TCR ACTIVATION IMPACT ON CHIMERIC ANTIGEN RECEPTOR THERAPY AGAINST ACUTE LYMPHOBLASTIC LEUKEMIA

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TCR ACTIVATION IMPACT ON CHIMERIC ANTIGEN RECEPTOR THERAPY AGAINST ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Tremendous progress has been achieved employing immunotherapy for B cell acute lymphoblastic leukemia (ALL), the most common diagnosis in children with cancer and a leading cause of cancer-related death. Recent trials using transfer of autologous T cells genetically modified to express chimeric antigen receptor T cells (CAR T) targeting the B cell restricted antigen CD19, have demonstrated remission rates of 80% in relapsed or refractory ALL. The feasibility of this therapy depends on the ability of the CAR T cells to expand following adoptive transfer, not always possible in this heavily pre-treated patient population. In these instances, allogeneic donors may be an alternative source to produce CAR T, however there is little known about the effectiveness of allogeneic CAR T cells (alloCART). These alloCART would have specificity for two antigens, one being the CAR target and the other derived from the endogenous T cell receptor (TCR) that could have reactivity against alloantigens. The impact of the endogenous TCR could potentially impact the efficacy of the alloCART against tumor and has
not been well studied. We established a murine model to specifically evaluate
the biology of alloCART and, more generally, the biology of CAR T cells
encountering TCR antigen. Using gender mismatched CART cells derived
from female donors to treat male recipients, we found significantly lower
survival due to poor leukemia clearance compared to gender matched female
recipients. We then controlled for TCR specificity of CAR T cells using TCR
transgenic CD4 or CD8 T cells targeted against male histocompatibility
antigens, and found that concurrent CAR and TCR stimulation induced a 6X
reduction in CART numbers, an increase in T cell exhaustion markers (PD-1,
Tim3, and Lag3) and 5X increase in apoptotic CAR T cells in the bone
marrow compared to CAR stimulation in the absence of TCR antigen. The
negative effects of the active TCR was predominantly in CD8 T cells.
Surprisingly, CD4 T cells, by dogma non-cytolytic, acquire cytolytic
capabilities through CAR activation, were able to clear leukemia without CD8
T cells and were less susceptible to concomitant TCR activation. Indeed, the
presence of TCR and CAR antigens led to increased CD4 CAR T expansion,
and better persistence than observed with CD8 CAR T. In addition, CD4 CAR
T cells produced higher levels of IL2, IFNγ, and TNFα compared to CD8 T
cells. These findings suggest that CD4 CAR T may have advantages over
CD8 CAR T cells, particularly when TCR antigen may be present such as with
the use of allogeneic donor cells. Future work will seek to examine the
downstream pathways in TCR vs. CAR and effects of TCR elimination.
DEDICATION

To my husband, Wyatt

For his unflagging support and encouragement through every failed experiment.

To my parents, Yunlong and Yun

For their eternal love and inspiration that helps me through all obstacles.

To my sister, Lixin

Whose intelligence and compassion encourages me to be better every day.
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The completion of my doctoral work could not have been achieved without the support of many individuals. I sincerely express my gratitude for all those who have encouraged and supported me through my training.

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Last, but certainly not least, my eternal love to my husband, Wyatt, who is there for me no matter what, who has supported me through my entire career thus far. He is my best friend who never fails to make me laugh even after the hardest days. Thank you for all of your hard work and dedication to support our own little family. I could not have achieved this PhD without you.
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<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>Allo-BMT</td>
<td>Allogeneic</td>
</tr>
<tr>
<td>Allo-HSCT</td>
<td>Allogeneic hematopoietic stem cell transplant</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplant</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of differentiation 3, T cell activation co-receptor</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4, glycoprotein on immune cells, binds MHC class II</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8, glycoprotein on immune cells, binds MHC class I</td>
</tr>
<tr>
<td>CD19</td>
<td>Cluster of differentiation 19, B cell antigen</td>
</tr>
<tr>
<td>CAR</td>
<td>Chimeric antigen receptor</td>
</tr>
<tr>
<td>CART</td>
<td>Chimeric antigen receptor T cell</td>
</tr>
<tr>
<td>CAR4</td>
<td>CD4+ chimeric antigen receptor T cell</td>
</tr>
<tr>
<td>CAR8</td>
<td>CD8+ chimeric antigen receptor T cell</td>
</tr>
<tr>
<td>CD28</td>
<td>Cluster of differentiation 28, T cell co-stimulatory signal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>E:T</td>
<td>Effector to target ratio</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GVH</td>
<td>Graft versus host</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft versus leukemia</td>
</tr>
<tr>
<td>HY-CAR4</td>
<td>Male antigen HY specific, CD4+, CAR T cell</td>
</tr>
<tr>
<td>HY-CAR8</td>
<td>Male antigen HY specific, CD8+, CAR T cell</td>
</tr>
<tr>
<td>ITAMS</td>
<td>Immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL2</td>
<td>Interleukin-2</td>
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<tr>
<td>IL7</td>
<td>Interleukin-7</td>
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<tr>
<td>IL10</td>
<td>Interleukin-10</td>
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<td>IL4</td>
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<td>Interleukin-5</td>
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<tr>
<td>IL6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible T cell costimulatory, also CD278</td>
</tr>
<tr>
<td>LAG3</td>
<td>Lymphocyte activation gene 3, also CD223</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death receptor 1</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>scFV</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIM3</td>
<td>T cell immunoglobulin and mucin-domain containing 3, also HAVCR2</td>
</tr>
<tr>
<td>VDJ</td>
<td>Variable, diversity, joining</td>
</tr>
<tr>
<td>VH</td>
<td>Heavy chain</td>
</tr>
<tr>
<td>VL</td>
<td>Light chain</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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INTRODUCTION

Leukemia is the leading cause of death in pediatric oncology, with acute lymphoblastic leukemia (ALL) being the most commonly diagnosed leukemia in children. (1, 2) Adoptive T cell immunotherapy using chimeric antigen receptor (CAR) provides a promising non-chemotherapy based treatment for refractory patients that relies on redirected-specificity of patient-derived T cells against tumor antigens. (3, 4) CARs are genetically engineered receptors that use the non-MHC-restricted specificity of monoclonal antibodies combined with TCR signaling and co-stimulatory domains to enable T cells to eliminate tumors based on cell surface antigens. T cells engineered with the anti-CD19 CAR has shown promising efficacy in treating patients with refractory ALL in phase I clinical trials, demonstrating complete remission rates as high as 90%. (5, 6)

CAR T cells generate potent anti-leukemic responses against surface tumor antigens through activation of the CAR receptor but generally retain the endogenous T cell receptor (TCR) that confers reactivity to other antigens presented on MHC molecules. This could have significant implications on the use of CAR T cells post-allogeneic hematopoietic stem cell transplantation (allo-HSCT), where the CAR T cells are derived from the allograft and have the potential to react against allo-antigens through the TCR. (7) The precursor frequency of infused CAR T cells specific for allogeneic antigens could be
relatively small in an transplant patient; However, CAR T cell expansion could be disproportionately driven through the endogenous TCR by tumor antigens, viral antigens, or normal antigens. Viral-specific CAR T cells rely on stimulation through the TCR by viral antigens for expansion of the CAR T cells both in vitro and in vivo.\((8–10)\) Despite enhanced expansion, the CAR T cells did not lead to significant clinical effects either against hematologic or solid tumors, suggesting the possibility of impaired CAR T cell function.\((11, 12)\) In the presence of both CAR and TCR antigens, it is not known which receptor will be dominant on the CAR T cell and how signaling through the endogenous TCR affects the efficacy of the CAR T cell against tumor. Understanding the effect of TCR activation on the efficacy of CAR therapy and the immunobiology of CAR and TCR signaling is important for the advancement of better CAR therapy.

The use of different subsets of T cells for adoptive immunotherapy has been explored.\((13, 14)\) Although many subsets of CAR T cells have been evaluated in many preclinical models, CD8\(^+\) CAR T cells alone\((15)\) or CD8\(^+\)/CD4\(^+\) CAR T cell combinations have been most commonly tested in different anti-CD19 CAR T cell clinical trials against ALL.\((5, 6, 16)\) With the use of xenograft and syngeneic murine models, we evaluate the efficacy of CD4\(^+\) (CAR4) and CD8\(^+\)(CAR8) individual product CAR T cells against ALL. We developed a clinically relevant syngeneic murine transplant model against a murine ALL in order to study the immunobiology of TCR signaling in CAR T cells. Here we report the comparable cytotoxicity and efficacy of CAR4 cells.
and the unique gene expression profiles of CAR activation in both CAR4 and CAR8 cells. Further, our findings demonstrate that TCR signaling induces elevated exhaustion markers and early apoptosis in CAR8 cells leading to T cell failure, while CAR4 cells maintain persistence and cytotoxicity against ALL in the presence of both TCR and CAR antigens.
CHAPTER I
BACKGROUND

1.1 Pediatric Acute Lymphoblastic Leukemia

Leukemia is the leading cause of death in pediatric oncology, with acute lymphoblastic leukemia (ALL) accounting for more than 30% of all pediatric cancers with over 6000 cases diagnosed in the United States annually.\(^{(1, 2, 17)}\) The rate of success of treatments for ALL has steadily increased for decades. Despite advancements in chemotherapy that has contributed to the 80% cure rate (disease free for 10 years), ALL remains a leading cause of death in pediatric oncology patients with relapsed and/or chemotherapy refractory disease.\(^{(18)}\)

As of the present, the incidence of ALL continues to increase by 1% per year, as it has been in the past 2 decades. Leukemia is most often caused by genetic alterations due to over expression of proto-oncogenes, hyperdiploidy, and chromosomal translocations that induce constitutively active kinases and transcription factors that leads to aberrations in the development of lymphoid cells\(^{(19–21)}\). This study in particular, is focused on the treatment of pre-B cell acute lymphoblastic leukemia, where the translocation of genes in a B cell progenitor leads the formation of fusion
proteins that normally contribute to the homeostatic control of cellular growth, cell renewal or growth factor signaling. (22–24) Karyotyping and genome-wide analysis has enabled researchers to identify the mutations and genetic abnormalities involved in lymphoid development that contributes to leukemia. In this study, we are focused on the treatment of pre-B cell ALL, induced by the translocation of HOX genes, E2a:PBX1 t(1:19) that represent about 25% of pre-B cell ALL cases. (25)

Initial treatment of ALL involves combination chemotherapy often involving the use of vincristine, doxorubicin, cyclophosphamide, cytarabine among many others. After initial intensive treatment, patients are then put on maintenance chemotherapy for up to 3 years with methotrexate and mercaptopurine to prevent disease relapse. The use of cranial radiation is sometimes prescribed for leukemia in the CNS compartment, which has significant toxicity on the intellectual capabilities and mental function of patients. (26)

For children with high-risk ALL and relapsed disease incurable by chemotherapy alone, treatment with allogeneic hematopoietic stem cell transplantation (allo-HSCT) can induce long term remission. Transplant often requires a matched family donor, and could induce 10-year remission in about 60% of patients; however, allo-HSCT is also associated with up to 24% transplant related mortality and other risks. (27, 28) One of the major restrictions of allo-HSCT that is over 70% of patients with relapsed ALL do not have HLA matched family donors who could provide the marrow donation. (29)
Studies have shown the anti-leukemic effect of allo-HSCT is depended on the graft-versus-leukemic (GVL) effects imparted by the donor T cells in the allograft.\(^{(30, 31)}\) Further clinical research aims to maximize the GVL effects while minimizing the potential graft-versus-host (GVH) response, a significant toxicity of allo-HSCT, where the allograft attacks the normal tissue of the patient. Adoptive immunotherapy with the use of genetically engineered receptors offers a superior alternative to standard therapy by redirecting T cell specificity towards antigens expressed on malignant cells.

1.2 T Cells

Our immune system is critical for the prevention of infections and disease. T cells are a type of lymphocyte that make up a part of our adaptive immune system. Billions of these highly specific cells circulate through our blood stream to detect foreign antigen and protect us from disease. T cells mediate cellular immunity by directly recognizing and eradicating diseased cells, including tumors. The importance of the immune system is emphasized especially in cancer due to the tumors abilities to evade immune recognition and to suppress immune reactivity in order to propagate and metastasize.\(^{(32, 33)}\)

T cells are conventionally divided into two subtypes by their expression of CD8 or CD4 receptors on their surface.\(^{(34)}\) CD8 T cells, or cytotoxic T cells, directly kill target cells through the release of cytotoxic granules. CD4 T
cells, or helper T cells, release cytokines for the recruitment of other immune
cells to the site of infection and aid in the expansion and activity of cytotoxic T
cells. T cells are able to generate very specific responses to antigens and
also develop memory responses in order to elicit rapid and stronger
responses to future infections.

T cells are activated through the T cell receptor (TCR) that gives the
cells the specificity to recognize foreign peptide. Every mature T cell has a
unique T cell receptor (TCR) that is generated during development in the
thymus. Each T cell expresses a different TCR that recognizes a different
antigen. A TCR is a heterodimer composed of two immunoglobulin-like
domains that formed from genetic rearrangement of multiple genes during
development. The TCR is comprised of a constant domain and variable
region that serves as the antigen-binding site for the receptor. The specificity
and immense diversity of the TCR comes from the random rearrangement
of the variable (V), diversity (D), junction (J), and constant (C) regions of the
TCR allele. The binding domain of the TCR or the variable domain, is formed
by VDJ recombination and results in the large magnitude of binding
permutations, in addition, extra base pairs could also be inserted at random at
the sites of recombination, which gives extra diversity to the receptors.

During development, T cell progenitors are selected through central
immunological tolerance based on their inactive response towards self-
antigens and active ability to bind MHC. (35, 36) While there are only 9
classical MHC molecules, each is highly polymorphic resulting in extremely
unlikely population compatibility. As illustrated in Figure 1A, antigens are only recognized by TCR when antigens are presented by a professional antigen presenting cell in the context of correct MHC molecules. TCR signaling consists of two signals. The T cell recognition of antigen through the TCR is the first signal to initiate a T cell mediated response. A second co-stimulatory signal is typically necessary to allow the T cell to maximally respond to an antigen. The CD28 co-receptor is the prototypical example of the second T cell signal. (37)

A T cell is activated upon binding of the TCR to an antigen presenting cells expressing the foreign antigen in the context of the major histocompatibility complex (MHC), along with co-stimulatory domains expressed on the T cell. CD8 receptors recognize MHC class I molecules, while CD4 receptors recognize MHC class II. Upon binding of the receptors, activation domains of the T cell activate downstream molecular pathways that induce T cell differentiation and immune reactions including proliferation of the T cell, migration to the site of infection, and the generation of T cell memory.

1.3 Adoptive T Cell Therapy

Scientists have harnessed the specificity and cytotoxic phenotypes of T cells against the treatment of cancer, including the use of tumor infiltrating lymphocytes (TILs). (38, 39) The adoptive cell transfer (ACT) of these tumor antigen specific T cells have been successful against the treatment of a few
cancers including melanoma. TILs can be found at the site of the tumor and often times, TILs are isolated directly from the tumor sample. These cells are then stimulated and expanded ex vivo, and re-infused back to the patient in large quantities and in an activated state. Treatment with TILs have been shown to induce long term remission in patients with melanoma and other virus-associated malignancies.

Clinical success with the use of TILs for adoptive T cell therapy in melanoma patients was encouraging. However, the isolation of TILs is challenging and the majority of cancers do not respond to TIL therapy due to immune escape of the tumor cells. Immune evasion has now been characterized as a hallmark of cancer. (32, 33) T cells require the presentation of tumor antigens in the context of MHC. A major mechanism of immune evasion is the downregulation of MHC complex expression by tumor cells in order to “hide” from immune detection. In addition, tumors are also able to suppress immune reactivity through the release of inhibitory cytokines, over expression of negative signals to T cells, and the recruitment and activation of immune suppressor cells. (32, 40) In an attempt to make adoptive cell therapy applicable to a variety of cancers without the restriction of MHC expression, genetically engineered chimeric antigen receptors (CAR) are now used to overcome these challenges.
Chimeric antigen receptors (CARs) are genetically engineered receptors that redirect T cells against tumor specific antigens independent of MHC restriction. The antigen binding portion of a CAR is derived from the variable binding domain of a monoclonal antibody that targets any extracellular antigen. Thus, CARs enable T cells to have antigen specificity derived from antibody-defined specificity that includes cognate antigen that normal T cells would be non-responsive to due to immune tolerance. In the CAR construct, the heavy and light chains of the antibody are linked together to form the single chain variable fragment (scFv) that binds to the target as shown in Figure 1. The scFv is linked to the activation domain of the T cell receptor complex, the CD3-zeta chain, that provides signal 1 in T cell activation. (41–44) The mechanisms of T cell activation through the CAR is not well understood. Studies have shown potential for CAR dimerization with endogenous TCR complex by the CD3-zeta domain contained in the CAR receptor that then illicit downstream T cell activation similar to native TCR activation.(45) Unpublished data suggest clustering of CAR on the T cell membrane when it interacts with CAR antigen positive cells. Data suggest formation of immunological synapse similar to that formed during TCR signaling. Upon clustering of receptors, immunoreceptor tyrosine-based activation motifs (ITAM) on CD3 subunits are activated. Then, activated ZAP70 and Lck leads to downstream cascade of events that ultimately results
in the activation of transcriptional changes that induce T cell proliferation and activation. Original first generation CARs included only the scFv and the CD3-zeta signaling domain which only demonstrated modest anti-tumor activity. To enhance the activation of T cell, second generation CAR constructs also includes the co-stimulatory domain of T cell activation such as CD28, 41BB, or Ox40. The co-stimulatory domains significantly increased CAR T cell expansion and persistence, thus enhancing CAR T cell function against tumor clearance. Third generation CARs containing two instead of one co-stimulatory domains have been evaluated, but whether or not third generation CARs exhibit better functionality over second generation CARs is unclear. Cytotoxic T cells expressing CAR receptors are reprogrammed to target tumor cells independent of MHC expression. This single chain fusion construct combines the specificity of an antibody with the potent cytotoxicity of a T cell in order to identify and eliminate tumor cells and could be developed to target all tumors with specific tumor antigen expression.
**a**
Antigen Presenting Cell

- Tumor antigen
- CD80 or CD86
- CD3 complex
- CD28

**b**
Antigen binding domain

- Light Chain
- Heavy Chain

Monoclonal antibody

**c**

- VL
- VH
- scFv

- Co-stimulatory domain CD28
- T cell activation domain CD3ζ
Figure 1. *Previous page*: T cell receptor signaling and chimeric antigen receptor design. (a) T cell signaling through T cell receptor (TCR). Signal 1 of T cell activation provided by TCR binding to antigen in the context of major histocompatibility complex (MHC) and activation of CD3-zeta activation domain. Signal 2 of T cell activation provided by binding of co-stimulatory domain (CD28, 41BB, Ox40 etc.). (b) Antigen binding motif of CARs are derived from the heavy (VH) and light (VL) chains of the variable binding domain of monoclonal antibodies. (c) VH and VL are linked to form the single chain variable fragment (scFv) that is the binding domain of the CAR. The binding domain is then linked to the co-stimulatory domain (in this case CD28) that provides the transmembrane domain of the receptor and signal 2 of T cell activation. The receptor also includes the intracellular domain of CD3-zeta signaling domain that provides signal 1 of T cell activation.
Consequently, CARs recognize antigens independent of MHC expression (Figure 2). This characteristic proves advantageous for CAR over TCR mediated therapy in that a tumor antigen specific TCR from one individual will not work in another individual should she lack the identical MHC molecule. In addition, a common method exploited by tumor cells is immune evasion by downregulating MHC expression so tumor associated antigens are not presented to the immune system. CARs can bypass this method of immune evasion because they are not dependent on MHC presentation of antigen.

**Figure 2. Chimeric antigen receptor directed killing of target cells.**

MHC independent, CAR directed killing. Antigen binding by antibody derived scFv binds to specific target on tumor cells, activates cytotoxic T cell that releases perforin, granzyme, and cytokines etc. that induce apoptosis in target cell.
1.5 CAR Immunotherapy in the Clinic

There are and has been numerous phase I/II clinical trials evaluating the efficacy of CAR therapy against hematopoietic malignancies. The first successful CAR construct used in clinical trials to treat acute lymphoblastic leukemia targeted the B cell-specific marker, CD19. CD19 is a B cell specific molecule that is a positive costimulatory molecule part of B cell receptor (BCR) signaling. CD19 is expressed on B cells from the pro-B-cell stage until the plasma cell stage, making it a good target for T cell immunotherapy against tumors across multiple cell stages of B cell development.\(^{[56–58]}\) Being a co-receptor of B cell signaling, CD19 is expressed only on B cells making it an attractive target for CAR therapy, with low off-target toxicity due to the specificity of the target. However, CD19 is not a tumor specific antigen as it is a normal signaling receptor on all B cells. Patients treated with anti-CD19 CAR therapy experience B cell aplasia due to deletion of both malignant and normal B cells expressing CD19.

T cells armed with CD19 CAR against pediatric ALL has been very successful in clinical trials. Remission rates against B cell malignancies reach 80% complete remission in patients with chemotherapy refractory and relapsed disease in phase I clinical trials.\(^{[5, 59–62]}\) Most patients currently treated with anti-CD19 CAR are heavily pre-treated and have experience multiple disease relapses, making therapy success even more remarkable. CD19 CAR T cells are potent and highly specific against CD19 positive
leukemia. Clearance of large leukemic burden is possible with a remarkably small dose of CAR T cells infused. However, complete remission of disease requires exponential expansion of the CAR T cells *in vivo* following infusion. Durability of the response depends on expansion of the T cells and also the ability of these T cells to persist long-term in the patient to prevent disease relapse. As previously noted in successful anti-CD19 CAR trials, response of patients often correlates with dramatic *in vivo* expansion of the CAR T cells.\(^{(5, 6, 62)}\)

To potentially boost the expansion of CAR T cells, investigators have used virus-specific T cells as initiating cells for generation of CAR T cells.\(^{(8, 9, 11, 63, 64)}\) Virus specific T cells has been observed to proliferate exponentially through the activation of the virus specific T cell receptor. Virus specific T cell are able to persist long term and provide antiviral activity *in vivo* in the presence of viral antigen.\(^{(12, 64, 65)}\) Investigators have modified virus specific T cells with CARs to induce potent anti-tumor capabilities with the expansion potential of virus specific T cells. Many patients receiving CAR therapy have received prior HSCT making them susceptible to viral infections. Virus specific CAR T cells have the potential to have activity against both the tumor and virally infected cells. However, the impact of simultaneous TCR signaling on the expansion and efficacy of the CAR T cells against malignancy is unknown.

Most CAR T cells have two specificities, one through the introduced CAR receptors, and also, through the endogenous T cell receptor (TCR) that
is still present on the surface of all T cells. Most CAR patients receive autologous CAR T cells where the TCR is tolerized to the patient. With the use of autologous cells from the patient themselves, there is no risk of graft-versus-host disease (GVHD), and a very small probability of the native TCR encountering antigen in the patient. However, the effect on the efficacy of CAR therapy is unknown with the use of virus specific CAR T cells where both receptors could be activated. The effect of the TCR could also have significant implications for the use of donor derived CAR T cells or CAR therapy in the setting of allogeneic hematopoietic stem cell transplantation.\(^{10, 66, 67}\) Although the precursor frequency of infused CAR T cells specific for TCR antigens could be relatively low following allo-HSCT, CAR T cells expansion may be disproportionately driven through the endogenous TCR, which has been observed particularly in the lymphophenic environments of all CAR patients receiving lymphodepleting regimens prior to CAR infusion.\(^{68–70}\) The response of CAR therapy after allo-HSCT has been lower than than patients receiving autologous transfers\(^{61}\), at the same time, small cohorts of patients receiving donor derived and virus specific CAR T cells have not responded as well as autologous patients\(^{9, 11, 66}\), but other clinical differences in the trial regimens may be the cause of the discrepancies. Given the potential for each receptor to affect the activity of T cells, a understanding of the immunobiology of CAR and TCR signaling will inform future optimization of CAR therapy.
1.6 Hypothesis

TCR signaling from chronic viral antigens has been thought to improve the survival and function of CAR transduced T cells but this notion has not been systematically tested. The goal of the project is to determine if an activated endogenous TCR has an effect on the efficacy of the CAR receptor in the presence of both antigens. We hypothesize that the presence of an active endogenous TCR impacts the function of CAR T cells for eradicating leukemia in the presence of both antigens. Additional goals of the project include identifying a CAR T cell product that is most effective at anti-tumor capabilities in the presence of one or both antigens for endogenous TCR and CAR receptors.
CHAPTER II
MATERIALS AND METHODS

2.1 Mice and Cell Lines

C57BL/6 (B6) CD45.1 (H-2^b congenic) mice were purchased directly from the National Cancer Institute-Frederick Animal Production Program (Frederick, MD) via the Jackson Laboratories (Bar Harbor, ME). MataHari (B6 Rag1^{−/−}, CD8^{+} TCRtg, H2-A^{b}-restricted, CD45.2^{+}) TCR transgenic mice against MHC class I-restricted HY^{AB} peptide NAGFNSNRAANSSRSS, and Marilyn (B6 Rag1^{−/−}, CD4^{+} TCRtg, H2-D^{b}-restricted, CD45.2^{+}) TCR transgenic mice against MHC class II-restricted HY^{DB} peptide WMHHNMDLI, purchased from the NIAID-Taconic repository (Rockville, MD) mice were previously described (71, 72). C57BL/6-Prf1 (perforin knockout) and NSG Mice (NOD/Shi-scid/IL-2Rnull) were purchased from jackson laboratories. E2aPbx murine acute lymphoblastic leukemia cell line was developed previously in lab with normal B6 mice with E2A-PBx translocation. NALM6 (DSMZ ACC 128) was modified to stably express GFP and Firefly luciferase by and was a kind gift from David Barrett (NALM6-GL; Children’s Hospital of Philadelphia). All murine cell lines and primary mouse cultures were cultured in Complete Mouse Media (CMM) consisting of RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, 1% l-glutamine (Invitrogen, Carlsbad,
CA) and 1% N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (Sigma-Aldrich, St. Louis, MO) and 1ml 2-Mercaptoethanol (Sigma-Aldrich). Human cell lines NALM6, REH (DSMZ ACC 22) and KOPN8 (DSMZ ACC 552) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Invitrogen). All animals were cared for under an animal use protocol reviewed and approved by the National Cancer Institute Laboratory Animal Safety Program-Animal Care and Use Committee (Bethesda, MD).

2.2 Retroviral Supernatant Production

293 GP cells were transfected for the production of CAR viral supernatant. Retroviral transfections follow general guidelines for transfection with lipofectamine reagent 2000 from Thermo Fisher (catalog number: 11668-030). Vector plasmid DNA is diluted in Opti-MEM media (9ug of vector plasmid to 1.5ml of Opti-MEM media. Lipofectamine 2000 is mixed with Opti-MEM at concentration of 60μl per 1.5 ml Opti-MEM. Two mixture are mixed together and incubated for 20 minutes at room temperature. Mixture is then added dropwise to plated 293GP cells and incubated for 6 hours at 37°C, 5% CO2 incubator. Change media after incubation with DMEM media plus 10% FBS. Supernatant was harvested at 24, 48 and 72 hours.
2.3 Generation of Murine CD19 CAR T Cells

Construction of Murine anti-CD19 CD28 CAR was previously described.(7) T cells were extracted from murine splenocytes using T cell enrichment column (R&D systems). Purified CD4 or CD8 T cell subsets were separated using untouched CD4 T Cell Isolation Kit or untouched CD8 T cell Isolation Kit (Miltenyi Biotec) prior to activation. Cells were then activated using anti-CD3/CD28 beads (Life Technologies) on day 1 using 3:1 beads:cell ratio with murine splenocytes then removed on day 3 after transduction. Cells were cultured with 30IU/ml IL-2 and 10ng/ml IL-7 for 5 days. Retroviral supernatants were produced by transfection of 293GP cell line using lipofectamine 2000 (Life Technologies) with plasmids encoding the CD19 CAR and pCL-Eco retroviral envelope DNA plasmid. Supernatants were collected 24, 48, and 72 hours after transfection. Activated T cells were transduced using retronectin (Takara) coated plates using combined viral supernatants on days 2 and 3. T cells were evaluated or infused on day 5. Transduction efficiencies were 80-90% for all CAR T cells used in experiments.

2.4 Generation of Human CAR T Cells

A CD19 CAR producer cell line, H3, was kindly provided by Dr. James Kochenderfer (Surgery Branch, NCI, NIH) and was cultured for viral containing supernatant as previously described [Kochenderfer JN, Feldman...
SA, Zhao Y, et al. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. J Immunother. 2009;32(7):689-702. The CAR employs a murine scFv targeting CD19 (FMC63) with CD28 and CD3zeta signaling endodomains and is identical to that in human clinical trial [Lee DW, et al Lancet 2014]. Human PBMCs from healthy donors were obtained from the Department of Transfusion Medicine, NIH Clinical Center, under an IRB approved protocol after informed consent in accordance with the Declaration of Helsinki. Bulk PBMCs were ficolled, aliquotted and cryopreserved until use.

Upon thawing, CD8\(^{+}\) or CD4\(^{+}\) human T cells were purified separately from the same donor using CD8 or CD4 negative selection kits (Miltenyi) following the supplied protocols. Purity was confirmed by flow cytometry. Separated T cells were activated using CD3/CD28 coated paramagnetic Dynabeads (Life Technologies) for 2 days in AIM-V media (Invitrogen) supplemented with 5% FCS, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, 2mM L-glutamine (Invitrogen), and 40 IU/mL recombinant human IL-2 (Roche). Retroviral supernatant was centrifuged on retronectin-coated, non-tissue culture treated 6-well plates for 2 hours at 2000g and 32\(^{\circ}\)C. Activated T cells were incubated for 24 hours on viral coated plates in fresh AIM-V supplemented media (rhIL-2 increased to 300 IU/mL) then transferred to freshly coated plates for a second 24-hour period. After 48 hours of viral exposure, T cells were replated in
fresh AIM-V supplemented media and expanded for 6 additional days before being cryopreserved.

2.5 Chromium Release Assay

Target cells were labeled with $^{51}$Cr (Perkin Elmer) at a ratio of 100uCi per $1 \times 10^6$ cells for 1 hour then washed thoroughly. $1 \times 10^4$ labeled target cells for each condition were co-incubated with effector T cells at various effector-to-target (E:T) ratios for 4 hours. Equal volumes of supernatant were transferred to LumaPlates (Perkin Elmer) and a Top Count Reader (Packard) was used to determine $^{51}$Cr activity. Specific lysis was determined by the formula: % lysis = (experimental lysis – spontaneous lysis) / (maximum lysis – spontaneous lysis) x 100. In blocking experiments, effector T cells were incubated for 1 hour with 100 ug/mL chloroquine (Sigma Aldrich) before co-incubation with target cells for 8 hours. Specific lysis was determined as before.

2.6 Bioluminescence Imaging

3 mg D-luciferin (Caliper Life Sciences) was injected into the peritoneum of NSG mouse. Four minutes post injection, mice were anesthetized and imaged for 30 seconds using a Xenogen IVIS Lumina machine (Caliper Life Sciences). Images were analyzed for bioluminescence expressed as
photons/s/cm²/steradian using Living Image Version 4.1 software (Caliper Life Sciences).

2.7 Flow Cytometry

FACS analysis for surface molecule expression was performed on an LSR II Fortessa flow cytometer (BD Biosciences, Hunt Valley, MD) and analyzed using FlowJo. The CD19 CAR was detected using biotinylated Protein L (Thermo Scientific) then conjugated to strepavidin-conjugated fluorophore (Catalog No. 12-4317). The following murine monoclonal antibodies were used for flow-cytometry: CD4 (clone RM4-5), CD8a (clone 53-6.7), CD45.2 (clone 104), CD45.1 (clone A20), PD-1 (clone J43), Tim3 (clone RMT3-23), Lag3 (clone C9B7W), B220 (clone RA3-6B2), CD19 (clone 1D3), CD25 (clone 3C7), and Fixable Viability Dye (Catalog No. 65-0866-18) from Affymetrix eBioscience, Biolegend, Bd Pharmingen, or R&D Systems.

2.8 CD107a Degranulation and Cytokine Assays

CD107a degranulation assays were conducted by co-incubating $5 \times 10^4$ T cells with $1 \times 10^5$ tumor cells that were stained with CellTrace Violet (Life Technologies) previously for 4 h in the presence of 2 μM monensin and 5μl CD107a antibody (clone eBioH4A3, eBioscience), and then evaluated by flow cytometry. Cytokine panel for CAR T cells were evaluated using Mouse V-
Plex Proinflammatory Panel (MR071) performed by core facility at NCI Frederick General Immunology/Cellular and Functional Assays Department. Supernatant was collected from CAR4 and CAR8 co-culture groups with CD3 depleted splenocytes from C57/B6 female mice. T cells were incubated with target cells at 1:1 ratio for 10 hours before supernatants were harvested.

2.9 In Vivo Caspase 3/7 Apoptosis Assay

Ex vivo analysis of bone marrow of leukemia bearing mice. ACK lysed bone marrow cells of biological replicates are stained with protein L for 1 hour followed by 3 separate washes. Cells are then stained with secondary antibodies and washed. Samples are then stained with live/dead staining and washed. Samples are then stained with caspase 3/7 dye CellEvent (Thermo Fisher catalog #C10423) for 25 minutes incubated in 37 Celcius degree incubator. Samples are then immediately analyzed using flow cytometer. All experiments have been repeated at least 4 times in the laboratory.

2.10 T Cell Depleted Bone Marrow Transplantation

Bone marrow cells were harvested from femurs and tibias of female B6 CD45.1+ mice and filtered through a 70-μm nylon filter. Cells are then ACK lysed and T cell depleted using CD3 microbeads (Miltenyi Biotec). Mice prior to transplant were lymphodepleted by 1000 rads (137Cs source) lethal total
body irradiation earlier the same day. Recipient mice were then transplanted with $4.5 \times 10^6$ T cell depleted bone marrow cell in serum free, phenol red free RPMI medium, through retro-orbital injection. Mice were weighed day of transplant and every two days after transplant. Transplanted mice were inoculated with E2aPBx two prior after transplantation. CAR T cells are made according to protocol and injected into BMT mice three weeks following transplant.

2.11 Xenograft and Syngeneic In Vivo Studies

NSG mice 8-12 weeks old were injected with $1 \times 10^6$ NALM6-GL cells via tail vein without preconditioning. After allowing for 3 days for engraftment, confirmed by bioluminescence, animals were injected with either activated but untransduced T cells (Mock) or CD19 CAR T cells from the same donor. Mice were monitored for disease burden by bioluminescence and euthanized at pre-determined endpoints.

B6 CD45.1 mice were injected with $1 \times 10^6$ E2aPbx (CD45.2\textsuperscript{+}) on day 1. Mice were then lymphodepleted by 500 rads total body irradiation (sub-lethal using $^{137}$Cs source) on day 4. On day 5, mice were retro-orbitally injected with $1 \times 10^6$ or $1 \times 10^5$ anti-CD19 CAR T cells from B6, Marilyn (CD45.2\textsuperscript{+}), or Matahari(CD45.2\textsuperscript{+}) mice. Mice in survival experiments were euthanized based on protocols approved by the NCI Bethesda Animal Care and Use
Committee. Takedown experiments were performed on different time points. Detection of CAR T cell was done by flow cytometry on bone marrow or splenocytes by congeneric markers (CD45.2) and Protein L (Life Technologies).

2.12 Microarray

CAR T cells generated from two TCR transgenic mouse strains, Marilyn(CD4) and Matahari(CD8) previously described.(71, 72) biological triplicate cultures of CAR T cells were co-cultured with CD3 depleted splenocytes harvested from, C57/B6 males (CD19+, HY+), C57/B6 females (CD19+, HY-), CD19 KO males (CD19-, HY+), and CD19KO females (CD19-, HY-) for ten hours. T cells are then isolated by mouse Pan T Cell Isolation Kit II(Miltenyi, CAT# 130-095-130). RNA isolated was performed using QIAGEN RNeasy Micro Kit (CAT# 74004). Above groups, plus unstimulated CAR4 or CAR8 T cells were assessed using The Affymetrix GeneChip Mouse Genome 430 2.0 array. The RNA extracts were sent to the microarray core facility in The Frederick National Laboratory for Cancer Research (FNLCR) for labeling and hybridization on microarray chips according to protocols specified by the manufacturers.
2.13 Statistical Analysis of Microarray Data

All statistical analyses were performed within R/Bioconductor statistical environment (www.bioconductor.org). These CEL files were normalized with the Robust Multichip Average methodology (RMA) in R Package Oligo (version 1.34.0). Each affy probe ID in the dataset was matched with the annotation file, GPL1261-14790, which is available from The National Center for Biotechnology Information (NCBI) GEO site. Differential expression p-values were determined with t-test function and false discovery rate (FDR) adjusted using the multtest bioconductor package (version 2.26.0).

2.14 Pathway Analysis and Heatmaps

Statistical results of differentially expression genes were analyzed through the use of QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity, IPA Fall Release, September 2015). Genes with p-values less than p=0.001 were used as input to run IPA core analysis which calculated significant canonical pathways and generated pathway figures. Heatmaps were created in R using the heatmap.2 function in gplots (version 2.17.0).
2.15 Statistics

Statistical tests were performed using GraphPad Prism version 6.0 for MacIntosh (GraphPad Software, San Diego, CA). Significance of survival was calculated using Kaplan-Meier survival curves by Wilcoxon signed-rank test. Significant differences between groups in *in vivo* T cells numbers, *in vivo* marker expressions and *in vitro* assays, were determined by unpaired Student t-test (not significant= p>0.05, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001).
CHAPTER III
RESULTS

3.1 Activation of T Cells Through Chimeric Antigen Receptor

The standard use of xenogeneic murine models is common for the evaluation of CAR activity by human CAR T cells in a heavily immunocompromised host. However, the eventual development of xenogeneic graft-versus-host disease in the mice elicited by the human CAR T cells against mouse tissue causes mortality in the mice. Toxicity of the human CAR T cells prevents the study of the persistence of the CAR T cells due to the shortened life span of the mice. In addition, the immunocompromised state of the NOD/SCID mice do not impart to the study of the biology of the CAR T cells in a normal system, where interactions between all cells in the immune system contributes to the overall immune response against the tumor. In order to study the biology of the CAR T cells, this study used a fully murine anti-CD19 CAR construct. The CAR models a clinical relevant CAR that is currently in clinical trials.\(^{(61)}\) It is an second generation CAR with a CD28 co-stimulatory domain, previously described (Figure 3a).\(^{(73)}\)

We have in our lab a pre-B cell ALL syngeneic murine model (E2aPBX) that has a very similar pathology and gene expression profile to human pre-B
a

![Diagram of molecular components]

CD19 scFv → CD28 Co-stim → CD3ζ

b

![Diagram of experimental timeline]

Leukemia infusion → Day 0 → 500 cGy Sub-lethal irradiation → CART infusion → Day 1 → T cell activation w/ anti-CD28/CD3 beads → Day 2 → Transduction → Day 3 → Remove beads → Day 4 → Day 5

C

![Graph of percent survival]

- 5E5 CART
- Mock
- 500 cGy only

Percent survival

Days
(a) Murine CD19 CAR construct previously described (59) is a second generation anti-CD19 CAR construct with a CD28 co-stimulatory domain. Light and heavy chains of variable binding domain of monoclonal antibody against murine CD19 are linked to form scFv. CAR construct uses CD28 transmembrane domain. (b) Timeline of murine in vivo experiments and CAR T-cell activation and transduction. Mice are injected with $1 \times 10^6$ murine Pre-B cell acute lymphoblastic leukemia (E2aPbx) previously described (67) on day 0. All mice receive 500 cGy irradiation as lymphodepleting regimen on day 3 and receive $1 \times 10^6$ CAR T cells on day 4. CAR T cells are generated from naïve T cell isolated from murine spleenocytes. All T cells are transduced with viral supernatant on days 1 and 2, and cultured with the addition of cytokines IL2 and IL7. Cells are activated with antiCD3/CD28 beads. (c) Murine syngeneic survival model with the use of polyclonal CD19 CAR T-cells against murine ALL, E2aPbx, after 500cGy irradiation as lymphodepleting regimen (n=5 per group). Survival of polyclonal CAR T cell treated leukemia bearing mice compared to leukemia bearing mice treated with GFP transduced T cells (Mock), and leukemia bearing mice that are not treated but irradiated (**p<0.01). Efficacy of polyclonal CAR T cells depends on lymphodepleting regimen. E2aPbx is an aggressive cell line that causes lethality in mice around day 25 post infusion despite irradiation prior to CAR infusion.
ALL, driven by the human gene translocation $E2aPBX1$ (Haiying Qin, unpublished). The E2aPBX cell line is a rapid growing murine ALL that grows in culture and is transplantable to syngeneic mice $\textit{in vivo}$. The leukemic progression mimics human ALL in that it first develops in the bone marrow but then infiltrates into the spleen and blood. Using this model, we have developed a robust syngeneic murine model to study the biology of CAR therapy using a murine anti-CD19 construct previously described$^{15}$. We have been able to recapitulate the curative ability of the anti-CD19 CAR murine model where anti-CD19 CAR T cell treated mice survived months longer than mice treated with GFP transduced T cells (Figure 3c). With the use of flow cytometry, I was able to detect CD19 CAR T cells in the spleen and bone marrow at 30 days post leukemia challenge when mice treated with GFP transduced T cells had already succumbed to disease.

Cancer immunotherapy has long focused on the use CD8 cytotoxic T lymphocytes due to their potent effector activities. Despite the attention on CD8 T cells as the main killer cells, CD4 “helper” T cells have also been found to have cytotoxic effector functions.$^{(74, 75)}$ In this study, we evaluated the killing potential of both CD4 CAR T cells (CAR4) or CD8 CAR T cells (CAR8). Polyclonal CD4 and CD8 T cells were enriched from splenocytes of C57/BL6 mice and separated by magnetic beads. T cells were then subsequently bead activated and transduced with anti-CD19 retroviral supernatant.

Despite the small percentage of CD4 T cells that are cytotoxic, when a CD4 T cell is stimulated through a chimeric antigen receptor, CAR4 cells
demonstrated a CD8-like cytotoxic phenotype with comparable killing efficacy as CAR8 cells. *In vitro* stimulation with E2aPbx cells induced CD107a expression by both CAR8 and CAR4 cells demonstrating degranulation capacity (Figure 4a). CD107a is a marker only expressed on the surface of lytic granules inside T cells. When T cells are activated, the granules fuse with the cell membrane and releases its contents including, cytokines, granzymes and other lytic molecules. During this process, the CD107a could be detected on the surface of the T cells by flow cytometry. The killing potential of murine CAR4 and CAR8 cells were also evaluated by caspase 3/7 assay. Expression of caspase 3/7 on apoptotic tumor cells were evaluated after 4 hour incubation with CAR4 or CAR8 cells. Both T cell types demonstrated equivalent *in vitro* potency against murine ALL (Figure 4b). The cells were then evaluated for their capabilities of cytokine production. Cytokine production upon stimulation with CD19+ murine ALL *in vitro* was more robust by CAR4 than CAR8 cells, with significantly higher amounts of IFNγ, IL2, TNFα, IL6, IL12, and IL4 in cell culture supernatants (Figure 4c). However, CAR8 cells produced significantly more of the immunosuppressive cytokine IL10, than CAR4 cells.
**a**

CD107a

**b**

% Caspase3/7+ cells

E:T Ratio

% Live Tumor

**c**

IFNγ IL2 TNFα IL6 IL12 IL4 IL5 IL1β IL10

TH1 TH2
Figure 4. *(Previous page)*: Murine CAR4 and CAR8 demonstrate comparable in vitro activity and *in vivo* persistence. (a) CAR4 and CAR8 cells demonstrating degranulation post stimulation through CAR receptor. CD107a expression (red) on CAR4 and CAR8 T-cells, after 4-hour incubation with CD19⁺ E2aPbx compared to isotype control of same incubation (grey). (b) Murine CAR4 and CAR8 killing against murine ALL, E2aPbx. Expression of caspase 3/7 on murine CAR4, CAR8 or combined cells following 4 hrs incubation with murine ALL and overall leukemic cell survival measured by cell viability dye, 7AAD. (c) Pro-inflammatory cytokine production of CAR4 and CAR8 cells after 4 hrs incubation with CD19⁺ murine ALL. Supernatants of triplicate co-cultures were harvested and analyzed for release of pro-inflammatory cytokines. CAR4 cells produced significantly increased amounts of IFN-gamma, IL2, TNF-alpha, IL6, II12, II4, II5, and IL1-beta. (*P<0.5, **P<0.01, ***P<0.001, ****P<0.0001) CAR4 cells expressed a Th1 cytokine profile, while CAR8 cells expressed a Tc1 cytokine profile when stimulated through the CAR receptor.
3.2 Syngeneic Murine CAR4 and CAR8 Cells Demonstrate Comparable *In Vivo* Efficacy with Durable Remissions

We next tested the *in vivo* efficacy of CAR4 and CAR8 cells against the eradication of murine acute lymphoblastic leukemia, E2aPbx. $1 \times 10^6$ CAR4 or CAR8 cells were given 4 days after leukemia injection and 1 day after 500 cGy of irradiation (Figure 3b). CAR4 cells alone were effective at maintaining long-term remissions in the mice compared to mice treated with CAR8 alone and mice treated with CAR4 plus CAR8 combination products. (Figure 5a) To evaluate if the efficacy is still comparable at a non-curative dose, mice were treated with $1 \times 10^5$ CAR T cells. At this dose, all three groups still demonstrated comparable efficacy, demonstrating that CAR4 cells alone are capable of eradicating leukemia and induce long-term remission. (Figure 5b)

Next I evaluated whether CD4 cell killing was also perforin dependent as with CD8 cells. Perforin is a cytolytic protein found in the granules released by cytotoxic T cells that introduce holes in the target cells. The open holes in the cell membrane of the target cells then allow for granzyme and cytokines to enter the cell. To evaluate the whether or not perforin plays a part in the killing of CAR4 and CAR8 cells, T cells were generated from perforin KO mice and used to treat leukemia bearing mice. Increased leukemia-induced mortality in mice treated with perforin deficient CAR8 cells compared to CAR4 cells suggests that the *in vivo* activity of CAR8 cells may be more dependent on perforin than CAR4 cells. (Figure 5c-5d)
Syngeneic System

Leukemia bearing B6

a

b

c

d
Figure 5. *(Previous page)*: Leukemia treatment with CAR4 and CAR8 cells *in vivo*. (a) *In vivo* analysis of the survival of leukemia bearing syngeneic mice following infusion of $1 \times 10^6$ CAR4, CAR8, or CAR4+CAR8 (5 x $10^5$ CAR4 plus 5 x $10^5$ CAR8 cells) polyclonal cells demonstrating comparable efficacy of individual products or combined products (n=5 per group). (b) *In vivo* analysis of low dose of $1 \times 10^5$ polyclonal CAR T-cells on day 4 into E2aPbx bearing mice, demonstrating comparable leukemia clearance with CAR4 cells alone at sub-curative dose (n=5 per group). (c) Treatment with polyclonal CAR4/CAR8 cells derived from perforin KO mice or WT B6 mice in WT recipients (n=5 per group).
Further, I evaluated the *in vivo* expansion of the CAR T cell in the recipient mice. I am able to detect the infused CAR T cells in the syngeneic murine system with the use of CD45 congenic mice for CAR T cell generation. Donor and recipient mice are of the same genetic background (C57/Bl6) but differs in the expression of the CD45 antigen. The expression of two different alleles (CD45.1 and CD45.2) allows for the differentiation of donor infused CAR T cells (CD45.2) from the recipient cells (CD45.1) detected through flow cytometry. For analysis of CAR T cell *in vivo*, bone marrows of the mice are harvested and processed. Cells are then stained with cell surface markers and analyzed by flow cytometry. Figure 6 demonstrates the example gating strategy to detect the CAR T cells. Live cells are first gated on with live/dead exclusion dyes. Infused cells are then separated by congenic markers (CD45.2). Of those cells CAR4 cells infused are CD45.2+ and CD4+, CAR8 cells are CD45.2+ and CD8+. From those gates, CAR4 and CAR8 cells can be analyzed for the expression of a variety of markers, such as CAR expression, exhaustion markers, subtype markers, etc.

By flow cytometry, I evaluated the early expansion and persistence of the infused CAR T cells in groups treated with CAR4, CAR8 and CAR4+CAR8 products in a syngeneic system. (from Fig. 5a) Both CAR4 and CAR8 cells can be detected in the bone marrow 7 days post-infusion. Interestingly, in this syngeneic system, when 1:1 mixed CAR4 + CAR8 cells are infused in the presence of CAR antigen only, CAR8 are the dominant population despite infusion of equal numbers of both CAR4 and CAR8 cells.
(Fig. 7), potentially due to a suppressive effects of IL10-producing CD8 cells on CD4 T cells(76, 77).

Figure 6. Flow cytometry gating on ex vivo analysis of CAR T cells in bone marrow. Analysis of CAR T cells ex vivo post infusion and treatment of mice. Bone marrow of mice are harvested and processed. Cells are then stained for cell surface markers and analyzed by flow cytometry. Infused CAR T cells are derived from congenic mice that express CD45.2 positive cells that differ from recipient mice that express CD45.1 positive cells. Live cells are first gated on by negative live/dead exclusion dye, then gated on positive CD45.2 cells to separate from endogenous T cells in the recipient. CAR T cells are further isolated by expression of CD4 and CD8 staining. From here cells are further analyzed by expression of apoptosis markers, CAR markers, etc to evaluate persistence, phenotype etc.
**CAR4**

**CAR8**

**CAR4+CAR8 1:1 mix**

---

**CD4**

---

**CD8**

---

**ns**

****
Figure 7. Murine CAR4 and CAR8 in vivo persistence. (a) Persistence of murine CAR4 and CAR8 cells in bone marrow of syngeneic recipients 14 days post-infusion following conditions identical to those presented for Figure 5a survival (**P<0.01, n=3). (1 X 10^6 CAR4, CAR8 or mixed products (5 X 10^5 CAR4 plus 5 X 10^5 CAR8)). Percentage of CAR4 or CAR8 cells found in the bone marrow of mice receiving CAR4 alone or CAR8 alone are not statistically significant. When equal amounts of CAR4 and CAR8 are co-infused, there are statistically significantly higher percentage of CAR8 than CAR4 in the bone marrow.
3.3 Efficacy of Gender Mismatched Donor CAR T Cells

To evaluate the potential involvement of TCR signaling and its effect on CAR8 cells, we utilized an allogeneic CAR T cell adoptive transfer in this syngeneic murine model by giving polyclonal gender mismatched CAR T cells derived from a female mouse to treat a male mouse. CAR T cells were made from female T cells and infused into either female mice (to resemble autologous infusions) and male mice (allogeneic infusion). After analyzing survival of the mice post infusion, we found significantly lower survival due to poor leukemia clearance in the male mice compared to gender matched CAR T treatment of female mice, which suggests potential TCR distraction and hindrance of CAR functionality in the presence of allogeneic antigens (Figure 8). Only a small percentage of donor female cells should recognize the allogeneic male antigens due to identical genetic background of the two mice. But previous research suggests disproportional expansion of allogeneic cells in the lymphopenic host that could exacerbate the TCR effect.\textsuperscript{(70, 78)} It is unclear is CAR T cells are distracted from trafficking to the site of the leukemia due to the broad expression of TCR antigen in the male mouse that could be recognized by the female CAR T cells. To determine if there is importance in the location specific expression of male antigens, we next performed bone marrow transplants to develop female mouse recipients with male derived bone marrow.
Figure 8. Murine polyclonal CAR T cell treatment with gender mismatched CAR T cells. (a) Survival analysis of syngeneic mice following infusion of $1 \times 10^6$ anti-CD19 CAR T-cells in gender matched recipients (female CART into female recipients) or gender mismatched recipients (female CART into male recipients) (**P<0.01, n=5).
3.4 HY Expression on Hematopoietic Tissues Alone

Following the previous observation, I next evaluated if the TCR distraction is site-specific to hematopoietic tissues where the leukemia resides. To restrict the expression of male antigens to the site of the tumor instead of the whole animal, CAR T cells were made from female or male mice and used to treat recipient mice after bone marrow transplantation (BMT). CD3+ T cell depleted transplants represent multiple conditions of TCR antigen expression, (1) no TCR antigen expression, (Female → Female BMT, [F→F]), (2) broad TCR antigen presentation but not in hematopoietic tissue, (Female → Male BMT, [F→M]), (3) restricted male TCR antigen expression in hematopoietic tissue, (Male→ Female BMT, [M→F]), and (4) additional control with no TCR antigen expression (Male → Male BMT, [M→M]). (Figure 9a) All groups except group (4) are treated with female derived CAR T cells. Group (4) is controlled with autologous transfer of male derived CAR T cells into Male recipients. Results indicate that broad expression of potential male antigen does not significantly impact CAR T cell efficacy (group 2) if no TCR antigen is present in the hematopoietic tissues where the leukemia reside (Figure 9b). As expected, the presence of TCR antigen in the hematopoietic tissues results in poor anti-leukemia responses as shown by the survival of group 3 in Figure 9. Group 3 represents female mice with male bone marrow cells. The presence of TCR antigen in the same tissue as the leukemia
negatively impacts the efficacy of the CAR against the leukemia. It is difficult to discern and isolate which clone of T cells are recognizing the allogeneic antigens, thus to control for TCR specificity we used TCR transgenic mice for future experiments.
a

Group 1
Female

Group 2
Female

Group 3
Female

Group 4
Male

Donor CAR T cells

b

Days

Percent survival

Group 1: BMT F-->F
Group 2: BMT F-->M
Group 3: BMT M-->F
Group 4: BMT M-->M
Group 5: F-F Activated T cells
Figure 9. (Previous page): Male antigen expression restricted to hematopoietic tissues. (a) Adoptively transferred polyclonal CAR T cells were infused into leukemia bearing recipients according to timeline outlined in figure 3, following bone marrow transplants 3 weeks prior. Experimental groups received lethal irradiation (1000 cGy) prior to infusion of CD3 depleted bone marrow cells. (1) (Female → Female BMT, [F→F]), (2) (Female → Male BMT, [F→M]), (3) (Male→ Female BMT, [M→F]), (4) (Male → Male BMT, [M→M]). (5) no transplant, treatment with activated T cells. (b) Survival analysis of female and male recipients after treatment with polyclonal anti-CD19 CAR T cells derived from females (groups 1-3) or males (group 5). (*p<0.05, n=5 per group). Mice are injected with 1 X 10^6 murine Pre-B cell acute lymphoblastic leukemia (E2aPbx) previously described on day 0. All mice receive 500 cGy irradiation as lymphodepleting regimen on day 3 and receive 1 X 10^6 CAR T cells on day 4. All T cells are transduced with viral supernatant on days 1 and 2, and cultured with the addition of IL2 and IL7. Cells are activated with antiCD3/CD28 beads.
3.5 HY-Specific CAR8 Cells Fail to Clear Leukemia in the Presence of TCR Antigen

The discrepancy in the durability of CAR-mediated responses between autologous syngeneic models and allogeneic models where allogeneic male-specific antigens contribute to lower survival suggested a possible effect of endogenous TCR signaling on CAR persistence and functionality. The effects are signified by decreased survival of mice treated by gender mismatched CAR T cells in a syngeneic system. To evaluate CART functionality in the presence of endogenous TCR antigen, I generated CAR4 and CAR8 cells from T cells with known specificity for the male minor histocompatibility antigen, HY, in order to control for both CAR and TCR specificity (Figure 10a). I was able to achieve over 90% transduction efficiency with the use of transgenic T cells in order to develop CAR T cells (Figure 10b).

To determine the impact of endogenous TCR signaling on the efficacy of CART cells in vivo, I adoptively transferred HY-specific CAR4 and CAR8 cells into leukemia-bearing male (HY+, with TCR and CAR antigens present) and female (HY-, CAR antigen only, no TCR antigen) mice (Figure 10c). With the use of CAR8 cells in the female recipients, CAR8 cells performed as expected and eradicated leukemia, which resulted in prolonged survival of mice. However, in the presence of TCR antigen (male recipients), CAR8 cells
completely failed to eradicate leukemia and survival of mice was not better than mice treated with mock T cells. The surprising results, is that in both female and male recipients, HY-specific CAR4 cells eradicated leukemia and the majority of mice survived past 100 days. CAR4 cells were not susceptible to the distraction by signaling through the endogenous TCR, and the CAR receptor was still able to eradicate tumor. (Figure 10d)

To confirm that treatment failure and death of animals were due to leukemia progression. I examined the bone marrow of the mice 7 and 14 days post infusion to look for leukemia burden. I confirmed the presence of increased CD19+ leukemia at early time points (day +7 following adoptive cell transfer, Figure 11a) and later time points (day +14, Figure 11a) in male recipients of HY-specific CAR8 cells. Data shows similar burden of leukemia in mock treated mice as CAR8 treated mice in the presence of TCR antigen.
a

CD8 HY (UTY) TCR transgenic mice

TCR:HY

CAR:CD19

HY-specific CAR8

CD4 HY (DBY) TCR transgenic mice

TCR:HY

CAR:CD19

HY-specific CAR4

b

HY-specific CAR4

HY-specific CAR8

CD19 CAR

Leukemia bearing Male recipient (TCR antigen -)

Leukemia bearing Female recipient (TCR antigen -)

c

HY TCR transgenic mice

CD4+ (DBY)

CD8+ (UTY)

HY+ Male cells

TCR:HY

CAR:CD19

CD19+ Leukemia

Leukemia bearing Male recipient (TCR antigen +)

Leukemia bearing Female recipient (TCR antigen -)

d

HY-specific CAR4 Treated

HY-specific CAR8 Treated

Percent survival

0 30 60 90 120 Days

0 20 40 60 80 100

Percent survival

0 30 60 90 120 Days

0 25 50 75 100

Mock

+ TCR antigen (Male)

+ TCR antigen (Female)
Figure 10. (Previous page): HY-specific, TCR transgenic, syngeneic murine model (a) CAR T-cells are generated from HY-specific CD4⁺(Marilyn) or CD8⁺(Matahari) TCR transgenic mice specific to the male minor histocompatibility antigen, HY. (b) CAR transduction efficiency of the HY-specific T-cells with anti-CD19 CAR construct are over 90% for both CD4 (HY-CAR4) and CD8 (HY-CAR8) CAR T cells. (c) HY-specific CD4⁺ or CD8⁺ T-cells were transduced with CD19/CD28z murine CAR then administered on day 4 to E2aPbx-bearing syngeneic male (HY⁺) and female (HY⁻) recipients. CAR T cells have specificity against HY antigen in male recipients through endogenous TCR and specificity against leukemia in both recipients through CAR receptor. (d) Survival analysis of female (HY⁻) and male (HY⁺) recipients after treatment with HY-specific CAR4 or CAR8 cells. (**p<0.01, n=5 per group). Mice are injected with 1 X 10⁶ murine Pre-B cell acute lymphoblastic leukemia (E2aPbx) previously described on day 0. All mice receive 500 cGy irradiation as lymphodepleting regimen on day 3 and receive 1 X 10⁶ CAR T cells on day 4. CAR T cells are generated from naïve T cell isolated from murine spleenocytes of HY transgenic mice. All T cells are transduced with viral supernatant on days 1 and 2, and cultured with the addition of IL2 and IL7. Cells are activated with antiCD3/CD28 beads.
Leukemia in BM

7 days post infusion

CAR4 → HY-
CAR4 → HY+
CAR8 → HY-
CAR8 → HY+
Mock

CD19
B220

% Leukemia in BM

14 days post infusion

CAR4
CAR8
Mock

TCR antigen

- + - + -
Figure 11. *(Previous page)*: Leukemia burden in male (HY⁺) and female (HY⁻) recipients treated with HY-specific CAR4 and CAR8 cells (a) Leukemia burden in bone marrow, 7 and 14 days post T-cell infusion. (***p<0.001, n=3) (b) Flow cytometry plots demonstrating leukemia (E2aPbx, CD45.2⁺B220⁺CD19⁺) burden in bone marrow of HY⁺(male) or HY⁻ (female) recipients treated with CAR4 or CAR8 products compared to mock treated group demonstrating CAR8 T cell failure in male recipients leading to leukemia progression.
3.6 HY-Specific CAR T Cell Expansion *In Vitro*

I next focused on potential causes of CAR8 failure in the presence of TCR antigen. Enumeration of CAR T cells in the bone marrow seven days post infusion demonstrated decreased HY-specific CAR8 cells in HY⁺ males compared to HY⁻ females. In contrast, TCR activation induced significant HY-specific CAR4 expansion during the early expansion phase. HY-specific CAR4 cells were present in significantly increased numbers in males compared to females (Figure 12a). I next examined if the decreased numbers of CAR8 cells in the presence of TCR antigen is due to apoptosis by measuring the expression of apoptosis markers caspase 3/7. There was no significant increase in caspase 3/7 in the presence of TCR antigen for HY-specific CAR4 cells. However, there was significantly higher expression of caspase 3/7 in HY-specific CAR8 cells in the presence of TCR antigen compared to CAR antigen alone, with or without the presence of CD19⁺ leukemia, which suggested the increase in apoptosis markers caspase 3/7 is a TCR induced effect (Figure 12b).

3.7 CAR Expression of CAR T Cells in the Presence of TCR Antigen

Looking at the expression of CAR on the surface of HY-specific CAR4 and HY-specific CAR8 cells resulted in an interesting observation. The
expression of CAR on infused T cells are not equivalent between male and female recipients. Isolated CAR T cells are stained with biotinylated protein L, an immunoglobulin binding protein that binds to the kappa light chain of the CD19 scFV of the CAR construct. As a secondary antibody, cells are then stained with streptavidin that is couple with a fluorophore for flow detection. HY-specific CAR4 cell expansion was driven through TCR stimulation as seen in Figure 12, however, the presence of TCR antigen induced significant downregulation of CAR on the surface of the T cells (Figure 13). However, due to the rapid expansion, the number of CAR positive cells in males and females are still equivalent in CAR4 cells. The same downregulation was seen also in HY-specific CAR8 cells in the presence of TCR stimulation in the male recipients.
**Figure a**

- **Legend:**
  - ns
  - **
  - ****

- **Graph:**
  - % CART in BM 7 days post infusion
  - TCR antigen
  - CAR4
  - CAR8

**Figure b**

- **Legend:**
  - ****

- **Graph:**
  - Caspase 3/7 cells Fold Change (male vs female recipients)
  - Leukemia
  - CAR4
  - CAR8

**Table:**

- Car4
- Car8
- Tcr antigen
- CAR4
- CAR8
- CD45.2
- CD4' or CD8

**Diagram:**

- Single product infusion
- CAR4
- CAR8
- CD4 or CD8
- CD45.2

- F→F
- F→M
Figure 12. (Previous Page): CAR T cell expansion and apoptosis in the presence of TCR or no TCR antigen. *Ex vivo* analysis of the bone marrow of leukemia bearing mice groups (n=5) treated with HY-specific CAR4 or CAR8 cells 7 days post infusion. (a) CAR T-cell persistence in female (HY-) and male (HY+) recipients (**p<0.01, ***p<0.001), plus flow cytometry plot demonstrating the infused CAR T cells in the bone marrow. (b) Analysis of apoptosis of CAR4 or CAR 8 cells in male versus female recipients. Cleavable detection agent provides substrate for active caspase 3/7 in the cell. Fluorescent tag is released when substrate is cleaved to allow detection by flow cytometry. Data shows fold change in caspase 3/7 expression in CAR4 and CAR8 cells in bone marrow of female versus male recipients in the presence of TCR antigen (CD19⁺ HY⁺) compared to no TCR antigen (CD19⁺ HY⁻) (****p<0.0001). Data also compares same groups but in recipients without leukemia demonstrating the effects on apoptosis with only TCR antigen present. (****p<0.0001).
HY-specific CAR4

HY-specific CAR8

**ns**

** CAR MFI**

TCR antigen

- +

CAR4

CAR8

- +

** CAR MFI**

TCR antigen

- +

CAR4

CAR8
Figure 13. (Previous Page): CAR expression on HY-specific CAR T cells in vivo. Ex vivo analysis of the bone marrow of leukemia bearing mice groups (n=5) treated with HY-specific CAR4 or CAR8 cells 7 days post infusion. (a) Histogram of CAR expression of HY-specific CAR4 and HY-specific CAR8 cells. Female recipients without TCR antigen (red) are compared to male recipients that are HY+ (blue). (b) Bar graph represents CAR MFI of 5 recipients per group. (**P<0.01)
3.8 HY-Specific CAR T Cell Exhaustion in the Presence of TCR Antigen

The deletion of CAR8 cells in the presence of TCR antigen could be due to the over stimulation by TCR activation and the cells could be exhausted. T cell exhaustion was first described in the setting of chronic viral infections where the constant stimulation of antigen during a chronic infection induces loss of T cell effector function. The exhaustion phenotype is characterized by the expression of inhibitory receptors such as PD-1, Tim3, and Lag3. Eventually the cells lose effector function and apoptose. (79, 80)

I evaluated the expression of PD-1, Tim3, and Lag3 on CAR4 and CAR8 cells in the bone marrow. HY-specific CAR4 cells expressed comparable amounts of PD-1 in both male (HY+) and female (HY-) recipients. However, in the presence of both TCR and CAR antigens in male recipients, HY-specific CAR8 expressed significantly higher amounts of PD-1 compared to HY-negative female recipients (Figure 14a). There was no significant difference between PD-1 levels between CAR4 and CAR8 cells in female recipients (HY-). In addition, HY-specific CAR8 cells expressed significantly higher amounts of other exhaustion markers, Tim3 and Lag3 in male recipients (HY+) compared to female recipients (HY-) (Figure 12b-c). In contrast, HY-specific CAR4 cells expressed significantly lower amounts of Tim3 and Lag3 in the presence of TCR and CAR antigen compared to CAR antigen alone (Figure 14b-c) which suggest a potential protective effect of TCR stimulation on the exhaustion profile of CAR4 cells.
In addition to being an exhaustion marker, PD-1 is also considered an activation marker for T cell activation and has been used as a marker to isolate tumor antigen reactive T cells for immunotherapy.\(^{(81, 82)}\) Use of congenic markers allows for the analysis of the endogenous T cells in the recipient and the infused CAR T cells separately. In female recipients both HY-specific CAR4 and CAR8 cells (CD45.2\(^+\)) expressed higher levels of PD-1 compared to endogenous T cells (CD45.1\(^+\)), demonstrating a level of PD-1 expression through activation of the CAR (Figure 15). However, when CAR8 cells are stimulated through the TCR and the CAR, the PD-1 level is almost four fold higher than in a female recipient with only CAR stimulation.

Taken together, these results demonstrate that simultaneous CAR and TCR stimulation on CAR8 cells induces increased expression of exhaustion markers and may limit expansion through increased apoptosis resulting in diminished \textit{in vivo} activity whereas this effect was not seen in CAR4 cells receiving dual stimulation.
Figure 14. (Previous page): Exhaustion phenotype of HY-specific CAR4 and CAR8 in the presence of TCR and CAR antigens. 

Ex vivo analysis of the bone marrow of leukemia bearing mice groups (n=5) treated with HY-specific CAR4 or CAR8 cells 7 days post infusion. (a) histogram of PD-1 expression by flow cytometry. Red=female recipients without TCR antigen HY, Blue= male recipients with TCR antigen HY. PD-1 MFI of CAR4 and CAR8 cells between male (HY⁺) and female (HY⁻) recipients represented by dot plot. (b) Percent Lag3 positive CAR4 or CAR8 cells in bone marrow and (d) percent Tim3 positive CAR4 or CAR8 cells in bone marrow of treated mice. (ns=P>0.05, **=P<0.01, ***=P<0.001, ****=P<0.001)
**a**

CD4

PD-1

CD4.2

ENDOGENOUS CD4 T CELLS (CD45.1+)

INFUSED CAR4 CELLS (CD45.2+)

**b**

CD8

PD-1

CD4.2

ENDOGENOUS CD8 T CELLS (CD45.1+)

INFUSED CAR8 CELLS (CD45.2+)
Figure 15. *(Previous page)*: PD-1 expression on infused CART and endogenous T-cells in the same recipient. (a) Infused CAR T-cells (CD4⁺/CD8⁺, CD45.2⁺) in the bone marrow are CAR positive 7 days post infusion. Endogenous T cells represented in left box on flow plot (CD4/CD8 positive, CD45.2 negative), infused CAR T cells represented in right box. (b) PD-1 expression of HY-specific CAR T-cells (CD45.2⁺) in the presence of CAR antigen or both CAR and TCR antigens compared to endogenous T-cells in the same recipient (CD45.2⁻).
3.9 Combination Treatment of Murine CAR4 and CAR8 T Cells Results in Partial Rescue of Exhausted Phenotype of CAR8 Cells in the Presence of TCR Antigen

To determine if poor efficacy of CAR8 cells in the presence of both TCR and CAR antigens could be overcome by co-administration of CAR4 cells, we infused equal numbers of HY-specific CAR4 and CAR8 cells into male and female leukemia bearing mice. At day 7 the increased expansion of CAR4 cells in the presence of TCR antigen seen when CAR4 cells were given alone was preserved when CAR4 cells were co-infused with CAR8 cells (Figure 16). Co-infusion of CAR4 cells improved expansion of CAR8 cells in the presence of TCR antigen compared to CAR8 cells alone (Figure 16 vs Figure 12a). Furthermore, co-infusion of CAR4 cells partially lowered the overexpression of PD1 and Tim3, but not Lag3 (Figure 17a-17c) seen on CAR8 cells in the presence of TCR and CAR antigens. This was associated with significantly reduced leukemic bone marrow infiltration when CAR8 cells were given with CAR4 cells to leukemia-bearing HY⁺ male recipients compared to CAR8 cells given alone (Figure 17d). Thus, that the negative effects of TCR signaling on CAR8 cells can be mitigated by the presence of CAR4 cells resulting in improved expansion and reduced expression of exhaustion markers on CAR8 cells, which enabled early leukemia clearance.
Figure 16: Co-infusion of HY-specific CAR4 and CAR8 cells. (a)

Analysis of bone marrow CAR T-cells 7 days post infusion of CAR4 or CAR8 alone treated mice (single product infusion) compared to CAR4 plus CAR8 combination treated mice (5 x 10^5 CAR4 plus 5 x 10^5 CAR8) in the presence of TCR antigen (*p<0.05, **p<0.01, n=5).
Figure 17. *(Previous page)*: Co-infusion of HY-specific CAR4 and CAR8 cells improves CAR8 persistence and exhaustion. Analysis of bone marrow CAR T-cells by flow cytometry 7 days post infusion of combination treated mice (5 x 10^5 CAR4 plus 5 x 10^6 CAR8) in the presence of TCR antigen compared to CAR4 or CAR8 single treatment. (a) PD-1 MFI expression of single infused CAR4 or CAR8 cells compared to CAR8 cells in the presence of CAR4 cells. (b) percent Tim3 positive cells in bone marrow, and (c) percent Lag3 positive CAR4 or CAR8 cells in bone marrow compared between co-infusion or single infusion. (d) Leukemia burden of combination treated mice compared to single product. Infused leukemia characterized by expression of CD45.2 positive, B220 positive and CD19 positive. (Unpaired T test: ns=P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)
3.10 Long-Term Effects of TCR Engagement in CD4 CAR T Cells

During the first two weeks after infusion, TCR stimulation increased proliferation of CAR4 cells with decreased Lag3 and TIM3 expression and no elevation of PD-1 expression compared to CAR8 cells resulting in improved persistence and long term survival suggesting that the negative effects of TCR signaling on CAR4 cells was less pronounced. I next assessed the status of persistent CAR4 cells in TCR antigen-expressing hosts at a later time point after adoptive transfer. The percentage of HY-specific CAR4 cells in HY⁺ male recipients 60 days following clearance of leukemia (Figure 18a) was significantly lower than in HY⁻ female recipients. In addition, CAR4 cells also expressed increased caspase 3/7 (Figure 18b) and significantly higher PD-1 (Figure 18c) in male recipients at day 60 compared to female recipients. This suggests that the negative effect of the TCR stimulation on CART cells also affects CAR4 cells but this occurs later than for CAR8 cells and does not prevent early anti-leukemic activity. However, these findings have implications for sustained protection against leukemia recurrence.
With Leukemia

No Leukemia

**PD-1 MFI**

- TCR antigen
- CAR4 (CD45.2+)

<table>
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<tr>
<th>TCR antigen</th>
<th>Leukemia</th>
<th>CAR4 in Bone Marrow</th>
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<tbody>
<tr>
<td>-</td>
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**Endogenous CD4 (CD5.1+)**

- + TCR antigen CAR4 (CD5.2+)
- - TCR antigen CAR4 (CD5.2+)

**% CAR4 cells**

- TCR antigen
- Leukemia

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<th>TCR antigen</th>
<th>Leukemia</th>
<th>CAR4 in Bone Marrow</th>
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**% Caspase 3/7 + CAR4**

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**With Leukemia**

**No Leukemia**

<table>
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<th>Leukemia</th>
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**PD-1**

- Endogenous CD4 (CD5.1+)
- + TCR antigen CAR4 (CD5.2+)
- - TCR antigen CAR4 (CD5.2+)
Figure 18. (Previous page): Presence of TCR antigen reduces long-term persistence of CAR4 cells. (a) Percentage of CAR4 cells in bone marrow of male (HY⁺) and female (HY⁻) recipients at day 60 post infusion (*p<0.05, n=5). (b) Percent of caspase 3/7 positive CAR4 cells in bone marrow of recipients at day 60 with or without leukemia compared to endogenous CD4 T-cells (CD45.1⁺) in the same male recipient (*p<0.05, **p<0.01). (c) PD-1 expression of CAR4 cells in male (HY⁺) compared to female (HY⁻) recipients with or without leukemia present at day 60(***p<0.001, ****p<0.0001).
3.11 Gene Expression of CAR4 or CAR8 Cells Stimulated Through TCR, CAR or Both Receptors

To better understand the differences between the in vivo biology of CAR4 and CAR8 cells, we compared the activation profiles of HY-specific CAR4 and HY-specific CAR8 cells 10 hours following CAR (CD3-depleted female splenocytes containing CD19⁺ B cells) TCR (CD3-depleted male splenocytes from CD19-deficient mice) vs CAR + TCR (CD3-depleted male splenocytes) stimulation in vitro (Figure 18).

Analysis of cytokines in cell culture supernatants of co-culture used for microarray analysis confirmed activation of cells through TCR, CAR, or TCR+CAR stimulation and confirmed marked differences between CAR4 and CAR8 cells (Figure 20) demonstrating that CAR4 cells are more robust producers of cytokines with the exception of IL-10 that was primarily produced by CAR8 cells.
a

T cell depleted splenocytes (B cells, DCs, other antigen presenting cells) + CD4 or CD8 CAR T cells

Pan T cell isolation

RNA isolation

b

<table>
<thead>
<tr>
<th>HY-Specific CART cells</th>
<th>Splenocyte Stimulation</th>
<th>TCR (HY)</th>
<th>CAR (CD19)</th>
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<td>CAR4</td>
<td>CD19 KO Male</td>
<td>+</td>
<td>-</td>
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<td></td>
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Figure 19. (*Previous page*) *in vitro* stimulation of CAR4 and CAR8 through TCR, CAR, or both, for microarray and cytokine analysis. (a) Experiment setup of *in vitro* stimulation for cytokine release assay and microarray. CAR T cells generated from two TCR transgenic mouse strains, Marilyn(CD4) and Matahari(CD8) were stimulated through CAR, TCR, both or neither receptors. Biological triplicate cultures of CAR T cells were co-cultured with CD3 depleted splenocytes harvested from, C57/B6 males (CD19⁺, HY⁺), C57/B6 females (CD19⁺, HY⁻), CD19 KO males (CD19⁻, HY⁺), and CD19KO females (CD19⁻, HY⁻) for ten hours. Supernatant was collected for cytokine analysis. CAR T cells are then separated from splenocytes by untouched mouse Pan T Cell Isolation Kit II. RNA isolation was performed using QIAGEN RNeasy Micro Kit (CAT# 74004) and sent for microarray analysis. Above groups, plus unstimulated CAR4 or CAR8 T cells were assessed using The Affymetrix GeneChip Mouse Genome 430 2.0 array. (b) CAR T-cells are stimulated with CD3 depleted splenocytes from different CD19/HY expressing donors in three biological triplicates.
Figure 20. (Previous page): *in vitro* CAR4 and CAR8 cytokine profile when stimulated through TCR, CAR or both. (a) Experiment setup of *in vitro* stimulation for cytokine release assay and microarray (Fig 17). CAR T-cells are stimulated with CD3 depleted splenocytes from different CD19/HY expressing donors in three biological triplicates. Cytokine levels *in vitro* of CAR4 or CAR8 cells post 10-hour stimulation through TCR, CAR, or both receptors, demonstrating CAR T-cell activation.
We next assessed the global gene expression of CAR4 and CAR8 cells following \textit{in vitro} stimulation. Principal component analysis demonstrated that CAR stimulation resulted in a more significant change in CAR4 cells than CAR8 cells compared to anti-CD3/CD28 bead expanded cells incubated with CD19^−/HY^− CD3 depleted splenocytes (Figure 21). Furthermore, CAR4 and CAR8 cells displayed expression profiles distinct from one another. CAR8 cells stimulated through both the endogenous TCR and CAR receptor expressed a very similar profile to CAR8 cells stimulated through the endogenous TCR alone suggesting a TCR dominant effect in the presence of both TCR and CAR antigens (Figure 21b).

Gene set pathway analysis through Ingenuity demonstrated a difference in apoptotic genes (P<0.001 cutoff on gene list) that differed between CAR4 and CAR8 cells supporting the difference seen in \textit{in vivo} data. CAR8 apoptotic profile differed significantly from CAR4 profile. There is an increase expression in pro-apoptotic genes in CAR8 cells (Havcr2 (Tim3), Pdcd1, Pawr) stimulated through the CAR over CAR4 cells stimulated through CAR. Despite an increased expression of anti-apoptotic genes, Bag3, Tnfap8, and Aatf in CAR8 cells stimulated through the TCR, there was an over expression of pro-apoptotic genes, Casp3, Pdcd1, Havcr2, Apitd1, BAK1, BIK in CAR8 cells stimulated through the TCR, or TCR+CAR. The overall expression demonstrated a difference between CAR4 and CAR8 cells but a distinct similarity between CAR8 cells stimulated through TCR and TCR+CAR (Figure 22).
Comparisons of the relative expression of T cell activation genes resulted in different clustering of all groups. Data suggested differential T cell activation through CAR receptor in both CAR8 and CAR4 cells (Figure 23). Pathway analysis of T cell activation through the CAR suggests that the downstream activation of CAR stimulation is similar to TCR stimulation but without TCR involvement. CAR4 cells stimulated through the CAR receptor induced significantly higher expression of ICOS, which augments in vivo persistence and effector function\(^{(83, 84)}\), compared to CAR8 cells (Figure 22). The stimulation through the TCR also resulted in more differentiated global gene set compared through CAR stimulation between CAR4 and CAR8 cells. Suggesting a more similar global transcriptional profile between CAR4 and CAR8 cells when stimulated through CAR (Figure 24). CAR and TCR stimulation profile differed between CAR8 and CAR4 cells in many genes involved in persistence and function (Gzma, Eomes, Ras2, Irf4, Jun, Fos), homing and migration, metabolism, and inhibition, suggesting a difference in the downstream activation of the two subsets of T cells, although the result also displays a profile very similar between CAR8 cells stimulated through TCR or both receptors (Figure 21). Data demonstrates that activation of CD4 or CD8 T cells differ significantly through TCR stimulation and also simultaneous CAR stimulation, that leads to differences in apoptosis, T cell activation and potentially other downstream effects.
Figure 21: Gene expression profile of CAR4 and CAR8 cells stimulated through CAR, TCR or both receptors. (a) Dendrogram and (b) Principal Component Analysis of global transcriptional profile of three biological replicates of CAR4 and CAR8 cells evaluated 10 hours after initial stimulation with CD3 depleted splenocytes detailed figure 17.
Figure 22. (Previous page): Gene expression profile of CAR4 and CAR8 cells stimulated through CAR, TCR or both receptors. (a) (b) Heatmap of z-score value of apoptotic, homing and migration, metabolism, effector, activation, and inhibitory receptor genes(80) compared between CAR4 and CAR8 cells stimulated through CAR, TCR or both receptors.
CAR4 T cell activation
b

CAR8 T cell activation

T Cell Receptor Signaling
Figure 23. (Previous pages): T cell activation pathway through CAR stimulation. Pathway analysis with use of Ingenuity Pathway Analysis software demonstrating the genes upregulated in the T cell activation pathway when (a) CAR4 or (b) CAR8 cells are activated through the CAR receptor. Pathway shows genes normally involved in T cell activation through the T cell receptor (grey) and overlaid are active genes when T cell is activated through chimeric antigen receptor (red).
Figure 24. (Previous page): Volcano plots of (a) global transcriptional profile of CAR4 cells stimulated through the CAR versus CAR8 cell stimulated through the CAR plotted by Pvalue versus fold change. (b) Global transcriptional profile comparison between CAR4 cells stimulated through TCR versus CAR8 cells stimulated through TCR. Colored dots represent differentially expressed genes between CAR4 and CAR8 cells at predetermined cutoff (i.e. fold change (fc) = ± 3, -log10 (P value) = 5.
3.12 Human CAR4 Cells Provide Superior Long-Term in Vivo Leukemic Control Compared to CAR8 Cells

In collaboration with Dr. Daniel W. Lee who provided data on the evaluation of human CAR4 and CAR8 cells, we evaluate the comparative efficacy of human CAR4 and CAR8 cells, we used a second-generation anti-CD19 CAR with a CD3ζ signaling domain and CD28 co-stimulatory domain that induced remission in 70% of patients with refractory B cell ALL when infused as a mixed CAR4/CAR8 product.\(^6\) Cytotoxicity of CAR4 cells in vitro against the pre-B cell ALL cell lines NALM6, KOPN8 and REH was comparable to CAR8 cells (Fig. 25a). Declines in the lytic capacity of both CAR4 and CAR8 cells in the presence of chloroquine suggest both subsets, at least partially, utilize the perforin pathway for target killing, which correlates with murine perforin data (Fig. 25b). We next evaluated the in vivo efficacy of CAR4, CAR8 and 1:1 mixed CAR4+CAR8 cells. Despite faster leukemia clearance of luciferase-expressing NALM6 by CAR8 compared to CAR4 at early time points (median time to clear, 2 days vs 4 days, Fig. 24), relapse was seen in 2/5 of the CAR8 treated mice on day 25 while mice treated with CAR4 cells or mixed CAR4 + CAR8 cells survived and remained disease free (Fig. 26a). The long-term survival advantage of CAR4 treated animals was associated with persistence of CAR4 cells in peripheral blood on day 55. Furthermore, in animals treated with mixed CAR4 + CAR8 cells, only the
CAR4 cells remained detectable at late time points (Fig. 26c). Thus, CAR4 cells demonstrate comparable cytotoxic activity to CAR8 cells and mediate durable remission *in vivo* which is associated with longer persistence. The development of T-cell-mediated xenogeneic GVHD impacts the *in vivo* biology of CAR T-cells in xenograft models. The lowered survival of CAR8 cells in the human xenograft models is most likely due to the presence of xenogeneic antigens that is detected by the endogenous T cell receptors of the CAR T cells. This data validates that the murine data, that activation of the TCR negatively impacts the efficacy of CAR therapy especially with the use of CAR8 cells.
Figure 25. (Previous page): Human CAR4 and CAR8 cells demonstrate comparable cytolytic activity but CAR4 have superior in vivo persistence in murine xenograft model (a) In vitro $^{51}$Cr release assay evaluating the cytolytic activity of mock or CD19-CD28z transduced human CD4 and CD8 CAR T-cells against human ALL cell lines, KOPN8, REH, and NALM6. (b) In vitro $^{51}$Cr release assay against NALM6 for both CAR4 and CAR8 cells with the addition of chloroquine (CHQ) to evaluate perforin-dependent killing. (c) In vivo treatment with $5 \times 10^6$ total human CAR4 or CAR8 only products, or $2.5 \times 10^6$ CAR4 plus $2.5 \times 10^6$ CAR8 combination treatment (**P<0.01, n=5 per group). (data from collaboration with Dr. Daniel Lee and his students Tasha Lin and Elizabeth Gardner.)
Figure 26: Human CAR4, CAR8 or combination cell treatment *in vivo*

(a) Bioluminescence images from mice inoculated with $5 \times 10^6$ human CAR4, CAR8, or 1:1 mixed products ($2.5 \times 10^6$ CAR4 + $2.5 \times 10^6$ CAR8) in xenograft model against human ALL, NALM6. (b) Relapse of CAR8 treated mice due to increase in luciferase positive leukemia. (c) Flow cytometry analysis of CAR T-cells in bone marrow showed presence of only CAR4 cells in mice treated with CAR4 only or 1:1 ($2.5 \times 10^6$ CAR4 + $2.5 \times 10^6$ CAR8) CAR4:CAR8 treated mice on day 55. (data from collaboration with Dr. Daniel Lee and his students Tasha Lin and Elizabeth Gardner.)
CHAPTER IV
DISCUSSION

T-cells expressing chimeric antigen receptors targeting the CD19 protein on the surface of B-lymphoid malignancies have demonstrated potent activity in patients resistant to standard chemotherapy.\(^{(5, 60, 61)}\) Based on the high remission induction rates, one of the current goals of CAR T-cell therapy is to improve response rates and evaluate approaches to sustain long-term remission. A major challenge is to determine to what extent the \textit{in vivo} biology of CAR-expressing T-cells can be extrapolated from our understanding of T-cell biology induced by T-cell receptor stimulation. There will likely be substantial differences between CAR- and TCR- stimulated T-cells given the artificial nature of the CAR construct. Indeed, a number of studies suggest this\(^{(85, 86)}\) but are limited as they were performed \textit{in vitro} or relied on the study of human T-cells in immunodeficient mice where xenogeneic GVHD may confound results. Using a syngeneic murine model, we studied the \textit{in vivo} behavior of CD19-targeted CAR T-cells in the presence of B-cell ALL and identified important differences between CD4 CAR T-cells and CD8 CAR T-cells that were particularly revealed in the presence of endogenous T-cell receptor stimulation. These models not only allowed for the study of \textit{in vivo} CAR T-cell biology but are also relevant in ongoing clinical CAR protocols, including the use of allogeneic CAR T-cells\(^{(10, 66, 87)}\) where
TCR reactivity is more likely. The importance of CD4 T-cells to an effective *in vivo* immune response has been well described\(^{(68, 88–91)}\). Although cancer immunotherapy has focused mostly on cytolytic effects of CD8\(^+\) T-cells\(^{(92, 93)}\) and CD4 T-cells have been considered primarily as cytokine producing helper cells, there have been reports of CD4 T-cells mediating cytotoxicity through effector pathways typically ascribed to CD8 T-cells\(^{(68, 74, 94, 95)}\). In our studies, we found that one of the features of CD4 T-cells equipped with a chimeric antigen receptor was evidence of target killing using perforin/granzyme pathways with retention of robust cytokine production. Collectively, the data we present from both human and murine CAR T-cells suggest that CAR4 cells can utilize cytolytic pathways but that *in vivo* leukemia clearance is not dependent on perforin, which is a potential advantage of CAR4 cells over CAR8 cells. Indeed, in all of the experiments described here, CAR4 cells were equally effective at clearing leukemia as CAR8 cells. CAR4 cells demonstrated slower kinetics of leukemia clearance in the xenograft model, suggesting that CAR8 cells may be important initially, particularly in the setting of high-burden or rapidly progressing malignancy.

Although published data is lacking thus far, a general concept that is emerging, especially for ALL, is that durability of remission may require CAR persistence. Such persistence has been considered a benefit of immune-based therapy where the potential for a memory response against a malignancy exists. It is unclear whether the principles of memory T-cell
generation in the context of TCR stimulation translates to CAR-expanded T cells. Our data demonstrate an advantage of CAR4 cells in their capacity to persist in vivo compared to CAR8 cells, suggesting the potential for enhanced tumor memory response. Importantly, this finding was true for both the xenograft model and the syngeneic model where immune biology may be less affected by xenogeneic reactivity.

The reduced functionality of CAR8 cells in the presence of TCR antigen is suggestive of the negative effects of hyperactivation and deletion that has been previously described for CD8 T-cells, particularly in the allogeneic setting. Furthermore, we found that CAR8 cells were more likely than CAR4 cells to display features typical of exhausted T-cells in vivo in the context of TCR and CAR stimulation, including expression of negative regulatory receptors and reduced IL-2 production. These findings were further supported by the gene expression profiles of CAR8 cells. Interestingly, the gene expression data suggests that CAR4 cells stimulated through the CAR are more protected against exhaustion and apoptosis than CAR8 cells. This is particularly evident when CAR and TCR stimulation occurred concurrently. Nevertheless, TCR stimulation contributed to reduced long-term persistence of CAR4 cells. Finally, the infusion of CAR4 cells with CAR8 cells was able to partially restore the reduced recovery of CAR8 cells early during expansion in hosts where both CAR and TCR antigens were present. Taken together, the data suggest there may be advantages for CAR4 cells over
CAR8 cells despite the more potent immediate cytolytic activity of CAR8 cells shown in the xenograft model.

The major limitation of these studies is the extent to which our findings in the murine syngeneic model will translate directly to humans. The majority of the preclinical development of CAR T-cell therapy has occurred with the use of xenograft models that allowed for in vivo experiments using human T cells and tumor cells. However, these studies preclude careful assessment of in vivo CAR T cell biology due to the use of immunocompromised hosts and the presence of xenogeneic antigens. The murine TCR transgenic system used in this study is well established in the assessment of TCR biology (74, 97) and allows for the incorporation of antigen-positive and antigen-negative hosts in order to study the long-term effects of TCR activation with cognate antigen in an in vivo environment. Our model uses a relevant leukemia target (CD19), relevant tumor model (pre-B cell acute lymphoblastic leukemia), and non-cross reactive HY transgenic T cells, allowing for the precise study of CAR-mediated T-cell behavior in the presence of TCR or CAR antigens. Nonetheless, these findings will need to be validated in humans, likely in the context of clinical trials.

The primary intent of this work was to study CAR T-cell biology in vivo in a clinically relevant murine model with the use of CAR T-cells with known TCR specificity. This study demonstrates that the endogenous TCR is an active receptor on all infused CAR T-cells and can affect the functionality of the T-cells independent of the CAR. These findings have direct implications to
the use of allogeneic and viral specific CAR T-cell already in clinical trials, and predict that the use of CAR4 cells, which have lower susceptibility to the negative effects of TCR activation, could be beneficial in such settings. Furthermore, there is enthusiasm to develop an “off-the-shelf” CAR T-cell product, which includes eliminating the TCR to avoid complications of alloreactivity. However, T cell receptors may dimerize with CAR receptors (45) and contribute to proper CAR signaling. Although our results would support such a TCR-deficient CAR T Cell, further studies will be required to better understand the implications of this approach.

Clinical trials have thus far have established that CAR therapy can be very effective at inducing remissions in refractory leukemia. It is currently assumed that persistence of CAR T cells and generation of immunologic memory will be critical to establishing long term remissions needed for cure. This work exposes novel aspects of CAR T cell biology, with broad implications regarding T cell activation and persistence in the setting of CAR-and/or TCR-induced T-cell activation, with important implications for the optimization of CAR therapy in patients. In particular, these results illustrate the need for strategies to enhance CAR T-cells using exogenous factors or modifications to CAR design that may better take advantage of T-cell biology that may include the incorporation of additional downstream activation domains to booster T cell activation and persistence while minimizing T cell exhaustion or apoptosis.
CHAPTER V

Challenges and Opportunities of Allogeneic Donor-Derived CAR T Cells

Adapted from publication:


With kind permission from Lippincott Williams & Wilkins
5.1 Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been used as an effective treatment against acute leukemia since the 1970s (30, 31, 98). It is now well established that the efficacy of allo-HSCT is also partly dependent on the anti-leukemic effect derived from the allogeneic cells from the donor graft, also referred to as the graft-versus-leukemia (GVL). The majority of the GVL effect is mediated by donor T cells contained in the allograft (30) but is also seen following delayed infusion of donor T cells (donor lymphocyte infusion, DLI), which have the potential of inducing remission of hematopoietic malignancies that has relapsed or persists after allo-HSCT (99–102). The desired GVL effect from donor T cells is frequently countered by the adverse effect associated with the allogeneic T cell response against normal tissue, termed graft-versus-host (GVH) response. Further, the potency of the GVL effect is often not enough for complete eradication of malignancy, particularly in the case of lymphoid malignancies (102–103). Several methods have attempted to intensify the GVL effects of allo-HSCT while minimizing the effects of GVH in order to lower toxicity and improve the outcome of treatment with varying degrees of success (105). Genetically engineered T cells expressing receptors to redirected specificity toward antigens expressed on malignant cells have the potential to generate a very specific GVL response.
Chimeric antigen receptors (CARs) are fusion constructs composed of the variable binding domain of a monoclonal antibody with the activation domain of the T cell receptor, with additional co-stimulatory domains of T cell signaling (i.e. CD28, 4-1BB, Ox40), in second and third generation CARs (3, 106, 107). CARs allow for the redirection and activation of effector T cells towards any cell surface molecule upon binding by the antibody derived receptor, and are independent of MHC restriction (106, 107). Arming T cells with a CAR against CD19 has been very successful in recent trials in the treatment of B cell malignancies (5, 6, 59, 60, 62, 108, 109). In B-lineage acute lymphoblastic leukemia (B-ALL), remission rates of heavily pre-treated, relapsed/refractory patients reach as high as 70-90% after anti-CD19 CART treatment (5, 6, 62). A significant proportion of patients receiving CAR T therapy have received an allo-HSCT, thus for CAR treatment, the T cells collected for CAR T cell production are derived from the allograft. Despite this, GVHD has not been observed in the patients following CAR infusion, perhaps since the cells have been tolerized to the transplant recipient prior to collection. Despite the lack of GVHD present in the allo-HSCT recipients, the responses of post allo-HSCT patients appears to be inferior to those in patients receiving autologous CAR T cells (6). The discrepancy in response may be attributed to the quantitatively lower or qualitatively inferior lymphocytes at the time of collection resulting from prior chemotherapy regimens, or the qualitative defects of reconstituted allogeneic T cells in the recipient, or both (110–112). A potential opportunity associated with the
alloHSCT platform is the potential to use “healthy” T cells collected from the donor.

Although CAR T cells are often described as having redirected specificity through the CAR receptor (i.e. toward CD19), with the presence of the endogenous T cell receptor (TCR), it is perhaps more appropriately described as additional specificity. Following alloHSCT, the precursor frequency of T cells specific for allogeneic antigen (i.e. tumor antigens, normal antigen, viral antigens) has the potential to be relatively high, particularly if donor-derived T cells are used since these T cells will not have been tolerized in the recipient. In the presence of both TCR and CAR antigens presented to the CAR T cell, it is not known which receptor will be dominant and how signaling through the endogenous TCR affects the efficacy of the genetically engineered CAR receptor function. Further, there is the possibility of a bystander effect in which the alloreactive T cells may impact the function of T cells that do not possess specificity for alloantigens(113, 114) but do express the CAR. Thus, the use of allogeneic CAR T cells, particularly healthy donor derived CAR T cells, requires an understanding of CAR T cell function in the allogeneic setting.
5.2 Donor Derived CARs in the Clinic

Human CAR T cells are known to cause xenogeneic GVHD in immunocompromised mice\(^{115, 116}\). However, preclinical experiments suggest that the use of donor derived allogeneic CAR T cells in immunocompetent mouse models are safe, as demonstrated by low GVHD rates\(^{117}\). Currently, there is a paucity of clinical reports in the literature addressing the risks of GVHD and the activity of donor derived CAR T cells. Kochenderfer et al had reported a small cohort of patients receiving donor derived CD19-CAR T cells for relapsed hematologic malignancies following allo-HSCT. All patients had at least 1 prior DLI, with either no GVHD, grade 1 acute GVHD, or mild chronic GVHD. There was no GVHD following donor derived CD19 CAR T cells in this cohort, however, only 3/10 patients responding to the CAR T cells\(^{118}\). Of note, patients on this trial did not receive lymphodepleting agents prior to CAR infusion, which is known to be significant for efficacy of adoptive T cell immunotherapy although data on the importance of lymphodepleting therapy in the context CAR T cells is lacking. Nonetheless, it is difficult to draw conclusions regarding efficacy in this small series since all autologous CAR reports incorporate lymphodepletion prior to infusion.
A second report of donor derived allogeneic CAR T therapy utilized virus specific T cells (VST) to reduce the risk of GVHD. VSTs are selected by ex-vivo antigen-driven expansion of T cells and have been used post allo-HSCT for treatment of viral induced complications(119–122). VSTs have been described as an suitable backbone for different CAR T cells (for CD30, GD2, CD19 etc), with an endogenous TCR specific to viral antigens and additional specificity through the CAR receptor against the tumor target(65, 119, 123–125). The use of donor-derived VST-CD19 CAR was reported in a limited number of patients with no GVHD or cytokine release syndrome observed[38]. Here, expansion of CAR T cell was seen with viral infection or reactivation suggesting that TCR activation is able to enhance the expansion of infused CAR T cells. However, the increased CD19 CAR expansion did not lead to a decrease in normal or malignant B cell numbers suggesting the possibility of impaired functionality of the CAR T cells when activated through endogenous TCR.

Currently there are 6 trials on clinicaltrials.gov evaluating the use of allogeneic donor-derived CAR T cells (June 30, 2015, Table 1), with some preliminary data presented in abstract form. An ongoing trial from the MD Anderson group has treated 12 patients with pre-emptive DLI of donor-derived Sleeping-Beauty transferred CD19 CAR following allo-HSCT. Three patients, all with ALL, remained alive and in remission(127).
### Current Clinical Trials using Donor Derived Anti-CD19-Targeted CAR Modified T cells

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One difference between using unmanipulated polyclonal donor derived T cells and VST-CAR T cells is that a substantially smaller percentage of the polyclonal population would be predicted to encounter TCR antigen in an allo-recipient based on the precursor frequency reported for allo-specific TCRs. With the use of VST-CAR T cells, every T cell infused could potentially encounter a TCR and CAR antigen simultaneously in the presence of a viral infection or reactivation. If there were an advantage of one receptor over another, the prediction would be a much more pronounced effect with the use of VST-CAR T cells. VST-CAR T cells seem to benefit from enhanced long term expansion due to stimulation through TCR by viral antigens as compared stimulation solely through the CAR (119, 123, 124), but this expansion does not necessarily correlate with CAR efficacy, suggesting a distraction through activation of the TCR. To further understand the explanation for the suboptimal results with the use of allo-CAR T cells as well as to improve such approaches in the future, there is a need to better understand basic immunobiology of T cells in the allogeneic environment, and the interaction of the CAR and the TCR.
5.3 CAR Versus TCR: Immunobiology in the Allogeneic Recipient

The density of CAR receptors on the T cells, as well as target antigen density, are important to the functionality of CAR T cell therapy\(^{128}\). However, CAR density differs between different CAR constructs and varies heavily depending on the method used to genetically modify the T cell to express the CAR, and is not typically measured systematically in most CAR reports. The density of TCR on the T cell surface has been extensively researched, and found to be about 40,000 molecules on a single normal CD4+ T cell\(^{129}\). Investigators also found that CD8+ T cells are able to produce cytokines with as little as 500-1000 TCRs on the surface, but low receptor levels affect the potency and lytic activity of the T cell\(^{130}\). The efficacy of a CAR T cells has been reported to be co-dependent on the expression level of the CAR receptor and expression level of the target antigen\[^{40}\]. In the case of a second-generation CD20-CAR T cell it was found that direct binding of hundreds of target antigens was required to activate lytic capacity and thousands of antigens interactions required before the cytokine production occurred\[^{43}\]. It is possible that there are more TCRs than CARs on a surface of a given T cell, potentially resulting in a TCR advantage. TCR and CAR interactivity and dynamics are of special importance in the context of allogeneic or VST-CARs, where the presence of both antigens for a substantial proportion of T cells is likely.
The binding specificity of a CAR is typically derived from a monoclonal antibody and significant discrepancies in affinity and avidity between the CAR or the TCR and a target cell may exist thus affecting T cell efficacy mediated by either receptor. The inherently low affinity of most endogenous TCRs that survive thymic selection maintains high potency by allowing for a sufficient “on/off rate” (132–134). There is very little known about the importance of affinity for CAR function. High affinity binding by the antibody portion may not allow for frequent engagement and disengagement thus affecting signaling through the CD3ζ chain. Thus, T cells might prefer signaling through native TCR and confer higher response against TCR antigen than CAR antigen. Indeed, investigators have studied activation thresholds of T cells transduced such that there is similar surface expression of TCR and CAR receptors demonstrating that TCRs were more active in the presence low antigen levels than cells transduced with CAR. In fact in the presence of low antigen density, stimulation through TCR resulted in maximum INFγ secretion compared to ~50% less secretion by the CAR in the presence of low-density CAR antigen [47]. Again, these differences would be accentuated in the allogeneic environment due to the presence of both TCR and CAR antigens. Dominance of TCR signal could potentially lead to increased GVHD and lowered CAR-mediated GVL, although this has not been observed in the very limited number of patients treated with donor-derived CAR T cells as discussed above. Nonetheless, the interaction between the CAR and the TCR
has not been thoroughly studied and will be critical for the optimization of CAR T cells in the clinic.

5.4 Improving Donor Derived CARs Through T Cell Selection

Today, many major pharmaceutical companies are investing in the research and development of CAR T cell design and manufacturing. Ideally, a universal, off-the-shelf, product would offset the costs of individual cell preparation required in trials today\(^{(136)}\). These products would likely be analogous to the donor-derived CAR T cells currently being given after allogeneic HSCT in limited numbers of ongoing trials. A goal of generating off-the-shelf products would be to potentially administer these without the requirement for a prior HSCT from the same donor. To achieve this, methods to minimize the potential for donor cell rejection by the recipient and to reduce the alloreactive potential of the product are needed. The safety and efficacy of such off-the-shelf products remains to be systematically tested. Several approaches have been proposed to increase the efficacy of donor-derived allogeneic CAR T cells with the intention of developing general produced cell products available to patients not qualified for autologous infusions due to challenges such as low lymphocyte numbers or poor lymphocyte quality (and expansion \textit{in vitro}).
A primary goal of using virus-specific T cells for CAR production is the selection of a T cell product that does not contain an alloreactive TCR. An additional advantage of CAR products with a virus-specific TCR is the ability to utilize enhanced expansion of the T cells by stimulation through the native TCR using a vaccine approach (such as the varicella-zoster virus vaccine) following adoptive CAR T cell transfer (137). A clinical trial addressing this method is currently open for autologous anti-GD2 CAR T cells (NCT01953900).

Another method to enrich for non-allogeneic T cells is by using antigen-experienced memory T cells for CAR transduction(116, 125). It would be predicted that the vast majority of the T cell population with a memory phenotype is likely to have encountered antigens other than allogeneic antigens, and T cells carrying TCRs specific to allogeneic antigens would maintain their naive properties. Thus, selection for memory phenotype cells should enrich for a non-alloreactive repertoire. Indeed, memory T cells have been shown to have less potential to generate GVHD(138–140) in murine models, in part due to non-alloreactive TCR enrichment with evidence that memory cells are less likely to traffic to GVHD target tissues, such as the GI tract. One marker that can distinguish memory from naïve T cells is CD45RA. Several pre-clinical models have selected for CD45RA-CD62L+CD8+ central memory T cells(125) or CD45RA- T cells (116) to use for CAR production. Both techniques demonstrated good CAR transduction, in-vitro functionality
and in-vivo effect. In addition, the memory CAR T cells maintained anti-leukemic ability without causing xenogeneic GVHD, a major survival limitation with the use of immunodeficient mice treated with human T cells(116). There are currently several clinical trials using central memory T cells for anti-CD19 CAR based immunotherapy in the autologous setting against non-Hodgkin lymphoma or ALL (NCT02051257, NCT01815749, NCT02146924, NCT2153580, NCT01318317), and another using anti-IL-13Ra2 for glioma (NCT02208362). One trial is evaluating central-memory donor-derived CD19 CAR T cells following allogeneic transplant (NCT01475058, table 1).

Another source of T cells that could be used for CAR immunotherapy is the γδ T cell subset. These T cells recognize antigens that are distinct from the protein-derived peptides that comprise allogeneic antigens recognized by the αβ TCR. Thus, γδ T cells have less allogeneic potential but have been shown to mediate anti-tumor responses. Although they consist a minority of peripheral T cells, a single subtype (vδ2) can be expanded in vivo prior to T cell collection after stimulation with zolendronic acid(141). The MD Anderson group used a different expansion approach, with polyclonal γδ T cell transduction with CD19 CAR followed by ex-vivo expansion on an antigen-presenting layer expressing the CD19 target. This resulted in expanded polyclonal γδ T cells that demonstrated in vitro and in vivo effects in murine models, although the efficacy was not as dramatic as αβ CAR T cells in immunodeficient mice[54]. Interestingly, T cells derived from induced
pluripotent stem cells (originally induced from T cells) transduced with CAR have very similar gene expression to γδ T cells, providing general as well as CAR-specific antitumor response\(^ {143}\). Despite initial enthusiasm, recent discoveries demonstrate potential tumor-promoting effects of IL17-producing γδ T cells\(^ {144}\). Nonetheless, should methods for efficient selection for the IFNγ producing γδ T cells be elucidated, this T cell subset will be a potential candidate for CAR T cell production.

In addition to T cells, NK cells can be used for the generation of CAR products for immunotherapy. NK cells have been shown to contribute significantly to the GVL effect, especially in haplo-identical MHC mismatched/KIR-mismatched settings\(^ {145-147}\). Generally, NK Cells are considered to have lower allogenicity compared to T cells; nevertheless, NK-mediated GVHD has been reported\(^ {148}\). NK-CAR cells has shown preclinical efficacy against a variety of tumor-associated antigens \(149-153\). Major challenges with the use of NK-CAR cells include low persistence inherent to NK cell immunotherapy, and complex intracellular signaling machinery potentially not compatible or optimal when using of conventional T cell activation domains. The use of specific NK cell activation domains such as DAP12\(^ {151, 154}\) is currently being evaluated in preclinical studies.
To develop donor derived T cells without the effects of TCR activation, CAR T cells with CAR-only specificity have been generated by selectively deleting the endogenous TCR. With current gene editing technologies, the endogenous TCR could be excised through the use of nucleases such as zinc-finger nucleases (ZFNs)\(^{(155)}\), transcription activator-like effector nucleases (TALEN)\(^{(156, 157)}\), and the CRISPR/Cas9 system\(^{(158)}\). The absence of an endogenous TCR eliminates the possibility of GVHD or the potential distraction of TCR receptor signaling. Using the same techniques, MHC class I could be deleted on donor-derived, off-the-shelf T cells to avoid rejection of transferred cells\(^{(44)}\). Even though novel gene editing tools may prevent GVHD and rejection, significant potential issues remain to be fully tested. Absence of MHC may elicit an NK response against allogeneic T cells. Also, the functionality of a CAR containing a CD3\(\zeta\) domain has been shown to be dependent on its ability to dimerize with the endogenous TCR in order to activate downstream pathways through the interactions with the TCR/CD3 complex\(^{(41, 45)}\). The results of these studies suggest that the efficacy of CAR T cell activity and persistence could be diminished by eliminating or mutating the endogenous TCR or MHC with the use of nucleases.

Lastly, adding a suicide gene to the CAR construct may be beneficial in minimizing the risk of GVHD after allogeneic CAR T cell infusion. Inducible
caspase 9 (iC9) is an intrinsic activator of apoptosis that can be transduced into allogeneic T cells that are administered following allo-HSCT and can effectively abrogate GVHD\(^{159, 160}\). Preclinical models have shown that activation of the suicide iC9 in CAR therapy rescued mice from xenogeneic GVHD in a CD44v6-CAR model\(^{161}\). Ongoing clinical trials have incorporated the iC9 construct into CAR T cell products to provide a method to eliminate autologous CAR T cells in the event of potential off tumor toxicity (NCT02107963, NCT01822652, NCT02439788).

5.6 Conclusions

CAR T cells are one of the exciting achievements of current adoptive immunotherapy, with noteworthy clinical successes in treating ALL. Currently, many patients may experience lower efficacy due to general T cell defects from prior chemotherapy or allo-HSCT. The use of donor-derived cells, especially on route to off-the-shelf T cells needs to be evaluated. Many current studies address safety questions, with multiple techniques being employed to avoid GVHD. Little is known about TCR/CAR interactions and the effects TCR signaling has on therapy outcome. Better understanding of the immunobiology of CAR and TCR signaling and functionality is required before we determine the benefits or pitfalls of including or deleting the endogenous TCR for CAR therapy. Altogether, solving challenges in MHC-
matched allogeneic CAR T cells will pave the way for off the shelf universal CAR T cells.
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