}CD19^{+}$ and able to regenerate $CD34^{+}$ progeny within transplanted leukemia. However the frequency of LSC is more in lin^{neg} $CD34^{+}CD38^{neg}$ fraction than lin^{neg} $CD34^{+}CD38^{+}$ progenitors **(173).** More recent data indicate that LSCs has phenotypically distinct subpopulations and may vary from patient to patient **(190, 191).**

Ideally, therapeutic targets that specifically expressed on LSC but not on HSC need to be identified to spare HSCs from being targeted and to protect normal hematopoiesis in the patient. Therefore functionally defining LSCs and HSCs is crucial in identifying therapeutic targets that are specific to LSC **(177-180).** A detailed LSC phenotype have been described as $Lin^{neg}CD34^{+}CD38^{+}CD90^{neg}CD96^{+}CD45RA^{neg}$ which distinguish them from normal myeloid stem cells and progenitors **(174, 175).** LSC have been shown to overexpress other myeloid markers such as CD13, CD33 and CD123 which are weakly expressed on HSCs

(181). Recently several other markers have been shown to be expressed exclusively on LSC than normal HSC which include CLL1, CD32, CD45RA, TIM3, CD47 and CD25 **(182-186).** HSCs are generally regarded as being devoid of expressing markers specific to blood cell lineages. However recent studies suggests that HSCs promiscuously express lineage-specific genes prior to commitment including myeloid markers **(171-175).** This aspect has significant clinical importance since number of therapies targeting myeloid markers aim to selectively kill leukemic blasts while sparing normal HSCs. HSCs will be targeted along with leukemic cells if they also express myeloid markers along with leukemic blasts **(177-181)**. For example, clinical trials of AML therapy targeting CD33 by gemtuzumab ozogamicin (GO) antibody conjugated to a cytotoxic agent have been shown to have prolonged cytopenia despite successful tumor clearance **(177,182,183).** This may be due to targeted killing of HSCs by GO and many patients have relapses since LSCs are resistant to the toxins **(184).** CD123 expression was assessed on HSCs and progenitors in 3 normal bone marrow samples as described **(185, 186).**

Our *in vitro* data indicate that CAR T cells showed toxicity to lineage positive and negative cells from however the HSCs from cord blood are minimally lysed. CAR T cells able to lyse LSCs which are phenotypically similar to HSCs shows the potential CAR therapy to eradicate leukemic stem cells which are responsible for relapse and therapy failure. Previous trials targeting CD123 with mAbs cytokine modalities did not report BM suppression. However pre-clinical studies targeting CD123 by CAR T cells showed myeloablation in mice. This may be the result of ability of CAR T cells to eliminate $CD123_{dim}$ population compared to antibody therapies. These observations prompt us to develop careful risk free therapy for AML and using low affinity CARs may rescue hematopoiesis while successfully eliminating AML tumors. These *in vitro* data suggests that CAR therapy can be detrimental to normal hematopoiesis and CD123-specific CAR T cells need to be employed with rescue strategies such as myeloablation as conditioning regimen for HSC transplantation.

CHAPTER-V

General Discussion and Future Directions

The main aim of this dissertation is to develop novel adaptive immunotherapies for the treatment of AML and B-ALL patients by redirecting specificity of T cells by genetic modification through chimeric antigen receptors. This was tested in three different specific aims. In first specific aim we have shown that CARs (chimeric CARs) can be generated by mix and matching V_H and V_L chains from different mAbs, which is a novel finding because traditional CARs derive their scFv from a single mAb. Chimeric CARs successfully expanded on AaPC, Clone 1-CD123 to large numbers needed for the clinic and stably co-expressed CAR and suicide gene iCaspase 9. Chimeric CARs executed effector functions, antigen-specific killing and IFN-γ production. Second specific aim enabled us head to head comparison of CD123- specific CARs with co-stimulatory domains CD28 and CD137. It was reported that CD19-specific CARs containing CD137 endo domain mediated enhanced survival of T cells and increased anti-leukemic activity *in vivo* compared to CARs with CD28 in clearing B-ALL tumors **(41).** Contrary to this in our head-to-head comparison of CD123-specific CARs with co-stimulatory domains CD28 and CD137, we observed similar rates of target lysis with both constructs *in vitro*, though there was a trend towards better survival with the CD28-containing construct in our *in vivo* AML model. However it is difficult to compare CARs across research groups since each group has different protocols, CARs vary in their design, expression on the T cells, culture conditions for propagating T cells, antigen density on tumor, affinity of scFv, CD4:CD8 ratio in T cell cultures, cytokine support for the infused T cells, lympho depleting strategy, disease targeted, and timing of CAR T-cell infusion with regard to standard therapy such as bone marrow transplantation **(162).** These data supports the translation of preclinical methods into clinical trials for AML by targeting CD123. In the third specific aim we showed that *in vitro* targeting of LSCs, and normal hematopoietic cells by CAR T cells. Since CD123 is expressed on normal hematopoietic cells, careful planning is needed in clinical studies to prevent off target toxicities. The hinge is the least commented aspect of CARs though they make important contributions to the interaction of CAR with its cognate antigen, formation of immunological synapse and necessary interaction of CAR with other proteins for activation signal **(162).** Preclinical data suggests that the spacial location of epitope length and binding, flexibility and origin of the hinge domain are important variables in the design of CARs and has bigger impact on CAR activity than variation affinity to scFv **(163-166).** The affinity of the scFv for antigen also affects the density of antigen required for efficient killing (87). Though the effect of antigen density for CAR therapy is not yet well-defined, it appears that CAR T cells preferably target tumors with high antigen density, while cells with lower density are more resistant to CAR T cells (97,98). Importantly our studies CAR-10 **(Figure 6A)** with IgG4 hinge showed minimal lysis in normal BM cells compared to CARs 6 with CD8α hinge with same scFv **(Figure 11A).** This finding has to be validated further in chimeric CARs other than CAR-10 *in vitro and in vivo* to generate low affinity CARs with various combination of hinge and chimeric scFvs to rescue normal hematopoiesis. Thus by choosing different source of V_H and V_L chains and perhaps different hinge regions, we may be able to tune the activation threshold for CAR T cells further, especially if a wider range of antibody affinities is used than was chosen for these studies.

Future Directions

Though the CARs typically identified by their endo-domains, the other components of CAR also has crucial role in their function and clinical efficacy including hinge portion. Constant region of IgG4 and CD8α are frequently used hinge regions, however Fc region have been reported to engage Fc receptors and activate innate immune cells **(137).** To avoid off target activation of CARs and unwanted immune responses we have generated CD123 specific chimeric CARs by introducing L235E and N297Q mutations in the CH2 region of IgG4-Fc spacer or replacing IgG4-Fc hinge with CD8α. In our future studies these CARs will be evaluated *in vivo* for enhanced anti-tumor activity and persistence. Additional modifications to the *ex vivo* culture by reducing the number of stimulations (addition of AaPC) of γ- irradiated AaPC further improved persistence and anti-tumor effect in preclinical studies targeting CD19 (data not shown). Furthermore, clinical data involving CAR T cells have highlighted that the persistence of genetically modified T cells can be compromised in human applications due to recognition of the recipient's immune system to mouse elements of the scFv used to derive specificit through the CAR architecture which can be resolved by using humanized scFvs. CARs generated by lentiviral or retroviral vectors exhibit significant anti-tumor efficacy and *in vivo* persistence but sometimes results in on-target and off- target cyto-toxicities. Unlike lentiviral and retroviral vectors SB transposition is cost effective gene transfer system requires less production cost for manufacturing clinical grade T cells. SB transfected genes doesn't integrate at sites of active transcription, has been shown not to activate oncogenes

(144-149). The introduction of suicide genes such as iCapase9 may mitigate the risks by conditional ablation of T cells. However the efficacy of these strategies are limited by incomplete elimination of transferred T cells **(138).** In summary our data supports CARs can be derived from two or three mAbs specific to an antigen by combining VH and VL chains. This approach may allow us to derive affinity tuned CARs to target tumors with differential antigen density sparing normal cells expressing antigen at low levels. Recent studies demonstrated enhanced persistence for CARs with CD137 compared to those with CD28 endodomain, however our data shows that CARs activated through CD28 or CD137 showed similar efficacy *in vitro* and *in vivo*.

CHAPTER-VI

Materials and Methods

Primary samples and animal use

All patient samples used for this study were obtained after written informed consent was granted in accordance with protocols established and approved by the MD Anderson Cancer Center (MDACC) and Internal Review Board (IRB). The identities of all samples were kept private. Animals were handled in accordance with the strict guidelines established by the MDACC Institutional Animal Care and Use Committee (IACUC).

Generation of CD123 specific CARs with scFvs derived from two monoclonal antibodies

To generate CARs specific for CD123, we generated scFv by assembling V_H and V_L chains from four monoclonal antibodies 26292, 32701, 32703 and 32716 specific to CD123 (96) and then fused in frame to the human CD8 spacer and transmembrane domain, then the CD3ζ and CD28 endodomains. Of the 12 possible scFvs that could have been made, we chose five at random for further testing. (**Figure 6A**). These five mix-and-match scFvs were spliced into the existing anti-CD19 CAR construct above to generate CARs 5-9 (**Figure 6A**). CAR 10 has the same scFv as CAR 6, but uses the IgG4 spacer and CD28 TM. CARs 1-4 has scFvs derived from

V_H and V_L single MAb. CAR constructs were custom synthesized and cloned into SB system constructs, as described previously for CD19 CARs (46).

Construction of iCaspase 9 co-expressing CARs in SB transposons

For experiments testing the relative contributions of CD28 vs. CD137 signaling as the costimulation signal for CAR T cells, we chose the CAR10 scFv described above (Figure 6A), since we have previously engineered CAR constructs using these costimulatory domains fused to the IgG4 transmembrane domain. On the 5' side of the resulting CAR sequence, there is an in-frame inducible caspase 9 sequence (iCasp9), followed by a Furin element and F2A peptide sequence, which together make an auto-cleavage site within the protein, resulting in two mature proteins from the single polypeptide sequence. The iCasp9 element creates a chemically inducible suicide switch in CAR^+ T cells. The CAR constructs were custom synthesized and codon optimized by Geneart, (Invitrogen, Grand Island, NY) and cloned into SB vectors (Figure14). The sequence for both plasmids was verified by Sanger sequencing (DNA Sequencing Core, MD Anderson cancer center (MDACC).

Primary cells and cell lines.

The TF1 cell line was obtained from European collection cell cultures (ECACC). Molm13, MV411 and OCI-AML3 were kind gifts from Dr. Dean A. Lee (MDACC). EL4 cells were obtained from ATCC. RCH-ACV and Kasumi-2 were kind gifts from Jeffrey Tyner (Oregon Health Sciences University). OCI-Ly19 was a kind gift from Dr. Richard Eric Davis (MD ACC). K562-derived aAPC were obtained from Dr. Carl H. June (University of Pennsylvania) and further modified with mIL15 and TAAs ROR1 and CD123 (see below). The Nalm-6 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). PBMC for T cell transfections were obtained from healthy donors after informed consent and isolated by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare). All cell lines were maintained in complete RPMI media, 10% FBS and 1X Glutamax-100. STR DNA Fingerprinting was done to confirm the identity of all cell lines at MD Anderson's Cancer Center Support Grant (CCSG) supported facility "Characterized Cell Line Core."

Generation of CD123^{+} Clone1-APC and EL4

To generate activating and propagating cells (AaPC) to support expansion of CD123-specific CAR T cells, we modified K562-based AaPC originally obtained from Carl June (Clone 9) which express CD19, CD64, CD86, and CD137L, to express an IL15/IL15Rα fusion protein ROR1 and CD123 using the *Sleeping Beauty* (SB) gene transfer according to our published methods (46). This new AaPC line we termed Clone1-CD123. The same process was used to create EL4 cells expressing CD123. CD123^{+} cells were selected by hygromycin selection.

Electroporation and propagation of CAR T cells.

On day 0, 20 million PBMC were resuspended in 100 μL of Amaxa human T cell Nucleofector solution (Cat. no. VPA-1002; Lonza, Basel, Switzerla nd) mixed with 15 μg of transposon and 5 μg of transposase (pKan-CMV-SB11) and electroporated using Program U-14. The following day (day1) cells were counted, surface stained for CAR expression by Fc antibody and stimulated with γ-irradiated (100 Gy) Clone 1-CD123 at 1:2 ratio of T cells to AaPCs. Cultures were supplemented with recombinant human IL-2 50 units/ml and 30 ng/ml of recombinant human IL-21 (Pepro Tech). AaPCs added every 7 days and IL-2, IL-21 added monday-wednesday and friday schedule beginning of day1 of each 7 day T cell expansion cycle. T cell cultures were phenotyped every week to monitor CAR expression and outgrowth of NK cells (CD3^{neg}CD56^{+} population) usually occurred between

10 to 14 days after electroporation. If the percentage of NK cells exceeded approximately 10% total population, depletion of NK cells was carried out with CD56 beads (cat.no.130-050-401, Miltenyi Biotech) according to manufacture instructions. As a control, $5x10^6$ PBMC were mock transfected with nucleofector solution without CAR plasmid and were co-cultured on γ-irradiated (100 Gy) anti-CD3 (OKT3) loaded K562-aAPC clone #1 at a ratio of 1:1 in a 7-day stimulation cycle along with similar concentrations of IL-2 and IL-21 as CAR T cells.

Real time PCR to determine integrated CAR copy number

The number of integrated copies of CD123-specific CAR transgene was determined by isolating genomic DNA using AllPrep DNA/RNA Mini Kit, (Qiagen cat # 80204) as described in **(25).** Briefly about 50-100 ng of DNA amplified using Steponeplus Real-time PCR system (Applied Biosystems), forward primer (5'-CAGCGACGGCAGCTTCTT-3'), reverse primer (5'-TGCATCACGGAGCTAAA-3') and carboxyfluorescein (FAM)-labeled probe and (5'- AGAGCCGGTGGCAGG-3'). These primers hybridize to the CAR in IgG4 and CD28 transmembrane region. Genomic DNA from a genetically modified Jurkat T-cell (clone #12) containing 1 copy of CAR per cell from CoOpCD19RCD28/pSBSO DNA plasmid was used as positive control. No DNA (CAR^{neg}) T cells were used as negative control. Results were analyzed using GraphPad Prism software.

Immunophenotype of CAR$^+$ T cells

T cells were immunophenotyped using appropriate antibodies and isotype controls. Cells were stained for 30 minutes at 4^0C followed by 2 washes with FACS buffer (PBS, 2% FBS, 0.1% sodium azide). For intracellular staining cells were fixed and permeabilized for 20 minutes at 4^oC with BD

Cytofix/Cytoperm (BD Biosciences, San Diego, CA) followed by staining with appropriate antibodies. All samples were acquired on FACS Calibur (BD Bioscience) and analyzed with FlowJo software (version 7.6.3).

Multiplex Gene Expression Analysis of CAR T cells

On day 35 of co-culture of CAR T cells on AaPC about 10^5 were lysed in 17 µl of RLT buffer (Qiagen) and frozen at -80^0C. Cell lysates were thawed and analyzed immediately using nCounter analysis System (NanoString Technologies, Seattle, WA) with "lymphocyte codeset array" as described **(26).** Data was normalized to spike positive control RNA and housekeeping genes (ACTB, G6PD, OAZ1, POLR1B, POLR2A, RPL27, Rps13, and TBP)

Where 2 normalization factors were calculated and applied to the raw counts. Each normalization factor was calculated from the average of sum of all samples divided by the sum of counts for an individual sample. Total counts for LCA genes described in CD123-specific CAR$^+$ T cells were directly reported as normalized mRNA counts. Limit-of-detection (LOD) was calculated from the negative control counts and reported as the mean plus two times the standard deviation (mean+2xSD) and shown as dashed lines in graphs of mRNA data.

iCaspase 9 functional assay.

CAR^+ T cells with and without icaspase 9 were seeded in 24 well plate @ 10^6 cells/well**.** 1μM of chemical inducer of dimerization (CID) (AP20187; Clontech) was added, cells were harvested after 24 hours and surface stained with CD3, FC followed by annexin-V and 7-amino-actinomycin D (7-AAD) for 15 minutes according to the manufacturer's instructions (BD Pharmingen). Within 1 hour after staining, cells were analyzed by flow cytometry using BD FACS caliber

Chromium release assay.

The cytolytic efficacy of CAR^+ T cells with target cell lines was evaluated by 4-hour chromium release assay as described in **(20).** Briefly $5x10^3$ 51Cr-labeled target cells were incubated with CD123 specific CAR^+ T cells in complete medium or 0.1% Triton X-100 (company) to determine spontaneous and maximum ^{51}Cr release, in a V-bottomed 96-well plate. The mean percentage of specific cytolysis of triplicate wells was calculated from the release of ^{51}Cr using a Top Count NXT (Perkin-Elmer Life and Analytical Sciences, Inc.) as 100 × (experimental release - spontaneous release)/(maximal release - spontaneous release). Data was reported as mean ± SD

Flow cytometric killing assay

For T cell killing assays, target cells were labeled with PKH-26 (Sigma, cat.no PKH26PCL) according to manufacture instructions and co-cultured with CAR^{+}T cells at E:T ratio of 1:1 for 3 days without exogenous cytokines. 7-AAD was added prior to flow cytometric analysis to exclude dead cells, viable cells phenotyped by CD3 and PKH-26.

Cytokine production by CAR^{+} T cells.

Effector cells were incubated with target cells at T cell to target ratio of 1:1 for 24 hours. Cytokine production from CAR^{+} T cells in response to antigen was determined using LEGENDplex™ multi analyte flow assay kit (Biolegend, cat.no 790004) according to manufacture instructions.

Mice studies

The *in vivo* antitumor efficacy of CAR T cells in TF1 cells was assessed in NOD/SCID/IL-2R (NSG) mice transgenic for human interleukin-3 (IL-3), stem cell factor (SCF), and granulocyte macrophage colony-stimulating factor (GM-CSF) obtained from Jackson Laboratories. For bioluminescent xenograft models, the TF1 cell line was genetically modified to express enhanced firefly luciferase (effLuc) (Figure S7) by transduction with a pLVU3G effLuc-T2A-mKateS158A lentivirus construct and sorting cells for uniform mKate expression. On day 0, 12 mice were injected intravenously (i.v) with 2.5 million TF1-effLuc cells, then divided into three groups of 4 mice each. On day 5, mice were injected with 10^{7} cells CD123-

CD28 (group 2), or CD123-41BB CAR T cells (group 3) per mouse, or were given no cells (group 1). Tumor engraftment was confirmed by bioluminescent imaging (BLI) before T cell infusion. Additional T cell infusions were administered on days 11 and 20, and the tumor burden was assessed serially by BLI. To test *in vivo* efficacy of CAR T cells in B-ALL model pre B-ALL cell line RCH-ACV was modified with enhanced firefly luciferase same way as TF1 and infused into NSG mice on day 0 and CAR T cells were infused on day 1, 7, 14 and 21 followed by BLI and IL-2 (60,000 units/mice) infusion. The experiments were performed twice; one representative experiment is shown.

Isolation of Lineage^{+} and Lineage^{neg} cells from BM cells

To determine CD123 expression on normal BM cells we have isolated Lineage positive cells using biotin conjugated lineage antibody cocktail followed by positive selection with anti-biotin microbeads using LD Column unlabeled fraction collected lineage negative and labeled fraction lineage positive.

Ethics statement

All patient samples used for this study were obtained after written informed consent was obtained in accordance with protocols established and approved by the MD Anderson Cancer Center (MD Anderson) and Internal Review Board (IRB). The all samples were de-identified. Animals were handled in accordance with the strict guidelines established by the MD Anderson Institutional Animal Care and Use Committee (IACUC).

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VITA

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