The curative potential of chimeric antigen receptor T-cell therapy for B-cell malignancies

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THE CURATIVE POTENTIAL OF CHIMERIC ANTIGEN RECEPTOR T-CELL THERAPY FOR B-CELL MALIGNANCIES

by

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ABSTRACT

Few cancers arising in fluid organ systems can be cured with localized therapeutic modalities, such as radiation or surgical organ removal. Chemotherapy and hematopoietic stem cell transplants have long been employed as the standard of care for patients diagnosed with leukemias and lymphomas. Though research continues to propose new, more potent chemotherapeutic agents, a new paradigm of treating cancerous malignancies with tumor-specific monoclonal antibodies, adoptively transferred tumor-fighting cells, and other exogenously administered immunomodulatory agents, has emerged over the past decade. These immunotherapies have dramatically improved the outcomes of patients diagnosed with cancers of B lymphocytes, referred to as B-cell malignancies.

Though curative FDA-approved therapies for patients diagnosed with B-cell malignancies have yet to be established, recent research in the field of adoptive T-cell therapy has produced promising results. Tumor infiltrating lymphocyte therapy (TIL therapy), T-cell Receptor Therapy (TCR therapy) and Chimeric Antigen Receptor T-cell Therapy (CAR T-cell therapy) are the three most extensively studied adoptive T-cell immunotherapies in the context of B-cell malignancies. TIL and TCR therapies, in which patients are provided with either the patient’s own tumor-specific T-cells or T-cells expressing engineered, tumor-specific TCRs, respectively, enhance the patient’s immune
system to mount a more potent, anti-tumor response. However, these adoptive T-cell therapies do not change the mechanisms of the immune response.

Cancerous cells can evade immune attack and dampen immune responses to survive and thrive in the body. By down-regulating their expression of human major histocompatibility complex I (MHC I), for example, cancer cells escape T-cell recognition, which is dependent on MHC expression. A chimeric antigen receptor (CAR), is composed of an antibody-derived (B-cell derived) extracellular, antigen-recognition domain, and T-cell derived intracellular domains. CAR T-cells, therefore, exploit the cytotoxic nature of CD8+ T-cells, and the MHC independent recognition of B-cell receptors, to identify and destroy all cells expressing a specific target. Consequently, many of the cancer cell’s mechanisms of immune evasion are less effective in the presence of CAR T-cells. Progressive generations of CAR T-cell designs couple these receptors with costimulatory molecules to amplify the activation, efficacy, and potency of these cells in-vivo.

Over the past five years, phase I and IIa clinical trials have produced remarkable results in the treatment of advanced stage, high-risk B-cell malignancies, namely Acute Lymphoblastic Leukemia (ALL), Chronic Lymphocytic Leukemia (CLL), and Non-Hodgkin’s Lymphoma (NHL). However, the significant oncogenic risks and fatal adverse events associated with this therapy necessitate further research to improve safety and reliable clinical efficacy of CAR T-cell therapy. In spite of these risks, the adoptive transfer of CD19-targeting, CAR expressing, cytotoxic T-cells (anti-CD19 CAR-T-cells) has produced sustained, complete remissions in patients diagnosed with progressive,
advanced-stage, B-cell malignancies, for whom alternative treatments were not available. The unprecedented results of early clinical trials, as well as ongoing preclinical studies aimed at improving the design and production of CAR T-cells suggest a promising future for CAR T-cell therapy as a cure for B-cell malignancies.
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<td>CD</td>
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CNS .......................................................... Central Nervous System
CR .......................................................... Complete Remission
CRP .......................................................... C-Reactive Protein
CRS .......................................................... Cytokine Release Syndrome
CSF .......................................................... Cerebrospinal Fluid
CTLA-4 .................................................. Cytotoxic T-Lymphocyte Antigen 4
DLBCL .................................................. Diffuse Large B Cell Lymphoma
DLBCLs .................................................. Diffuse Large B Cell Lymphomas
DNA .......................................................... Deoxyribonucleic Acid
FCL .......................................................... Follicular Cell Lymphoma
GVHD .......................................................... Graft-versus-Host Disease
HSCT .................................................. Hematopoietic Stem Cell Transplant
HSCTs .................................................. Hematopoietic Stem Cell Transplants
HSPCs .................................................. Hematopoietic Stem Cells
IgK .......................................................... Immunoglobulin-K
Igλ .......................................................... Immunoglobulin-λ
IL .......................................................... Interleukin
IL-2 .......................................................... Interleukin-2
IL-6 .......................................................... Interleukin-6
IL-21 .......................................................... Interleukin-21
MAS .......................................................... Macrophage Activation Syndrome
MCL .......................................................... Mantle Cell Lymphoma
MHC ................................................................. Major Histocompatibility Complex
MHC I ............................................................ Major Histocompatibility I
MHC II ............................................................ Major Histocompatibility II
MHCs .............................................................. Major Histocompatibility Complexes
MRD ............................................................ Minimal Residual Disease
mRNA .............................................................. Messenger Ribonucleic Acid
MSKCC ......................................................... Memorial Sloan Kettering Cancer Center
NCI .............................................................. National Cancer Institute
NHL .............................................................. Non-Hodgkin’s Lymphoma
NHLs ............................................................. Non-Hodgkin’s Lymphomas
N/R .............................................................. Not Reported
PD ............................................................... Progressive Disease
PMBCL ........................................................ Primary Mediastinal B-Cell Lymphoma
PR ............................................................... Partial Remission
PRs .............................................................. Partial Remissions
RNA .............................................................. Ribonucleic Acid
SB .............................................................. Sleeping Beauty
scFVs ............................................................ single-chain variable fragments
SD ............................................................... Stable Disease
SMZL ........................................................ Splenic Marginal Zone Lymphoma
SMZLs ........................................................ Splenic Marginal Zone Lymphomas
Tc cell ............................................................ CD8+ Cytotoxic T-cell
TCM cell ................................................................. Central Memory T-cell
TCR .................................................................. T-cell Receptor
TCRs ................................................................ T-cell Receptors
TEM cell ............................................................. Effector Memory T-cell
TH cell ................................................................. CD4+ Helper T-cell
TIL ................................................................. Tumor infiltrating Lymphocyte
TILs ................................................................. Tumor infiltrating Lymphocytes
UPenn .............................................................. University of Pennsylvania
BACKGROUND

Lymphocyte malignancies refer to a set of diseases characterized by the uncontrolled division of abnormal lymphocytes. Cancers that affect lymphocytes fall into two main categories, leukemias and lymphomas. In leukemias, the original cancerous cells are located in the blood or bone marrow, while in lymphomas, the original cancerous cells are located in lymph nodes or other related tissues. In both cases, the cancer can proliferate outside the compartments of origin to affect multiple tissues and body systems (Ansell & Armitage, 2005; Shafer, 1966). Neoplastic cells in leukemias and lymphomas may be of B-cell or T-cell origin, and are referred to as B-cell or T-cell malignancies, respectively.

While the human body is typically capable of mounting an immune attack against threats to an individual’s health, cancer cells can evade the immune system’s defenses and replicative control mechanisms. Research continues to uncover the processes by which cancerous cells survive and thrive in the body, yet many cancers remain incurable. Though the development of many therapeutic agents has successfully prolonged the life of patients diagnosed with high-risk B-cell malignancies over the past decade, advancements in immunotherapy hold some of the greatest promise as future cures for B-cell cancers.

The Immune System

The immune system, which confers the body’s ability to resist harm, can be broken down into two overarching groups: innate immunity and adaptive immunity.
Innate immunity is genetically predetermined for each individual, while adaptive immunity is learned over an individual’s lifetime. As such, the mechanisms and magnitude of the innate immune response do not differ upon repeated exposure to the same pathogen. In contrast, the mechanisms of adaptive immunity permanently change with exposure to an antigen. Lymphocytes, cells that interact with antigens through antigen-specific receptors, confer the specificity of the adaptive immune system (Mak & Saunders, 2006a).

Lymphocytes, a subset of white blood cells that may reside in the general circulation or in lymphoid organs, consist of B lymphocytes (B-cells) and T lymphocytes (T-cells). B-cells are derived from bone marrow and can be activated by the presence of an antigen to differentiate into antibody producing plasma cells. T lymphocytes mature in the thymus, and comprise 60-70% of the total peripheral lymphocyte population (Germain, 2002; Pieper, Grimbacher, & Eibel, 2013).

The three commonly discussed subsets of lymphocytes are naïve lymphocytes, effector lymphocytes, and memory lymphocytes. Naïve lymphocytes are mature lymphocytes capable of differentiation, but have not yet been exposed to an antigen. Effector lymphocytes are short lived cells capable of immediately eliminating antigens. Memory lymphocytes are long-lived antigen specific cells that remain dormant until a secondary exposure of a pathogen (Chaplin, 2010).

Two main subsets with distinct functions exist within the memory lymphocyte populations: protective memory lymphocytes and reactive memory lymphocytes. Effector memory T cells (TEM cells) confer protective memory by migrating to the site of
antigenic stimulation and immediately mounting an effector response. Antigen exposure stimulates central memory T-cells (T\textsubscript{CM} cells), which confer reactive memory, to proliferate and differentiate into effector T-cells. T\textsubscript{CM} cells have little effector function (Lanzavecchia & Sallusto, 2000). Within the B-cell populations, plasma cells secrete antibodies against pathogens, serving the protective memory function, while memory B-cells confer reactive memory by proliferating and differentiating into plasma cells upon a repeated exposure to the antigen (Ochsenbein et al., 2000).

When a pathogen enters the body, a single clonal population of lymphocytes is capable of recognizing it. Upon recognition, only this clonal population of lymphocytes will be stimulated to proliferate and respond to the invader in a process known as clonal selection. Upon proliferation, some clones will differentiate into effector lymphocytes to respond to the immediate attack, while other clones will differentiate into memory lymphocytes. These memory lymphocytes do not participate in the initial attack against the pathogen, but remain in a resting state until triggered by a future exposure to the same pathogen. These memory lymphocytes are poised to mount a swift and vigorous response upon a second exposure to the pathogen (Chaplin, 2010).

**T-Lymphocytes**

The two most commonly discussed subsets of T-cells, determined by the cluster of differentiation (CD) expressed on the cell surface, are CD4+ helper T-cells (T\textsubscript{H} cells), and CD8+ cytotoxic cells (T\textsubscript{C} cells). Cell surface molecules CD4 and CD8 function as coreceptors for CD4+ helper T-cells, and CD8+ cytotoxic T-cells, respectively, by
determining the cell’s compatibility with cell surface proteins known as major histocompatibility complexes (MHCs) (Pearce, Shedlock, & Shen, 2004). Together, an antigen and major histocompatibility complex (MHC) form a pMHC unit, which can be recognized by T-cell receptors (TCRs). T_H cells release cytokines to activate phagocytic cells, such as macrophages, to destroy these ingested invaders, or activate B-lymphocytes to produce antibodies against these invaders following their recognition of a major histocompatibility II (MHC II) domain. T_C cells interact with the major histocompatibility I (MHC I) domain, which is expressed on most cells in the body, and are capable of directly destroying compromised cells (MacLeod, Clambey, Kappler, & Marrack, 2009; Swami, 2013). Consequently, T-cells are only capable of recognizing antigens as antigen proteins that are bound to MHCs, while B-cells do not require MHCs to identify an antigen (Mantegazza, Magalhaes, Amigorena, & Marks, 2013).

TCRs (figure 1) are essential to T-cell antigen recognition and activation. A mature, naïve, or memory T-cell that has not yet encountered its appropriate pMHC unit is considered activated when it has been stimulated, by the presence of a recognizable antigen, to proliferate and differentiate into an effector T-cell.

T-cell activation typically requires 2 signals. The first signal is a recognition signal, indicating that the T-cell receptor (TCR) has found its cognate antigenic peptide. The second signal, originating from the interactions between cosignaling molecules on both the antigen presenting cell (APC) and the T-cell, determines whether the T-cell will be activated in response to having found its cognate antigenic peptide.
Once a TCR interacts with its cognate antigenic peptide in a pMHC unit, CD3 chains associated with the TCR undergo a conformational change, which initiates an intracellular signaling cascade that alters the cytoskeletal structure of the cell (Alarcón, Gil, Delgado, & Schamel, 2003; Gomez & Billadeau, 2008; Kane, Lin, & Weiss, 2000).

Figure 1: T-cell Receptor. The most common TCR is a heterodimeric glycoprotein composed of one alpha (TCRα) and one beta chain (TCRβ), linked by disulfide bonds (Kuwana et al., 1987). Each of these chains has a constant region that recognizes relevant signaling molecules, a variable region that contains the binding site for the pMHC complex, a charged transmembrane protein that maintains the TCR’s location in the cell membrane, and a cytoplasmic tail (Mak & Saunders, 2006b). The constant region and charged transmembrane protein of the TCR are noncovalently linked to the CD3 complex, which is composed of 5 proteins: gamma (γ), delta (δ), and epsilon chain (ε), and two zeta (ζ) chains. Each of these CD3 proteins contain an immunotyrosine based activation motif to execute intracellular signaling upon TCR antigen recognition. The variable region of each alpha and beta chain contains four hypervariable regions which interact with different portions of the pMHC complex. Aside from the CD3 complex, T-cell surfaces often house many other invariant proteins and protein complexes as well (not shown) (Kuwana et al., 1987; Mak & Saunders, 2006b). Taken From (Anriar-commonswiki, 2010).
Costimulatory receptors on the T-cell surface recognize and bind compatible costimulatory surface molecules on antigen presenting cells (APCs) to initiate an intracellular signaling cascade. The identity of the costimulatory molecules determines whether this cascade inhibits or facilitates T-cell activation. Interaction of the costimulatory receptor Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) with its cognate costimulatory receptor, for example, inhibits T-cell activation, while the interaction of CD28 or CD137 with their respective cognate molecules serves as an essential second signal for T-cell activation (Acuto & Michel, 2003; Valk, Rudd, & Schneider, 2008).

Tc cell activation is typically preceded by Th cell activation, and APC activation. When the APC, usually a dendritic cell, presents a specific pMHC unit that is recognized by the Th cell, receptor interaction and costimulation results in the activation of both cells. The APC is now poised to costimulate the appropriate Tc cell, while the Th cell secretes cytokines that facilitate Tc cell activation. An optimal combination of a strong TCR-pMHC binding affinity, costimulation, and Th cell derived cytokine environment activates Tc cells. The new effector Tc cell can now identify and destroy MHC I bound foreign peptides in the body (Mak & Saunders, 2006c).

*Standard of Care for Treat B-cell Malignancies*

Multiple prognostic factors, such as the classification and clinical staging of a cancer, the presence of genetic aberrations, the patient’s age, and the patient’s health condition, assist physicians in determining the favorability of a patient’s outcome with standard treatments for B-cell malignancies. Typically, younger patients with early-stage,
Newly diagnosed patients are typically treated with a chemotherapeutic regimen that has been established as the standard of care for the condition and stage of the patient’s cancer. If the patient does not achieve a complete remission (CR) with first-line therapy, the patient will be treated with an alternative chemotherapy regimen, referred to as second-line chemotherapy. Once a complete remission is achieved, patients are typically treated with maintenance therapy, to prevent the recurrence of the cancer. If the patient’s cancer reemerges (relapses) after previously achieving complete remission, the patient is treated with salvage therapy. If a patient fails to achieve CR with second-line chemotherapy or salvage therapy, the patient may receive further chemotherapy treatments (not shown), or may proceed to receive alternative treatments.

**Figure 2: Overview of Chemotherapy Flow.** Newly diagnosed patients are typically treated with a chemotherapeutic regimen that has been established as the standard of care for the condition and stage of the patient’s cancer. If the patient does not achieve a complete remission (CR) with first-line therapy, the patient will be treated with an alternative chemotherapy regimen, referred to as second-line chemotherapy. Once a complete remission is achieved, patients are typically treated with maintenance therapy, to prevent the recurrence of the cancer. If the patient’s cancer reemerges (relapses) after previously achieving complete remission, the patient is treated with salvage therapy. If a patient fails to achieve CR with second-line chemotherapy or salvage therapy, the patient may receive further chemotherapy treatments (not shown), or may proceed to receive alternative treatments.
localized cancers that lack genetic aberrations have the most favorable prognoses, while older patients with disseminated, aggressive disease have the least favorable prognoses. Chemotherapy and hematopoietic stem cell transplantation remain the two most commonly employed, standard of care treatments for patients diagnosed with B-cell malignancies (Döhner et al., 2000; Hilden et al., 2006; Rai et al., 2004).

**Table 1. Common Terms in the Discussion of Cancer Therapies**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Complete Remission (Complete Response)</td>
<td>The eradication of all detectible cancer and the disappearance of all signs of the disease.</td>
</tr>
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<td>Complete Response Rate</td>
<td>The proportion of patients who experience a disappearance of the cancer with the treatment. Often expressed as a percentage.</td>
</tr>
<tr>
<td>Duration of Response</td>
<td>The period of time from the documented response to therapy, to the time of disease progression.</td>
</tr>
<tr>
<td>Event-free survival</td>
<td>Period of time from the patient’s enrollment into the trial, to detectible disease progression or the cessation of current treatment.</td>
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<td>Minimal Residual Disease</td>
<td>A disease that does not present with any morphological signs and symptoms, and can only be detected by highly sensitive laboratory technology (Hallek et al., 2008; S. Lee et al., 2000).</td>
</tr>
<tr>
<td>Objective Response Rate (ORR)</td>
<td>The proportion of patients whose cancer diminishes by a predefined amount with the treatment. Often expressed as a percentage.</td>
</tr>
<tr>
<td>Overall Survival (OS)</td>
<td>Period of time from the patient’s enrollment into the trial, to the death of the patient.</td>
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<tr>
<td>Progression free survival</td>
<td>Period of time from the patient’s enrollment into the trial, until detectible disease progression or death of the patient.</td>
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<tr>
<td>Progressive Disease (PD)</td>
<td>A state in which the cancer continues to grow.</td>
</tr>
<tr>
<td>Primary Refractory Cancer</td>
<td>Cancer that does not respond to, or is resistant to, one or more therapeutic agents,</td>
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Patients diagnosed with B-cell malignances are often treated with an initial course of chemotherapy intended to induce a complete remission (CR) in the patient, commonly referred to as induction therapy (figure 2). The success of most cancer therapies are often discussed in terms of the presence of the disease (table 1). Chemotherapeutic regimens are tailored to the patient’s disease type, extent of malignancy, and health status, among other factors. If the first regimen, known as first-line chemotherapy, fails to induce a complete remission of the cancer, the patient may undergo an alternative chemotherapeutic regimen, known as second-line chemotherapy. Complete remission refers to a state in which the treatment has successfully eradicated the cancer such that the cancerous cells are undetectable by conventionally used, highly-sensitive, detection methods. A wide array of chemotherapeutic agents are currently in use. These agents may be administered either alone as single agent chemotherapy, or in combinations as multi-agent chemotherapy. If a patient’s cancer does not respond to chemotherapeutic agents,

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<td><strong>Relapsed Cancer</strong></td>
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*Treatment may be discontinued for multiple reasons, such as the need for alternative therapies, or patient preference. (“NCI Dictionary of Cancer Terms,” n.d.).*
it is said to be refractory. A cancer may be refractory to one or more therapeutic agents (Friedberg, 2008; Lister et al., 1978).

Patients who achieve CR are treated with maintenance chemotherapy to prevent the reemergence of the cancer. If a patient experiences a revival of the disease after achieving complete remission with chemotherapy, the patient’s cancer is said to have relapsed. In some cases, the patient’s relapse is caused by the cancer developing a resistance to therapeutic agents. In this event, the cancer is referred to as relapsed and refractory cancer. Patients who have relapsed are treated with salvage therapy to eradicate the relapsed disease. Salvage chemotherapy may also be referred to as “third-line” or “fourth-line” chemotherapy (Gökbuget et al., 2012; Sud & Friedberg, 2008).

Monoclonal antibodies, such as rituximab, an antibody against the B-cell costimulatory molecule CD20, are gaining popularity as effective therapies against B-cell malignancies. These immunotherapeutic agents may be administered alone, or alongside chemotherapeutic agents in a chemoimmunotherapy regimen. Chemoimmunotherapy is currently used as first-line, second-line, maintenance, and salvage therapy regimens, and has successfully prolonged the lives of many patients diagnosed with B-cell malignancies (Brusamolino, 2009; Dotan, Aggarwal, & Smith, 2010).

In spite of recent advances in the development of novel therapeutic agents, the only truly curative, non-experimental therapies in use for the treatment of B-cell malignancies are hematopoietic stem cell transplants (HSCTs). In HSCTs, healthy hematopoietic stem cells administered to the patient engraft (settle) in the patient’s bone marrow, and restore healthy bone marrow function and normal hematopoiesis in the
patient. Stem cells can either be derived from a donor (allogeneic), or from the patient (autologous) (Gratwohl et al., 2003). HSCTs are associated with severe, potentially fatal adverse events, and are consequently considered high-risk procedures, with limited patient eligibility (Cornelissen & Blaise, 2016).

Prior to receiving allogeneic or autologous HSCTs, patients are treated with intense regimens of chemotherapy to decrease the patient’s tumor burden, and impair the patient’s immune mechanisms. The intense pre-treatments are very physically taxing, which further increases the danger of this already high-risk therapy, especially in elderly patients. As such, HSCTs are primarily explored as therapeutic options for young, fit patients (Wildes, Stirewalt, Medeiros, & Hurria, 2014).

Stem cells for autologous HSCTs are collected from the patient during a period of complete remission. Patients with large tumor burdens or refractory disease may fail to achieve remission with conventional therapies, and are therefore unable to receive autologous stem cell transplantation. This same patient group may be denied allogeneic hematopoietic stem cell transplants (allo-HSCTs) as well, as greater success is observed in patients who have achieved complete remission (Bacher et al., 2012; Doney et al., 2011).

Donor derived T-cells in allogeneic stem cells may engraft in the bone marrow, expand, and mount an immune response against the cancer, known as the graft-versus-cancer effect (Kolb, 2008). However, these T-cells are also capable of attacking the recipient’s healthy tissues, thereby initiating graft-versus-host disease (GVHD), a potentially fatal complication of allo-HSCTs. Another serious complication of allo-
HSCTs is host-versus-graft disease, wherein the recipient’s immune system mounts an attack against the donor graft (Holtan, Pasquini, & Weisdorf, 2014). Though autologous HSCTs are not associated with these adverse events, autologous grafts are incapable of initiating a T-cell mediated graft-versus-cancer attack (Kuruvilla, 2016).

Following the allogeneic hematopoietic stem cell transplant (allo-HSCT), patients may receive repeated donor lymphocyte infusions, which can be curative for some patients (Kubuschok, Held, & Pfundschuh, 2015). Many B-cell neoplasms in patients who are ineligible to receive stem cell transplants, or relapse after the administration of one or more stem cell transplants, are considered incurable. Further research is necessary to establish a curative treatment for such malignancies (Cornelissen & Blaise, 2016; van den Brink et al., 2010).

Adoptive T-cell Therapy

Immunotherapy is a method of treatment that enhances an individual’s own immune system, or utilizes components of the immune system, to eradicate the cancer. The objective of T-cell immunotherapy, also referred to as adoptive T-cell (ATC) therapy, is to utilize either autologous or allogeneic T-cells to confer the patient’s enhanced tumor-fighting abilities. The three most commonly discussed forms of T-cell immunotherapy are tumor infiltrating lymphocyte (TIL) therapy, T-cell receptor (TCR) therapy, and chimeric antigen receptor (CAR) T-cell therapy.

Tumor Infiltrating Lymphocyte Therapy: The objective of TIL therapy is to harvest, expand, and infuse a patient’s own tumor specific lymphocyte population to
eradicate malignancies. By expanding the TIL cell population in-vitro, the cells are able to proliferate away from the patient’s immunosuppressive tumor environment. This process allows for the repair of dysfunctional cell types, and for the selection of cells with the greatest tumor specificity. This therapy does not change the method in which the immune response against the malignancy is carried out. Rather, it simply provides the patient’s existing anti-tumor immune mechanisms with a greater population of the patient’s own cancer-fighting cells, to fuel a more robust immune response. In the typical course of this treatment, the patient’s tumor infiltrating lymphocytes (TILs) are harvested and expanded in a laboratory. The patient is then treated with chemotherapy and interleukin-2 (IL-2) infusions, followed by the infusion of the expanded TIL population (Sim et al., 2014). The success of phase I clinical trials was hindered by many patients choosing to drop out of the trial due to the intense preparative treatments (Besser et al., 2013, p.).

Disadvantages to TIL therapy include potentially fatal adverse events and the high cost associated with the therapy, which are common to most ATC trials (Hershkovitz, Schachter, Treves, & Besser, 2010). Additional disadvantages of this approach include an inability of the infused cells to persist in-vivo, as well as the lengthy time period required for the TIL expansion ex-vivo (Wu et al., 2012).

TIL therapy has been most extensively studied in chemotherapy-refractory metastatic melanoma patients. The results of phase I clinical trials suggest that TIL therapy may be a curative therapeutic option for this patient population, though its reported success in treating other malignancies is limited (Goff et al., 2010; Hershkovitz
et al., 2010). Phase II clinical trials are currently underway to further investigate the curative potential of TIL therapy.

**T-Cell Receptor Therapy:** TCR therapy is an engineered T-cell therapy in which TCR genes encoding for antigen-specific TCRs are transferred to primary T-cells. In TCR therapy, T-cells, often TIL cells, with high-affinity, tumor specific TCRs are isolated. The genes encoding the selected clone’s TCR alpha and beta chains are then transduced into primary T-cells, which are then expanded in-vitro and administered to the patient in an infusion (Schmitt, Ragnarsson, & Greenberg, 2009).

Challenges to the success of this therapy include inconsistent TCR expression in the engineered T-cells, an inability of the infused cell population to persist in the patient, and an inability to confer stable immunologic memory with this method (L. J. Cooper, Kalos, Lewinsohn, Riddell, & Greenberg, 2000; Dossett et al., 2009). This therapy is also associated with an increased risk of autoimmunity (Schmitt et al., 2009).

TCR therapy has primarily been studied in patients with hematologic neoplasms, with varying rates of success (Cavalieri et al., 2003). As investigations to enhance the safety and efficacy of this therapy continue, the anticipated success of TCR therapy’s curative potential continues to grow.

**Chimeric Antigen Receptor T-cell Therapy:** In CAR T-cell therapy, CD8+ T-cells are modified with a transgenic chimeric antigen receptor to produce a high affinity, tumor specific, cytotoxic T-cell. Certain tumors are capable of down-regulating their expression of human MHC I (HLA class 1), disrupting antigen presentation mechanisms, and rendering T-cells anergic, to escape MHC dependent antigen recognition by TCRs.
(Garrido & Algarra, 2001; R. Singh & Paterson, 2007). These mechanisms of immune evasion are less effective against CAR T-cells, thereby conferring CAR T-cell therapy’s advantage over TIL and TCR therapies (Zhou & Levitsky, 2012). CAR T-cell therapy exploits the cytotoxic nature of CD8+ T-cells, and the MHC independent binding ability and recognition of B-cell receptors, to identify and destroy all cells that express a specific cell surface marker. The ability of successfully designed CAR T-cells to persist in the body confers its advantage over monoclonal antibody immunotherapy, as CAR T-cells can potentially provide long-term, in-vivo, immunosurveillance (Davila & Brentjens, 2013). CAR T-cell therapy has been most extensively studied in B-cell neoplasms, namely Acute Lymphoblastic Leukemia (ALL), Chronic Lymphocytic Leukemia (CLL), and various Non-Hodgkin’s lymphomas (NHLs). The results of phase I clinical trials indicate that, with further research, CAR T-cell therapy may be truly curative for patients with very advanced, high-risk B-cell neoplasms.
Goals

As a “hot-topic” of research today, the results of CAR T-cell therapy preclinical studies and clinical trials continues to be published often, thereby preventing this thesis from providing a completely comprehensive overview of the most current research in this field. However, the intent of this thesis is to provide the reader with a greater understanding of the reasons for CAR T-cell therapy’s esteemed reputation in cancer research.

1) The first goal of this thesis is to provide the reader with a brief overview of the current methods of treating B-cell malignancies, and impress, upon the reader, the need for novel treatment modalities.

2) The second goal of this thesis is to provide an in-depth overview of current literature to characterize the various steps involved in designing, producing, and administering CAR T-cell therapy, and to illuminate points of discourse and areas of improvement within each of these steps.

3) A thorough investigation into the results of published clinical trials is presented to place, in context, the current significance of CAR T-cell therapy in the treatment of B-cell malignancies.

4) The final goal of this thesis is to evaluate the curative potential, and anticipated role of CAR T-cell therapy in the treatment of B-cell malignancies in the future.
THE STRUCTURE, DESIGN, PRODUCTION, AND ADMINISTRATION OF CAR T-CELLS

CAR T-cell Structure

The chimeric antigen receptor is so named because its components are derived from both antibodies and TCRs. Kuwana et al. published the first design of CAR T-cells composed of anti-tumor, antibody-derived single chain variable fragments (scFVs), coupled with a transcellular hinge domain, and a single TCR derived CD3ζ signaling domain in donor derived T_{H} cells (Kuwana et al., 1987). Subsequent designs of first-generation CAR T-cells utilized CD3γ, rather than CD3ζ, signaling domains, in Tc cell substrates, but continued to include a single extracellular recognition region coupled with a single intracellular signaling region (Eshhar, Waks, Gross, & Schindler, 1993; Gross, Waks, & Eshhar, 1989).

The recognition of the important role of costimulatory molecules in T-cell activation inspired the design of second (figure 3), third, and fourth generation CAR T-cells, which contain one, two, and three costimulatory domains, respectively, in addition to the first generation CAR T-cell construct (Finney, Akbar, & Lawson, 2004; Imai et al., 2004; Pulè et al., 2005a; J. Wang et al., 2007). Though second generation chimeric antigen receptors (CARs) are currently the most extensively studied CARs in clinical trials thus far, clinical trials are currently being conducted to evaluate the safety and clinical efficacy of third and fourth generation CAR T-cells.
Figure 3: Diagram of Second Generation CAR T-cells. Second generation CARs are composed of antibody-derived scFVs, a hinge transmembrane domain, a T cell-derived intracellular CD3ζ domains, as well as a single costimulatory domain (Finney et al., 2004; Imai et al., 2004). Reprinted from Molecular Oncolytics, Vol 3, Wang, Xiuyan, Clinical Manufacturing of CAR T Cells: Foundation of a Promising Therapy, 16015., 2016, with permission from Elsevier.

**Designing CAR T-cells**

The success of CAR T-cell therapy is entirely dependent on the ability of the CAR T-cells to target and destroy the cancer with minimal additional damage to the patient’s health. Therefore, it is critically important, when designing CARs, to ensure that:

1) The CAR specifically recognizes and targets cancer cells (on-target effect).
2) The CAR T-cells are capable of being effectively activated.

3) The CAR T-cells are capable of proliferating and persisting in the patient’s body for long periods of time.

4) The engineered T-cells are capable of stably and reliably expressing the CAR.

In theory, it is possible to create a CAR against any cell surface molecule, with the understanding that any cell expressing that marker will be destroyed. With this knowledge, it is crucial to select a target that is maximally expressed on cancer cells, and minimally expressed on healthy tissues. CAR T-cells currently being studied in most phase I and IIa clinical trials for B-cell malignancies recognize the CD19 cell surface molecule, which is only expressed on malignant and benign cells of the B-cell lineage. These cells are referred to as anti-CD19 CAR T-cells (Brentjens et al., 2011, 2013; Davila et al., 2014; Kochenderfer et al., 2010, 2012, 2013, 2015; Kusenda & Kovarikova, 2016; Porter et al., 2013, 2015; Porter, Levine, Kalos, Bagg, & June, 2011; Schuster et al., 2015).

The incorporation of one or more costimulatory molecules into the CAR T-cell structure is intended to enable adequate T-cell activation, to achieve an effector response capable of eradicating the patient’s cancer. CD137 (4-1BB) and CD28 are the most commonly utilized costimulatory domains in CAR T-cell design. In second generation CAR T-cells, which have only one costimulatory domain, the superiority of these domains continues to be disputed. Preclinical models reveal that CD28 coupled CAR T-cells have greater anti-tumor effects against B-cell Acute Lymphoblastic Leukemia (B-ALL) (Posey et al., 2013). Other preclinical studies propose that CD28 coupled CAR-
cells are more likely to trigger rises in IL-2 and tumor necrosis factor-α (TNF-α) levels, compared to CD137 coupled CAR T-cells, thereby causing natural regulatory T-cell mediated-inhibition against the CAR T-cells’ effector functions (D. W. Lee et al., 2014). Progressive generations of CAR T-cell models, incorporating two or three costimulatory domains, are designed to improve CAR T-cell activation and function.

Maximizing proliferative potential and minimizing T-cell exhaustion are critical factors in prolonging T-cell persistence in-vivo. Most phase I clinical trials to date selected for CD3+ T-cells, which consists of many different T-cell subsets (Kalos et al., 2011; Kochenderfer et al., 2010; Savoldo et al., 2011). However, new evidence suggests that cytotoxic effector T-cells, T<sub>CM</sub> cells, T<sub>EM</sub> cells, and naïve T-cells are not equal in their ability to resist T-cell exhaustion. Therefore, the selection of the correct T-cell type to undergo genetic modification is important in conferring CAR T-cell persistence (C. Berger et al., 2008; Klebanoff et al., 2005). Utilizing T<sub>CM</sub> cells or naïve memory T-cells will impart greater persistence and proliferative advantages to the engineered T-cells in-vivo (Hinrichs et al., 2009, 2011; Nguyen et al., 2016). Other methods to enhance T-cell activation are employed in the manufacturing process (C. Berger et al., 2008; Hinrichs et al., 2009, 2009, 2011).

Manufacturing CAR T-cells

Leukocytes are first collected through a process called leukapheresis, wherein peripheral blood mononuclear cells are taken from a sample of the blood, and the remainder of the blood is returned to the patient. If the leukocytes are derived from the
patient who will later receive the CAR T-cell infusion, the product is autologous. If a donor contributes these cells, the resulting product is allogeneic. The existence of harvestable T-cells is, therefore, a prerequisite for creating CAR T-cells. Physicians schedule leukapheresis per the patient’s other treatments to ensure that the patient’s lymphocyte population is sufficiently large enough to harvest.

**Figure 4: Autologous CAR T-cell Manufacturing.** Leukapheresis is used to obtain white blood cells from the patient (step 1) (Xiuli Wang et al., 2016). The leukapheresis product is then subjected to counterflow centrifugal elutriation (Step 2) (Powell et al., 2009) and immunomagnetic selection beads (not shown) to enrich for specific T-cell phenotype(s) (Xiuyan Wang & Rivière, 2016). The selected T-cells are then activated with antibody-coated beads (step 3) (Porter et al., 2006) before genes encoding the CAR design are transferred to the T-cell substrate by viral vectors (step 4), transposon/transposase system (not shown), or mRNA transfer (not shown) (Cartellieri et al., 2014). The genetically modified T-cell population then expands in a bioreactor (step 5). Following the washing and concentration of the newly expanded CAR-T cell population (step 6), the product is subjected to quality control measures (step 7), before being cryopreserved and shipped to the patient’s treatment location (step 8). The thawed CAR T-cell product is then intravenously administered to the patient. Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: Nature Reviews (Fesnak, June, & Levine, 2016).
The product of leukapheresis contains a combination of leukocytes, including B-cells, T-cells, and APCs, among other white blood cell types. Lymphocytes are commonly enriched by counterflow centrifugal elutriation, which exploits the difference in cell sizes to select for lymphocytes in the leukapheresis product (Banfalvi, 2008; Powell et al., 2009). Specific subsets of T-cells, most commonly CD3+ T-cells, are then enriched for by either positive or negative immunomagnetic selection beads (Zhu et al., 2016). Bead-based systems to specifically select for T<sub>CM</sub> cells and naïve T-cells have also been established (Casati et al., 2013; Xiuli Wang et al., 2012). The enriched T-cell population is then capable of undergoing the downstream activation steps, or may be cryopreserved for future use (Campbell et al., 2015; Xiuyan Wang & Rivièrè, 2016).

Many biotechnology companies have facilitated the use of beads-based T-cell activation, namely antibody coated magnetic beads and antibody coated nanobeads, as common methods of T-cell activation (Cartellieri et al., 2014; Terakura et al., 2012, p. 19). Antibody coated magnetic bead systems covalently couple superparamagnetic, APC-sized, inert beads to antibodies. Anti-CD3 and anti-CD28 antibodies, involved in signal 1 and signal 2 of T-cell activation, respectively, are most often utilized in beads-based activation of CD3+ T-cell populations. This system simultaneously allows for the selection and the activation (steps 2 and 3, figure 4) of the desired T-cell population (Smith et al., 2015). These beads must be removed prior to continuing with the downstream steps in CAR T-cell manufacturing process (Matheson, Peden, & Lehner, 2014). Antibody coated nanobeads use a biodegradable polymeric nanomatrix coupled to anti-CD3 and anti-CD28 antibodies to activate T-cells using the same process as the
antibody coated magnetic beads described above. The biodegradable nature of these nanobeads confers their primary advantage - they do not need to be removed prior to continuing with the downstream steps in the manufacturing process (Casati et al., 2013).

Genes encoding the chimeric antigen receptor must then be transferred into the selected T-cell substrate population. Gamma retroviral transduction and lentiviral transduction are the two most commonly used methods of gene transfer in CAR T-cell production. However, transposon/transposase systems are gaining popularity as the potential for mainstream administration of CAR T-cell therapy becomes an increasingly realistic goal. The objective of choosing one of these three methods is to efficiently transfer CAR encoding genes, and to ultimately produce a T-cell that is capable of stable CAR expression (Cartellieri et al., 2014).

Gamma retroviral transduction is the most extensively studied method of gene transfer in engineered T-cells. Unlike lentiviral transduction, gamma retroviral vectors are only capable of transducing dividing cells. Both gamma retroviruses and lentiviruses are retroviruses, and therefore contain a ribonucleic acid (RNA) genome (Yi, Jong Noh, & Hee Lee, 2011). In retroviral transduction, scientists create vectors with RNA genomes encoding for the desired CAR. When the virus enters the T-cell, its RNA genome is reverse transcribed to generate deoxyribonucleic acid (DNA) that viral integrase enzymes then insert into the cellular genome. The T-cell is now capable of encoding and expressing the CAR (Cepko & Pear, 2001). Although retroviral transduction has proven largely successful, potential genotoxicity and insertional mutagenesis resulting from
genomic integration, particularly genomic integration into known cancer related genes, remains a concern (Field et al., 2013; Q. Liu et al., 2015).

Transposon/transposase systems are currently being investigated to improve gene transfer rates in CAR T-cell production. This system involves the transfection, non-viral introduction via electroporation or lipotransfection, of DNA into the T-cell, which poses less immunogenic risk than viral transduction, to induce stable CAR expression (Monjezi et al., 2016). In the “sleeping beauty” (SB) transposon system, the CAR encoding DNA transposon, a sequence of synthetic DNA that is capable of changing its position in a genome, is introduced into the cell. The SB transposase enzyme inserts the transposon randomly into the T-cell genome in a “cut-and-paste” manner (Ivics, Hackett, Plasterk, & Izsvák, 1997). Notably, this random integration results in a potential mutagenic risk, and it therefore poses a potential oncogenic risk as well. Utilizing mRNA-derived transposase and supercoiled DNA vectors, known as “minicircles,” instead of plasmids, as the transposon’s source increases gene transfer rates while decreasing T-cell toxicity. Minicircles also have the benefit of lacking antibiotic resistance genes (Monjezi et al., 2016). Studies have also shown success using the “piggyBac” transposon/transposase system as an alternative to the SB system (Manuri et al., 2010). Further investigations are necessary to establish the clinical efficacy and safety of SB and “piggyBac” transposon/transposase systems in CAR T-cell production.

After successful retrovirus-mediated gene transfer, CAR T-cell expansion most commonly occurs in a bioreactor (Themeli, Rivière, & Sadelain, 2015). CAR T-cells produced by transposon/transposase gene transfer must be expanded by recursive
artificial APC (aAPC) stimulation, where an environment of artificial APCs (aAPCs), cytokines IL-2 and interleukin-21 (IL-21), and cells expressing a variety of costimulatory molecules enhance the population’s expansion (Suhoski et al., 2007).

Table 2: Quality Release Tests for CAR T-cells Based on Method of Gene Transfer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concerns</th>
<th>Retroviral Gene CAR T-cell Products</th>
<th>Transposon/Transposase System CAR T-cell Products</th>
</tr>
</thead>
</table>
| Safety    | Presence of harmful agents in final product. | • Mycoplasma  
• Sterility  
• Replication-competent retrovirus or lentivirus  
• Endotoxins  
• Aberrant transgene insertion | • Mycoplasma  
• Sterility  
• Endotoxins |
| Purity    | Abundance of unwanted remnants of the patient’s initial leukapheresis sample and/or the manufacturing processes. | • CAR T-cells %  
• non-CAR T-cells %  
• Residual Tumor Cells  
• Activation and/or Selection Beads | • CAR T-cells %  
• non-CAR T-cells %  
• aAPCs |
| Identity  | Percentage of CAR T-cells, in comparison to other undesired components, in the bulk product. | CAR T-cells % | CAR T-cells % |
| Potency   | Cytotoxic potential of the newly engineered CAR T-cells. | • In-vitro cytotoxic lymphocyte assay  
• IFN-γ secretion assay | • In-vitro cytotoxic lymphocyte assay  
• IFN-γ secretion assay |
While various quality control measures are built into every step of the manufacturing process, the product after expansion is washed and concentrated, then subjected to quality release testing. Such tests typically evaluate the safety and purity of the T-cell product (table 2). Once quality release tests are completed, the CAR T-cell product is cryopreserved and transported to the patient’s site of care. The entire process of manufacturing CAR T-cells can be completed in 5-10 days (Fesnak et al., 2016).

**Administering CAR T-cell Infusions**

Physicians may choose to provide patients preparative, lymphodepleting chemotherapy prior to the CAR T-cell infusion. The importance of pre-treatment continues to be disputed in current literature (Brentjens et al., 2011; Gattinoni et al., 2005). A general point of concern when pre-treating patients with chemotherapy is the difficulty in discerning if, and to what extent, the patient’s response is caused by the chemotherapy, or the engineered T-cell infusion. However, many groups have suggested an inverse relationship between tumor burden and the success of CAR T-cell therapy. Therefore, pre-treating patients with chemotherapy may decrease the patient’s tumor burden prior to the CAR T-cell infusion thereby enhancing the efficacy of CAR T-cells in the patient (Davila & Brentjens, 2013; Kochenderfer et al., 2012). Yet, chemotherapy-refractory patients, by definition, will not receive substantial benefits from pre-treatment. Many groups have reported that preparative chemotherapy had little to no effect on the tumor burdens of relapsed and/or refractory patients with B-cell neoplasms prior to the
CAR T-cell infusion (Kalos et al., 2011; Kochenderfer et al., 2012; Porter et al., 2015, 2011). Additionally, complete responses have been achieved in patients who did not receive chemotherapy, indicating that the outcomes of CAR T-cell therapy may not be enhanced by preparative treatment (Grupp et al., 2013; Kochenderfer et al., 2013). Further investigations aimed at establishing the effects of preparative chemotherapy, and a standard regimen of pre-treatment, have yet to be performed.

The cryopreserved CAR T-cell product is thawed and intravenously infused into the patient’s blood stream. The infusion process can take up to four weeks, and must be tailored to each individual patient’s current treatment regimen (Turtle et al., 2016). The first few weeks post-infusion are characterized by rapid activation and proliferation of the CAR T-cells in the patient’s body. Patients experience most cytokine related adverse events during this time, with the greatest severity of complications occurring at the peak of the CAR T-cell population’s expansion. Varying correlations between the persistence of CAR T-cells in-vivo and the extent of B-cell malignancy eradication have been reported in phase I clinical trials. In practice, widely accepted guidelines indicating clinically appropriate dosages of CAR T-cells have not been established. There is evidence to suggest that the dose of infused CAR T-cells should be altered per the patient’s tumor burden after pre-treatment (Davila & Brentjens, 2013; Kochenderfer et al., 2012).
COMMON ADVERSE EVENTS IN THE COURSE OF CAR T-CELL THERAPY

While design and manufacturing processes impart significant risks to CAR T-cell therapy for B-cell malignancies, the most significant adverse events reported in phase I clinical trials are related to elevations in cytokine levels following CAR T-cell infusion (figure 5). The most commonly observed adverse events following CAR T-cell infusion are cytokine release syndrome (CRS), macrophage activation syndrome (MAS), neurological toxicities, tumor lysis syndrome, and B-cell aplasia.

**Cytokine Release Syndrome**

Most patients experience CRS to some degree following CAR T-cell infusion. The activation and proliferation of T-cells after the infusion results in an elevation in the levels of circulating cytokines that causes the systemic inflammatory response known as CRS. T-cell activation and proliferation are necessary mechanisms in ensuring T-cell persistence and efficacy (Fitzgerald et al., 2016; D. W. Lee et al., 2014). Though laboratory markers cannot be reliably used to diagnose CRS, high levels of C-reactive protein (CRP) and ferritin may be observed, along with drastic elevations in cytokine levels (Barrett, Teachey, & Grupp, 2014; Davila et al., 2014; Klinger et al., 2012).
Figure 5: Potential Risks and Common Complications of CAR T-cell Therapy. Though rarely observed in CAR T-cell patients to date, insertional oncogenesis and anaphylaxis are notable risks of the CAR T-cell manufacturing process. The gene-transfer step in CAR T-cell production has the potential to disrupt genetic material and/or gene structure, causing harmful mutagenesis (Sadelain, 2004). Mouse-derived CAR genes and/or the novel structure of recombinant proteins may stimulate an immune response against the infused cells, further damaging the patient’s health (Maus et al., 2013). On-target, off-tumor toxicity occurs when the CAR T-cell target is expressed on healthy tissues, which are then attacked and damaged by the CAR T-cells. In anti-CD19 CAR T-cell therapy, the destruction of the CD19-expressing cell population leads to B-cell aplasia. CRS is the most common adverse event following CAR T-cell infusion. Many, though not all, neurological toxicities are symptoms of CRS. The onset CRS and many neurological toxicities correlate temporally with the peak of T-cell expansion following CAR T-cell infusion (Fitzgerald et al., 2016). Taken from (Bonifant, Jackson, Brentjens, & Curran, 2016).

Patients may exhibit symptoms of CRS hours to days after CAR T-cell infusion. CRS has a heterogenous presentation, and patients may exhibit a wide array of symptoms from high fever to organ failure, that range in severity from mild to fatal (Roskos, Davis, &
Schwab, 2004). Regardless of the severity of CRS, patients are typically hospitalized for observation and treatment (Fitzgerald et al., 2016; Maude, Teachey, Porter, & Grupp, 2015). Careful surveillance of the patient’s condition and immediate treatment of cardiovascular dysfunction and respiratory distress is critical in the treatment of severe CRS. The ultimate objective of treating CRS is to minimize life-threatening, toxic symptoms without interfering with mechanisms of T-cell activation and proliferation (D. W. Lee et al., 2014).

Tocilizumab, an antibody that blocks the receptor of the pro-inflammatory cytokine interleukine-6 (IL-6), has been proven to successfully treat CRS related fever and blood pressure changes over a period of 1-3 days. Tocilizumab can potentially inhibit T-cell activation and proliferation, though this has not yet been proven in a clinical study (Maude et al., 2015). Tocilizumab may also hinder the recognition and treatment of MAS (Shimizu et al., 2012). Consequently, the administration of tocilizumab is restricted to severe CRS cases. Corticosteroids, which are commonly used to treat shock and sparingly used to treat severe CRS, can successfully reverse CRS symptoms, but have the documented effect of eliminating the CAR T-cell population over time (Davila et al., 2014; Kochenderfer et al., 2013; Maude, Barrett, Teachey, & Grupp, 2014). In life threatening CRS cases, patients may be treated with immunosuppressive therapy at the expense of the CAR T-cells’ viability, leading to a relapse of the cancer (Maude et al., 2015).
**Macrophage Activation Syndrome**

MAS is primarily recognized as a potentially fatal condition resulting from systemic inflammatory disorders, particularly in pediatric patients. Patients suffering from MAS often exhibit high fevers, enlargement and/or insufficiencies of the liver and spleen, deficiencies in blood cells (pancytopenia), coagulopathy, and neurological toxicities. Similar to CRS, MAS is the byproduct of cytokine elevations produced by rapid T-cell activation and proliferation. In the context of CAR T-cell therapy, CRS is often discussed as transforming into MAS (Grupp et al., 2013). The finding that both MAS and CRS produce similar cytokine elevation profiles supports this assertion (Maude et al., 2015; Teachey et al., 2013, 2016). However, investigators have yet to establish a concrete relationship between CRS and MAS in the context of CAR T-cell therapy.

**Neurological Toxicities**

Following CAR T-cell infusions, patients may present with a number of neurologic toxicities. The most commonly occurring encephalopathies include confusion and loss of alertness (obtundation) (Fitzgerald et al., 2016; Kochenderfer et al., 2015). These neurological toxicities present later than septic shock-associated encephalopathies, and are believed to be related to the patient’s CRS, although it should be noted that pre-treatment with tocilizumab did not prevent the incidence of neurotoxic events. In most cases, these neurotoxic events are low-grade and self-limiting (Fitzgerald et al., 2016). Severe neurological toxicities are less commonly observed in patients receiving CAR T-cell therapy, but may include seizures, an inability to speak or to comprehend speech.
(aphasia), muscle twitches, and unilateral facial paralysis, among other complications. In a select few cases, severe neurological toxicities were found to be caused by CAR T-cells penetrating the blood-brain-barrier to reside in the cerebral spinal fluid (Hu et al., 2016). However, the cause for most of observed encephalopathies, both mild and severe, following CAR T-cell infusions remains unknown (Davila et al., 2014; Fitzgerald et al., 2016; Kochenderfer et al., 2012, 2015).

**Tumor Lysis Syndrome**

Tumor lysis syndrome, a common complication of many hematologic cancer therapies, occurs when numerous tumor cells release their contents upon destruction. Patients often present with a variety of metabolic abnormalities ranging from mild to fatal in severity, shortly after the therapy takes effect. The severity of tumor lysis syndrome is positively correlated to the patient’s tumor burden. In managing tumor lysis syndrome, maintaining proper renal function and preventing cardiac and neuromuscular dysfunction is of critical importance (Howard, Jones, & Pui, 2011). In CAR T-cell therapy trials, cases of tumor lysis syndrome were typically observed when CAR T-cell populations peaked in-vivo (Grupp et al., 2013).

**B-cell Aplasia**

The primary objective of treating B-cell neoplasms with B-cell targeting CAR T-cells is to eliminate cancerous B-cells by eradicating the patient’s entire B-cell population. Therefore, B-cell aplasia, the absence of B-cells, is an expected result of
successful anti-CD19 CAR T-cell therapy that correlates with the infused cells’ persistence and potency in-vivo. Sufficiently potent anti-CD19 CAR T-cells eliminate the plasma cell population as well, resulting in a state of insufficient antibody production known as hypogammaglobulinemia. Though B-cells are important components of the body’s immune system, B-cell aplasia is a manageable condition that is treated with infusions of antibody precursors known as gamma globulins (Dai, Wang, Lu, & Han, 2016).

THE RESULTS OF SECOND GENERATION, ANTI-CD19 CAR T-CELL THERAPY PHASE I AND IIA CLINICAL TRIALS IN PATIENTS DIAGNOSED WITH B CELL MALIGNANCIES

Second generation, anti-CD19 CAR T-cells are the most extensively studied CAR T-cell construct in clinical trials for patients with B-cell neoplasms. Most phase I and IIa studies with this T-cell design have been conducted in populations of patients diagnosed with high-risk, relapsed and/or refractory B-cell malignancies, for which no curative treatment options were available. Anti-CD19 CAR T-cells have shown the greatest curative potential in B-cell Acute Lymphoblastic Leukemia (B-ALL) patients, with notable success in treating B-cell Chronic Lymphocytic Leukemia (B-CLL), B-cell Non-Hodgkin’s Lymphomas (B-NHL, lymphomas that do not express Reed Sternberg cells), and Multiple Myeloma.
Acute Lymphoblastic Leukemia

B-ALL is a neoplasm of B lymphocyte precursors, known as lymphoblasts, that typically affects children. While most ALL patients are diagnosed with precursor B neoplasms, 15-20% of patients are diagnosed with precursor T-cell neoplasms (S. L. Cooper & Brown, 2015; Hochberg, El-Mallawany, & Cairo, 2014; Shustov, 2012).

Newly diagnosed B-ALL is typically associated with a favorable prognosis in children; approximately 95% of pediatric patients achieve complete remission with standard therapies. However, B-ALL is associated with very poor prognoses in newly diagnosed adults, as well as in patients of all ages diagnosed with relapsed and/or refractory disease (Benjamin & Stein, 2016; Ottmann et al., 2007; Pui & Evans, 2006; Raetz & Bhatla, 2012; Thomas et al., 2010). Allo-HSCT is considered the only curative treatment for patients who fail to achieve sustained complete remissions with blinatumomab (anti-CD19 monoclonal antibody) incorporated chemoimmunotherapy, although eligibility for this treatment is limited. B-ALL that has relapsed or fails to adequately respond to allo-HSCT is considered incurable (Doney et al., 2011; Maude, Frey, et al., 2014).

Phase I clinical trials investigating the effects of second generation anti-CD19 CAR T-cell therapy have yielded successful results in treating children and adults with B-ALL (table 3). Nearly every patient in these clinical trials experienced CRS and B-cell aplasia (Brentjens et al., 2011, 2013; Dai et al., 2015; Grupp et al., 2013; Maude, Frey, et al., 2014)(Grupp et al., 2013; Maude, Frey, et al., 2014). Although preclinical studies showed more potent anti-leukemic effects of CD137 coupled anti-CD19 CAR T-cells in the
eradication of B-ALL, both CD137 expressing CAR T-cells and CD28 expressing CAR T-cells have been studied extensively in clinical trials.

Table 3. Summary of Phase I Clinical Trials for Second Generation, Anti-CD19 CAR T-cell Therapy in Patients Diagnosed with B-ALL

<table>
<thead>
<tr>
<th>Publication</th>
<th>Trial Location</th>
<th>Number of patients</th>
<th>Costimulatory Molecule</th>
<th>Complete Response Rate</th>
<th>Longest Duration of CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Grupp et al., 2013)</td>
<td>UPenn</td>
<td>2</td>
<td>CD 137 (4-1BB)</td>
<td>100%</td>
<td>11+ months</td>
</tr>
<tr>
<td>(Maude, Frey, et al., 2014)</td>
<td>UPenn</td>
<td>30</td>
<td>CD 137 (4-1BB)</td>
<td>90%</td>
<td>24+ months</td>
</tr>
<tr>
<td>(Brentjens et al., 2013)</td>
<td>MSKCC</td>
<td>5</td>
<td>CD28</td>
<td>100%</td>
<td>N/R a</td>
</tr>
<tr>
<td>(Davila et al., 2014)</td>
<td>MSKCC</td>
<td>16</td>
<td>CD28</td>
<td>88%</td>
<td>N/R a</td>
</tr>
<tr>
<td>(D. W. Lee et al., 2015)</td>
<td>NCI</td>
<td>20</td>
<td>CD28</td>
<td>70%</td>
<td>5+ months</td>
</tr>
</tbody>
</table>

a Patients who achieved complete remission went on to receive allo-HSCT. Not Reported (N/R). University of Pennsylvania (UPenn). Memorial Sloan Kettering Cancer Center (MSKCC). National Cancer Institute (NCI).

Most adult patients enrolled in these trials were diagnosed with either relapsed and/or drug-refractory (chemotherapy-refractory and/or blinatumomab-refractory) disease, while children enrolled in these studies were diagnosed with relapsed and/or drug-refractory disease, or relapsed disease after receiving allo-HSCT (Grupp et al., 2013; Maude, Frey, et al., 2014). Unlike the childhood B-ALL investigations, where CAR T-cell therapy is explored as a curative option in children whose cancer relapsed
after receiving allo-HSCT, the objective of CAR T-cell therapy in an adult population is to prepare patients to receive allo-HSCT (Brentjens et al., 2013).

In children who have previously undergone allo-HSCT, the patient-derived T-cell substrates are of the donor’s lineage, and are therefore considered allogeneic. Interestingly, none of the patients who received these allogeneic CAR T-cells, as the result of having undergone an HSCT in the past, developed GVHD (Grupp et al., 2013; Maude et al., 2015). However, a study investigating donor-derived CAR T-cells in adult B-ALL patients found that CAR T-cells engineered from T-cells directly derived from the donor, rather than patient-derived donor lymphocytes, are capable of causing GVHD (Dai et al., 2015).

Patients with higher disease burdens at the time of the engineered T-cell infusion experienced more severe CRS, which typically progressed to MAS (Brentjens et al., 2011; Fitzgerald et al., 2016; Grupp et al., 2013). A small number of patients experienced encephalopathy as well. Though every patient in these studies achieved B-cell aplasia, Maude et al. reported prolonged B-cell aplasia lasting more than a year after CAR T-cell clearance in their large population of both children and adult relapsed and refractory ALL patients (2014). In contrast, Brentjens et al.’s results show an increase in normal B-cell lymphopoiesis associated with the decline of the CAR T-cell population (2011). The difference in these results cannot be attributed to the CAR T-cell design, as both studies utilized CD19-28z CAR T-cells.

Following CAR T-cell infusion, CAR T-cells were found in the cerebrospinal fluid (CSF) of twenty-one patients across two clinical trials. Only 2 of these patients were
diagnosed with central nervous system (CNS) leukemia prior to the modified T-cell infusion (Grupp et al., 2013; Maude, Frey, et al., 2014). Although central nervous system (CNS) involvement is relatively uncommon in B-ALL, it is responsible for 6% of B-ALL relapses. The 1-10% of B-ALL patients with CNS involvement are considered part of the high-risk B-ALL group who often undergo intrathecal therapy (medications administered directly into the spinal canal), cranial irradiation, and allo-HSCTs (Lazarus et al., 2006). The finding of CAR T-cells in the CSF is therefore significant, as their presence may impart immune protection in the CNS, thereby preventing the relapse of B-ALL in the CNS (Pullen et al., 1993). This claim is supported by the finding that two patients with prior CNS leukemia did not experience a relapse of CNS leukemia after the CAR T-cell infusion (Maude, Frey, et al., 2014).

Most B-ALL relapses in these clinical trials were due to insufficient CAR T-cell persistence in-vivo, or due to the emergence of an escape variant. The administration of steroids to manage severe CRS symptoms may have caused the diminished persistence of CAR T-cells in these patients. Though repeated CAR T-cell infusions were found to enhance the persistence in these patients, few patients treated with steroids received this protocol (Davila et al., 2014; Maude et al., 2015). Reasons for this include a lack of eligibility to receive repeated infusions, personal preference, and/or the trial did not examine the effects of repeated CAR T-cell infusions. Relapses unrelated to problems of persistence resulted in a CD19- escape variant cancer. It is possible for patients to have B-cell precursors that lack CD19 (CD19- precursors). In such cases, though anti-CD19 CAR T-cells eliminate CD19+ cells, a cancerous CD19- escape variant, related to the
original CD19+ lineage, can proliferate and result in a relapse of the cancer (Maude et al., 2015).

The CR rates of the newest FDA approved chemotherapy drugs used to treat B-ALL are less than 25% (Maude, Frey, et al., 2014). In contrast, the complete remission rates of CAR T-cell therapy, reported by these clinical trials, is between 70-100%, with documented sustained remissions of up to two years. The results of these studies are therefore significant, and suggest that, with further research, CAR T-cell therapy may be widely employed as a curative treatment for CD19+ ALL.

Multicenter phase I clinical trials, as well as phase IIa clinical trials, are currently underway to further investigate the clinical success of CD19 CAR T-cell therapy in B-ALL patients (Curran et al., 2015).

**Chronic Lymphocytic Leukemia**

The most commonly diagnosed adult leukemia, CLL, is a cancer of mature B-cells (Goldin & Caporaso, 2007; Jemal, Siegel, Xu, & Ward, 2010). Though the presentation and clinical course of CLL varies greatly, abnormalities in the structure or function lymph nodes (lymphadenopathy) commonly accompany a CLL diagnosis (Parikh & Shanafelt, 2016).

The widespread use of chemoimmunotherapy has greatly improved the overall response and progression free survival rates of CLL patients treated with standard of care therapies (Thompson et al., 2016). Despite the remarkable success of chemoimmunotherapy, disease progression is typically anticipated, and often inevitable.
Though allo-HSCT is considered a curative therapy for CLL patients, CLL primarily affects elderly patients, many of whom are ineligible for allo-HSCT. As such, CLL is widely considered an incurable disease (Rozovski et al., 2015).

Four major phase I clinical trials have been conducted by various academic organizations to investigate the therapeutic effects of second generation anti-CD19 CAR T-cell therapy in CLL patients (table 4). Patients enrolled into these studies typically had very advanced CLL that failed to respond to multiple other forms of therapy. Most patients had refractory CLL, CLL that has relapsed multiple times, and/or CLL with chromosomal abnormalities or mutations that rendered their disease aggressive and severe (Brentjens et al., 2011; Kalos et al., 2011; Kochenderfer et al., 2012, 2013; Porter et al., 2015).

Studies that treated patients with lymphocyte depleting chemotherapy prior to administering the CAR T-cell infusion revealed that reductions in CLL tumor cell frequency were either not present or negligible after receiving the preparative chemotherapy. Such results ensure that any observed cancer eradication can be attributed to the efficacy of CAR T-cells, rather than to the anti-tumor effects of the preparative chemotherapy. Every study reported marked reductions in adenopathy, and a significant reduction or clearance of CLL in the bone marrow within one month after the infusion, in patients who exhibited a clinical response. (Brentjens et al., 2011; Kochenderfer et al., 2012; Porter et al., 2011). These studies showed that the clearing of the tumor was related to engineered T-cell trafficking to tumor sites in the lymph nodes, bone marrow, and liver less than 2 days after CAR T-cell infusion (Brentjens et al., 2011).
Kochenderfer et al.’s study published in 2013 investigated the outcomes of donor-derived CAR T-cells in both B-CLL and B-NHL patients whose cancer relapsed after allo-HSCT. Only patients who had not developed GVDH, or who had developed only mild, grade 1 acute GVDH after receiving a donor lymphocyte infusion, were enrolled in this study. T-cells were derived directly from the patient’s lymphocyte transplant donors, rather than the patients themselves. Interestingly, although some patients received transplants from HLA-matched sibling donors, while other patients received transplants from unrelated donors, none of the patients who received the allogeneic CAR T-cell infusion developed GVHD (Kochenderfer et al., 2013).

Table 4. Summary of Phase I Clinical Trials for Second Generation, Anti-CD19 CAR T-cell Therapy in Patients Diagnosed with B-CLL

<table>
<thead>
<tr>
<th>Publication</th>
<th>Trial Location</th>
<th>Number of patients enrolled</th>
<th>Costimulatory Molecule</th>
<th>Pre-treatment</th>
<th>Number of patients who Achieved SD (longest duration of SD)</th>
<th>Number of patients who Achieved PR (longest duration of PR)</th>
<th>Number of patients who Achieved CR (longest duration of CR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Brentjens et al., 2011)</td>
<td>MSKCC</td>
<td>8</td>
<td>CD28</td>
<td></td>
<td>Group 1: No&lt;sup&gt;a&lt;/sup&gt; 1 (4+ months)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(Kochenderfer et al., 2012)</td>
<td>NCI</td>
<td>4</td>
<td>CD28</td>
<td>Yes</td>
<td>1 (6 months)</td>
<td>2 (7+ months)</td>
<td>1 (15+ months)</td>
</tr>
<tr>
<td>(Kochenderfer et al., 2013)</td>
<td>NCI</td>
<td>4</td>
<td>CD28</td>
<td>No</td>
<td>1 (3 months)</td>
<td>0</td>
<td>1 (9+ months)</td>
</tr>
<tr>
<td>(Porter et al., 2015)</td>
<td>UPenn</td>
<td>14</td>
<td>CD 137</td>
<td>Yes</td>
<td>N/R&lt;sup&gt;b&lt;/sup&gt; 4 (13+ months)</td>
<td>4 (53+ months)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Dose escalation study in which group one was treated with dose-escalating CAR T-cell therapy doses without preparative chemotherapy, and group 2 was pre-treated with dose-escalating chemotherapy prior to a constant dose of CAR T-cell infusion.

<sup>b</sup>Stable disease status not reported. Four patients are alive with disease. Longest reported duration of patient alive with disease is 26 months.
Early studies report elevations in cytokine levels and the clinical presentation of what is now referred to as CRS. In a study published in 2011, the death of one patient within two days after receiving the CAR T-cell infusion was attributed to a possible pre-existing infection (Brentjens et al., 2011). It is possible that the patient’s symptoms and abnormal cytokine profile may have been associated with undiagnosed CRS. Though few studies report severe cases of CRS, a better understanding of CRS and the use of tocilizumab could have aided these early clinical trials in improving the patient’s CRS symptoms and promoting CAR T-cell persistence.

It was generally observed that the development of CRS resulted in a delayed clinical response to the CAR T-cell infusion (Kalos et al., 2011; Kochenderfer et al., 2013; Porter et al., 2015, 2011). A small number of patients who developed severe CRS went on to develop MAS (Porter et al., 2015).

Notably, two patients in a study conducted at the National Cancer Institute (NCI) did not experience any cytokine mediated toxicities. However, neither of these patients achieved sustained remissions after receiving CAR T-cell infusions, and both received alternative treatments for their progressing CLL (Kochenderfer et al., 2013). CAR T-cells were not detected in one of these two patients after infusion, supporting the suggestion that some level of CRS is necessary for the proliferation and persistence of the CAR T-cell population (Davila et al., 2014; Kochenderfer et al., 2013). Other common adverse events reported in this study include tumor lysis syndrome and neurologic toxicities. The onset of tumor lysis syndrome also further delayed the patient’s clinical response to the engineered T-cell infusion (Kochenderfer et al., 2013; Porter et al., 2011). The few
documented occurrences of neurologic toxicities were transient, and likely caused by CRS-associated high fevers (Porter et al., 2015).

Brentjens et al. report that the anti-CD19 CAR T-cell infusion in three of the four patients studied greatly reduced the tumor burden and/or lymphadenopathy without the onset of B-cell aplasia (2011). However, CAR T-cell therapy failed to induce complete or partial remissions in any of these patients, suggesting that B-cell aplasia is a necessary anti-leukemic event in the successful course of CAR T-cell therapy (Brentjens et al., 2011; Nazimuddin et al., 2013). This is supported by Porter et al.’s finding that long term B-cell aplasia is directly related to long term CAR T-cell persistence, which subsequently allows for both effector and central memory CAR expressing T-cells to partake in long term immunosurveillance (Porter et al., 2015).

Nearly every patient who failed to respond to the CAR T-cell infusion progressed or died within a year following the infusion. This result further establishes the very advanced nature of CLL in patients enrolled in these studies. In CLL patients who initially achieved partial remissions (PRs), the cancer ultimately progressed for one of three reasons: 1) A preexisting condition, unrelated to the patient’s CLL, resulting in either necessary treatment that interfered with the CAR T-cell population persistence, or the patient’s death. 2) The patient’s CLL transformed into another cancer type (histologic transformation) that was unresponsive to the CAR T-cell therapy. 3) The CAR T-cells failed to persist in-vivo. None of the patients who achieved CR following the engineered T-cell infusion relapsed. The only death of a patient who achieved CR with CAR T-cell therapy is attributed to a complication following surgery for basal cell carcinoma,
unrelated to the patient’s CLL (Brentjens et al., 2011; Kochenderfer et al., 2012, 2013; Porter et al., 2015).

The use of the CD28 costimulatory domain in CAR T-cell design in Kochenderfer et al.’s study, published in 2012, is particularly significant, as CAR T-cell infusions were followed by the administration of IL-2 until the patient’s cytokine mediated toxicities necessitated its cessation (Kochenderfer et al., 2012). Preclinical studies have shown that CD28 costimulation elevates IL-2 levels, and that high IL-2 can impede the effector activity of CAR T-cells (D. W. Lee et al., 2014; Milone et al., 2009). Additionally, patients treated with IL-2 alone have been shown to experience cytokine toxicities (Panelli et al., 2004). Although the course of cytokine elevations in patients treated with both CAR T-cells and IL-2 differs from temporally from the typical course of IL-2 related cytokine storm, it is possible that the combined effects of CD28 costimulation and exogenous IL-2 administration exaggerated cytokine elevations in these patients (Kochenderfer et al., 2012; D. W. Lee et al., 2014). It is therefore possible that withholding IL-2 treatment might have enhanced the outcomes of this trial.

The results of most phase I clinical trials investigating CAR T-cell therapy in CLL patients reveal that disease progression relates temporally with the loss of the detectible engineered T-cell populations in the patient. Disease progression also coincided with lymphadenopathy reduction (Brentjens et al., 2011; Kalos et al., 2011; Kochenderfer et al., 2012). Although complete remissions were only induced in 6 of the 30 enrolled patients, the ability to induce lasting, complete remissions in patients who do not require further therapy is a significant stride in treating this “incurable” cancer. More
significant still, is the ability to induce remissions in patients who relapsed after receiving allo-HSCTs, as these transplants were considered the only curative treatment option for CLL. Research has, thus far, proven that CAR T-cell therapy is capable of ablating large tumors in patients with rapidly progressing, advanced, CLL.

Dr. Porter’s research group at the University of Pennsylvania is currently conducting a phase II dose-optimization trial to further study the clinical efficacy of CAR T-cell therapy in the treatment of CLL (Porter et al., 2013).

Non-Hodgkin’s Lymphomas

The great majority of published results from phase I clinical trials investigating the therapeutic efficacy of second generation CAR T-cell therapy in non-Hodgkin’s Lymphoma (NHL) has come from Kochenderfer’s research group at the National Cancer Institute (table 5). These trials utilized CD28-coupled anti-CD19 CAR T-cells, and comprised a study population of patients diagnosed with Mantle Cell Lymphoma (MCL), Follicular Cell Lymphoma (FCL), Splenic Marginal Zone Lymphoma (SMZL), and Diffuse Large B-cell Lymphoma (DLBCL). Most patients in these trials experienced some degree of CRS. With the exception of notable neurologic toxicities, all adverse events resolved within three weeks after the CAR T-cell infusion (Kochenderfer et al., 2012, 2013, 2015).
Table 5: Summary of Phase I Clinical Trials for Second Generation, Anti-CD19 CAR T-cell Therapy in Patients Diagnosed with NHL

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>Number of patients</th>
<th>Number of patients who achieved complete remission</th>
<th>Number of patients who achieved partial remission</th>
<th>Longest recorded period of complete remission (months)</th>
<th>Longest recorded period of partial remission (months)</th>
<th>Longest recorded period of stable disease (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMZL</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>23+</td>
<td>0</td>
</tr>
<tr>
<td>PMBCL</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>N/R</td>
<td>22+</td>
<td>1</td>
</tr>
<tr>
<td>DLBCL (not otherwise specified)</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>9+</td>
<td>1</td>
<td>11+</td>
</tr>
<tr>
<td>Follicular Cell Lymphoma</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>18+</td>
<td>0</td>
</tr>
<tr>
<td>Mantle Cell Lymphoma</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3+</td>
<td>3+</td>
</tr>
</tbody>
</table>

N/R = not reported

MCL is non-Hodgkin’s B-cell cancer that primarily affects elderly patients, and occurs more often in men than in women. The highly aggressive nature of this cancer renders it very difficult, in many cases impossible, to cure. While patients with low-risk MCL tend to respond well to therapy, most patients present with advanced MCL at their initial diagnosis (Geisler et al., 2010). A widely accepted, standard of care chemoimmunotherapy protocol to treat MCL has not yet been established (Bernstein et al., 2013; Delarue et al., 2013; Geisler et al., 2008; Romaguera et al., 2005). Younger patients diagnosed with MCL typically receive intensive chemoimmunotherapy followed
by autologous HSCT, while older patients may be treated less intensive, single agent or combination chemotherapy, or chemoimmunotherapy (Kluin-Nelemans et al., 2012; Y. Liu, Zhang, & Zhong, 2015; Ruan et al., 2015). Despite varied success of salvage chemoimmunotherapy, recently developed kinase inhibitors, and immunomodulatory agents, autologous HSCT is hailed as the only potentially curative therapy for high-risk MCL (Y. Liu et al., 2015; M. Wang et al., 2012).

The incidence of FCL, a relatively common, slow-growing (indolent) NHL, is positively correlated with advancing age. Patients often do not present with apparent symptoms at the time of their initial diagnosis (Solal-Céligny et al., 2004). In certain cases, FCL may undergo a histologic transformation in which this indolent cancer becomes a very aggressive lymphoma, such as diffuse large B-cell lymphoma (Giné et al., 2006).

The indolent nature of FCL renders the “watch and wait” approach to treatment ideal for patients with early stage cancer. In this case, therapy will not be initiated until the cancer progresses. Patients who undergo treatment often receive radiation or induction chemoimmunotherapy, and maintenance therapy with a combination of rituximab and cancer-targeting radioactive particles (Krause, Debus, & Neuhof, 2011). Aggressive salvage therapy regimens followed by autologous stem cell transplants are becoming more common methods of treating relapsed FCL. In patients for whom autologous stem cell transplantation has failed, allo-HSCT may be approached as the next therapeutic option. Further therapeutic options for FCL patients who relapse following allo-HSCT do not yet exist (Tarella et al., 2015).
Splenic marginal zone lymphoma is a rare, low-grade lymphoma that typically affects the elderly, accounts for less than 1% of non-Hodgkin lymphomas (Zucca, Bertoni, Roggero, & Cavalli, 1998). SMZL arises in the spleen and invades the bone marrow, peripheral blood, lymph nodes, and less commonly, the liver, as it progresses (Franco, Florena, & Iannitto, 2003). Due to its rare occurrence and indolent nature, few prospective trials have been conducted to investigate successful treatments for this neoplasm. As such, a definitive standard of care for SMZL has yet to be established. However, in nearly one third of the SMZL diagnosed patients, the cancer progresses so slowly that treatment is never required (Franco et al., 2003). The “wait and watch” approach is often employed in the treatment of these patients; therapy is not administered until the patients exhibit concerning symptoms of disease. In cases where SMZL is caused by hepatitis C infection, treating the patient for hepatitis C can effectively cure the patient of SMZL (F. Berger et al., 2000; Catovsky & Matutes, 1999a; Dreyling et al., 2013).

Four treatment modalities are commonly employed in cases where SMZL directed treatment is necessary. Splenectomy, the most effective treatment for slowly progressing, early stage SMZL, is the first of four possible methods of treatment (Catovsky & Matutes, 1999b). Disadvantages to this approach include, long-term immunosuppression resulting from the loss of the spleen, and the consequent increased risk of infection (Lenglet et al., 2014; Xing et al., 2015). Single agent rituximab therapy, the second standard treatment method, has replaced chemotherapy as the preferred treatment in patients with non-metastasized SMZL who are ineligible for splenectomy (Dreyling et
Chemoimmunotherapy, the third modality of treatment, is the preferred course for patients with advanced stage and/or aggressive SMZL at the time of diagnosis (Bennett, Yegena, Dave, & Schechter, 2008). Although there are several documented cases of patients achieving complete remissions, an optimal, curative combination of chemotherapeutic agents for aggressive induction therapy has not yet been established (Franco et al., 2003). Pancytopenia, among other conditions, can render a patient ineligible to receive chemotherapy. The fourth treatment modality, irradiation, is the preferred method for treating newly diagnosed SMZL patients who are ineligible to receive chemotherapy. Although the benefits of splenic irradiation have been reported in only a small population of patients, studies show that low-dose radiotherapy can diminish the presence of various signs and symptoms of the cancer (El Weshi et al., 1998). Chemoimmunotherapy regimens are often administered as salvage therapy for relapsed and/or refractory SMZL patients.

Kochenderfer et al.’s investigation, published in 2012, studied the effects of CD28 coupled CAR T-cells in four CLL patients, as well as in three FCL patients, and one SMZL patient. The concerns of possible IL-2 mediated unfavorable outcomes, noted in the analysis of CLL patients enrolled in this trial, apply to NHL patients as well. Although investigators in this study assert that patients with severe CRS did not present with infections that could be concretely linked to sepsis via elevated cytokine levels, it must be noted that one FCL patient died of viral infection, bacterial infection, and cerebral infarction, among other conditions. In light of this patient’s late-stage, progressing cancer, it is possible that the patient’s death is unrelated to the CAR T-cell
infusion. Later investigations studying the extremely varied clinical presentation and physiological effects of CRS indicate that it is possible that CRS, or other adverse events that are commonly observed after CAR T-cell infusion, may have contributed to the patient’s death soon after the engineered T-cell infusion (Fitzgerald et al., 2016).

Ultimately, this study produced PRs in two of the three FCL patients, revealing that it is possible to achieve a PR lasting greater than eighteen months with CAR T-cell therapy in this patient population. Additionally, the longest duration of B-cell depletion in this group was found to last 36 weeks. The significance of the period of B-cell deletion, and its relevance to engineered T-cell persistence, was not determined in this study. Notably, one FCL patient who achieved PR with a first round of CAR T-cell therapy relapsed within six months of a second CAR T-cell infusion for unknown reasons. As such, the relationship between this relapse and the persistence of CAR T-cells, B-cell aplasia, or the possibility of an escape variant, was not established in this study. The SMZL patient’s progress was reported in a later publication by this same group (Kochenderfer et al., 2012).

The most commonly occurring NHL, DLBCL is consequently the most extensively studied type of lymphoma with regards to CAR T-cell therapy. DLBCL is a highly aggressive cancer characterized by proliferating neoplastic masses in the lymph nodes, bone marrow, spleen, and less commonly, other organs (Schneider, Pasqualucci, & Dalla-Favera, 2011). Subtypes of DLBCL are named according to their location of origin, and the treatment for DLBCL varies according to the designated subtype. Primary Mediastinal B-Cell Lymphoma (PMBCL), for example, is a DLBCL originating in
thymic B-cells. The treatment for PMBCL differs slightly from treatments for other diffuse large B-cell lymphomas (DLBCLs). Though a standard treatment protocol for PMBCL has not been established, chemoimmunotherapy and radiation are commonly administered (Dunleavy & Wilson, 2015).

While some elderly patients diagnosed with DLBCL may undergo radiation therapy for bulky disease (Held et al., 2014), multi-agent chemoimmunotherapy have become the norm in the treatment of DLBCL. Although the four-year survival rate for patients initially diagnosed with DLBCL is around 65%, approximately one-third of patients diagnosed with DLBCL will develop relapsed or refractory disease. While the prognosis for patients with relapsed disease is poor, the prognosis for patients with chemotherapy refractory and/or rituximab refractory DLBCL is poorer still. If eligible, patients with relapsed and/or refractory disease may receive autologous HSCT following salvage therapy. Although patients with drug-refractory DLBCL are less likely to achieve CR with salvage therapy, studies have shown that autologous HSCT in this patient group are comparable to the results of this treatment modality in chemotherapy-sensitive relapsed patients (Guglielmi et al., 1998; Kewalramani et al., 2000). Patients with chemoimmunotherapy-sensitive cancer who have relapsed within one year after induction therapy (early relapse) have lower overall survival rates and progression free survival rates compared to late relapsing patients (Hamadani et al., 2014; Mounier et al., 2012). An effective, curative therapy for early relapsed DLBCL and refractory DLBCL has yet to be established.
A 2013-published study investigated the success of CAR T-cell therapy in two DLBCL patients and 4 MCL patients, all of whom have disease that has relapsed after receiving allo-HSCT (Kochenderfer et al., 2013). In contrast to Kochenderfer et al.’s study published in 2011, patients in this clinical trial did not receive lymphodepleting chemotherapy prior to CAR T-cell infusions, or IL-2 treatment following the infusion, and donor lymphocytes, rather than autologous lymphocytes, served as the T-cell substrate (Kochenderfer et al., 2013).

Notably, zero NHL patients in this study experienced high-grade adverse events or GVHD following the infusion. Only one MCL patient who was diagnosed with stable disease before enrollment achieved partial remission with CAR T-cell therapy. One MCL patient with partial regression at the time of enrollment was found to have stable disease persisting longer than three months after CAR T-cell infusion, and the malignancy status of the remaining four patients did not change with this therapy (Kochenderfer et al., 2013). The results of this study reveal that a single course of second generation CD28 coupled anti-CD19 CAR T-cell therapy is not a cure for MCL. It is possible, however, that the repeated administration of CAR T-cell infusions may improve the malignancy status of patients with MCL.

The progress of the SMZL patient from Kochenderfer et al.’s 2012 publication was reported again in 2015. Kochenderfer’s group studied a single SMZL patient, who received three therapies prior to CAR T-cell infusion (Kochenderfer et al., 2015). The ability of CAR T-cell therapy to induce a partial remission lasting longer than 23 months is therefore a significant advancement in the treatment of high-risk SMZL.
Although Kochenderfer et al.’s 2015 study population was comprised of NHL and CLL patients with varying risks levels of malignancy, each patient received between two and twelve prior therapies for their cancer. All but two patients in this study had chemotherapy refractory disease. The patients without chemotherapy refractory disease were diagnosed with relapsed disease after receiving autologous HSCT (Kochenderfer et al., 2015).

Two PMBCL patients and one DLBCL patient in this study exhibited notable adverse events that have not been previously reported in CAR T-cell therapy clinical trials for NHL patients. Neurologic toxicities unique to this study include aphasia, unilateral facial paresis, confusion and severe myoclonus that resolved within two weeks after the T-cell infusion, a mild tremor that resolved within one month post-infusion, and facial spasms and apraxia that resolved within 20 days post CAR T-cell infusion. Though the cause for these adverse events remains unknown, the low and intermediate malignancy-risk PMBCL patients were able to achieve CR and PR, respectively, while the high risk DLBCL patient achieved a status of SD after the CAR T-cell infusion (Kochenderfer et al., 2015).

Kochenderfer’s 2015-published study reported the results of anti-CD19 CAR T-cell infusions in a total of eleven DLBCL patients. Four of these patients were diagnosed with PMBCL, and one DLBCL patient acquired this lymphoma as the result of a Richter histological transformation of CLL. Autologous stem cell transplantation has long been considered the only curative therapy for high-risk DLBCL (Kewalramani et al., 2000). The successful results of this study are, therefore, remarkable. A PMBCL patient who
was diagnosed with stable disease after 6 cycles of chemoimmunotherapy, followed by radiation therapy, and an additional two cycles of chemoimmunotherapy, achieved a complete remission, lasting greater than 22 months following CAR T-cell therapy. In another example, a patient with high-risk chemotherapy-refractory DLBCL who received five prior therapies was able to achieve a CR lasting longer than 9 months. All but one patient, a high-risk PMBCL patient with SD lasting one month, achieved either PR or CR with CAR T-cell therapy in this trial (Kochenderfer et al., 2015). The results of this study indicate that CAR T-cell therapy may be curative for high-risk DLBCL.

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>3-month overall response rate</th>
<th>Progression free survival rate at 11.7 months (median follow up)</th>
<th>Response duration at 11.7 months (median follow up)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLBCL</td>
<td>54%</td>
<td>43%</td>
<td>83%</td>
</tr>
<tr>
<td>MCL</td>
<td>50%</td>
<td>N/R</td>
<td>N/R</td>
</tr>
<tr>
<td>FCL</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 6. Results of Schuster et al.’s Phase IIa Clinical Trial

The results of a Phase IIa clinical trial conducted at the University of Pennsylvania on a population of twenty-one DLBCL patients, fourteen FCL patients, and three MCL patients treated with CD137 coupled anti-CD19 CAR T-cells are presented in table 6. The study population consisted of patients with high-risk relapsed or refractory lymphoma for which no curative treatments were available. Only one patient in this study
developed severe CRS, and one patient experienced a severe neurologic toxicity (encephalitis). Although the extent of the malignancy for each patient following the therapy was not reported, treatment of high-risk FCL patients with anti-CD19 T-cells has shown the greatest promise in this trial (Schuster et al., 2015).

Ongoing multi-center phase I clinical trials, as well as phase IIa clinical trials are being conducted to further evaluate the potential curative effects of CD19 CAR T-cell therapy in NHL patients (Locke et al., 2015).

**Multiple Myeloma**

Multiple myeloma is a cancer of plasma cells that presents with greatest incidence in elderly patients of African descent. Multiple myelomas account for approximately 15-20% of all diagnosed hematologic malignancies, making it the second most commonly occurring hematologic neoplasm (Becker et al., 2007). The malignancy originates when genetic material is aberrantly altered during the maturation and differentiation of stem cells along the B-cell lineage within the bone marrow. Consequently, as the malignant plasma cell population expands, it invades the bone marrow and the bone, causing masses of plasma cells (plasmacytomas) that develop on bones. Patients with plasmacytomas are commonly treated with radiation (Krause et al., 2011).

In the course of multiple myeloma progression, the expanding malignant plasma cell population produces large amounts of the abnormal immunoglobulin known as myeloma protein (M Protein). The abundance of M protein in blood can lead to kidney damage, dangerously viscous blood, and dampened immune responses. Due to the slow
growing nature of multiple myeloma, as well as the concerning toxicities, patients may not receive treatment until harmful levels of M protein are observed (Kusenda & Kovarikova, 2016).

Novel chemoimmunotherapeutic agents, as well as the routine administration of allo-HSCT, when applicable, have shown great success in prolonging the survival of relapsed and refractory myeloma patients, achieving a 17-month median survival from the first diagnosis of relapsed disease (Rosenzweig & Krishnan, 2016). The mechanism behind the neoplasm’s repeated recurrence, in spite of these therapies, is the persistent presence of minimal residual disease over long periods of time, following treatment with standard therapies (Hsu et al., 1997).

A published case study of a single patient with persistent disease following autologous stem cell transplantation revealed that second generation CD137 coupled anti-CD19 CAR T-cells may be beneficial in the treatment of multiple myeloma. Interestingly, multiple myeloma is considered a CD19- cancer. While only 0.05% of the patient’s malignant plasma cells expressed CD19, this level of expression was enough to obtain a significant response to the CAR T-cell infusion. In this study, the patient received myeloablating chemotherapy, followed by autologous stem cell transplantation, and finally a CAR T-cell infusion. The successful expansion of the engineered T-cell population in-vivo did not cause CRS in this patient, although cytokine level rises were observed. B-cell aplasia was observed earlier than it would normally occur with chemotherapeutic agents alone, indicating successful on-target effects of the CAR T-cells. The patient regained normal hematopoietic function, and non-malignant B-cell
development following the decline of the CAR T-cell population, indicating that the prolonged persistence past 47 days of CAR T-cells in-vivo is not required for the curative success of this therapy (Garfall, Maus, Hwang, et al., 2015).

Further investigations conducted by Garfall et al. following the same protocol of chemotherapy, autologous stem cell reinfusion, and CAR T-cell infusion achieved complete remissions in two of three evaluable multiple myeloma patients (Garfall, Maus, Lacey, et al., 2015). The results of these studies indicate that CAR T-cell therapy, in conjunction with autologous stem cell transplantation, can be curative for multiple myeloma patients.

THIRD AND FOURTH GENERATION CAR T-CELLS

The addition of a second and/or third costimulatory molecule(s) to the CAR structure has successfully enhanced CAR T-cell activation in preclinical models (Pulè et al., 2005b; J. Wang et al., 2007). The initial results of a phase I/IIa clinical trial using third generation anti-CD19 CAR T-cells coupled to both CD28 and 4-1BB costimulatory molecules in eleven patients with relapsed and/or refractory B-cell neoplasms, for whom no other curative options were available, are less promising. Six patients in this study were pre-treated with mild chemotherapy. The study revealed that the combination of two costimulatory molecules enhanced T-cell activation. However, the six patients relapsed within three months of receiving the infusion. The causes for these relapses are currently being investigated in this ongoing clinical trial (Enblad et al., 2016). While Enblad et al.’s results do not indicate any additional clinical benefits when compared to second
generation anti-CD19 CAR T-cells, third generation anti-CD20 cells have shown improved cytotoxic effects and in-vivo persistence when compared to their second generation counterparts (Till et al., 2012). Third and fourth generation CAR T-cell designs continue to be investigated in both in-vitro models, as well as in clinical trials.

ALTERNATIVE CAR TARGETS

The decreased B-cell expression of CD19 in many lymphomas, as well as relapses of CD19- ALL following CAR T-cell infusions, necessitates the study of other CAR targets. Research suggests that designing CAR T-cells against targets other than CD19 may also improve the safety of CAR T-cells. CAR T-cell targets currently under investigation include cell surface markers, immunoglobulin light chains, and other tumor associated proteins (table 7).
<table>
<thead>
<tr>
<th>Alternative CAR T-cell Therapy Targets</th>
<th>Suggested for Patients Diagnosed With...</th>
<th>Reasoning</th>
<th>Status of Current Investigations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD20</td>
<td>• B-NHL</td>
<td>Rituximab has been a successful therapy against B-cell malignancies (Vitolo et al., 2012).</td>
<td>Phase I Clinical Trials (Till et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>• B-ALL</td>
<td>• Rituximab does not cause hypogammaglobulinemia (DiLillo et al., 2008).</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• CD20 expressed on most B-NHL and B-ALL cells (Davis, Czerwinski, &amp; Levy, 1999; Thomas et al., 2009).</td>
<td></td>
</tr>
<tr>
<td>CD30</td>
<td>• NHL</td>
<td>Expressed on NHLs, Reed Sternberg Cells, B and T-cell leukemias (Di Stasi et al., 2009; A. Hombach et al., 1998, 2001).</td>
<td>Phase I Clinical Trials (C.-M. Wang et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>• Hodgkin’s Lymphoma</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>• B-cell Leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• T-cell Leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig Light Chains</td>
<td>• B-cell Neoplasms</td>
<td>Malignant B-cells express an abnormal IgK/ Igλ ratio; this property can be exploited to target cancerous B-cells (Bergón, Miravalle, Bergón, Miranda, &amp; Bergón, 2005).</td>
<td>Preclinical Studies with Xenograft models</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Targeting Ig Light chains would not induce B-cell aplasia, or hypogammaglobulinemia, thereby protecting the patient’s infection-fighting abilities (Vera et al., 2006).</td>
<td></td>
</tr>
<tr>
<td>BCMA</td>
<td>• Multiple Myeloma</td>
<td>Plasma cells lack significant CD19, CD20, CD22, and Ig light chain expression (Craig &amp; Foon, 2008).</td>
<td>Phase I Clinical Trials (Carpenter et al., 2013; Fesnak et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• B-cell maturation antigen (BCMA) is almost exclusively expressed on plasma cells.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Can eliminate plasma cells without inducing B-cell aplasia (Novak et al., 2004).</td>
<td></td>
</tr>
</tbody>
</table>
CD138

- Multiple myeloma
- CD138 is expressed on plasma cells (Kawano et al., 2012).
- Malignancy regressed in four out of five evaluable patients in an ongoing clinical trial with second generation 4-1BB coupled anti-CD138 CAR T-cells.

Phase I clinical Trials (Guo et al., 2016).

ROR1

- NHL
- B-ALL
- Abnormally expressed receptor tyrosine kinase-like orphan receptor (ROR1) is a characteristic of many CLLs, mantle cell lymphomas, B-ALLs (Hudecek et al., 2010).

Phase I Clinical Trials (Deniger et al., 2015).

CD22

- DLBCL
- FCL
- NHL
- B-ALL
- Many NHLs and B-ALLs express CD22 (Polson et al., 2010).

Phase I Clinical Trials (Fesnak et al., 2016; Haso et al., 2013).

CD33

- AML
- Monoclonal anti-CD33 antibodies have been investigated as a therapy for Acute Myeloid Leukemia, a cancer caused by acquired mutations in bone marrow stem cells (Estey, Estey, & Estey, 2012; Jurcic, 2012).

Phase I Clinical Trials (O’Hear, Heiber, Schubert, Fey, & Geiger, 2015; Q. Wang et al., 2015, p. 33)

The revolutionary success of rituximab in the treatment of B-cell neoplasms inspired the anticipated success of anti-CD20 CAR T-cells. Rituximab has not been shown to cause CD20- neoplastic escape variants or severe hypogammaglobulinemia, suggesting that anti-CD20 CAR T-cells may prove to be safer and more effective alternatives anti-CD19 CAR T-cells (DiLillo et al., 2008). However, early clinical trials failed due to unstable CAR expression, inadequate in-vivo persistence, and a failure to mount a significant response against CD20+ B-cells in-vivo (Till et al., 2008). Second generation anti-CD20 CAR T-cells that succeeded in inducing partial remissions in
DLBCL patients caused notable on-target off-tumor damage at sites of the cancer’s metastasis (Y. Wang et al., 2014). Though clinical trials of third generation anti-CD20 CAR T-cells failed to obtain high levels of stable CAR expression, the therapy induced a response in MCL and indolent NHL, and was well tolerated by most patients in the study (Till et al., 2012). It is possible that transferring CAR genes through methods other than retroviral or lentiviral transduction will induce more stable expression of engineered receptors on anti-CD20 CAR T-cells, thereby supporting the future success of this target.

While CD30 is expressed on many NHLs, Hodgkin’s Lymphomas, and B and T-cell leukemias, it is also expressed on healthy progenitor cells and hematopoietic stem cells (HPSCs), allowing for potentially harmful on-target off-tumor effects of anti-CD30 CAR T-cell therapy. In in-vitro preclinical studies, anti-CD30 CAR T-cells significantly eliminated both non-Hodgkin’s and Hodgkin’s lymphoma cells. In-vivo preclinical studies revealed that the eradication of lymphoma cells with anti-CD30 CAR T-cells is not coupled to B-cell aplasia (Di Stasi et al., 2009; A. Hombach et al., 1998, 2001). HPSCs were found to only minimally express CD30 in these studies, and thus both normal lymphopoiesis and hematopoiesis was conserved following engineered T-cell induction (A. A. Hombach et al., 2016). Phase I clinical trials to study anti-CD30 CAR T-cells in lymphoma patients are currently being conducted.

Despite the great clinical successes of CD19 targeting CAR T-cells, the search for a target that will confer maximal on-tumor effects with minimal additional toxicity continues. B-cells express a light chain of either immunoglobulin-K (IgK), or immunoglobulin-λ (Igλ). The ratio of IgK/Igλ-expressing B-cells is maintained within a
narrow range in healthy humans, and deviation from this ratio is indicative of malignant clonal expansion (Bergón, Miravalles, Bergón, Miranda, & Bergón, 2005). Light chains, therefore, present an attractive target for CAR T-cell therapy. Second generation anti-IgK CAR T-cells with a CD28 costimulatory domain have been shown to successfully eradicate human lymphocyte-derived B-cells (Vera et al., 2006). These promising results indicate that future clinical trials will likely investigate the curative potential of light-chain-targeting CAR T-cells in patients with advanced B-cell malignancies.

Abnormally expressed receptor tyrosine kinase-like orphan receptor (ROR1), a protein known to assist cancerous cells in evading natural immune responses, is expressed on many B-cell malignancies and solid tumors (Hudecek et al., 2010). Anti-ROR1 CAR T-cells successfully eradicated chemotherapy-refractory B-CLL and MCL in non-human primate models, and is currently being studied for its safety and clinical efficacy in a phase I clinical trial (Deniger et al., 2015; Hudecek et al., 2010, p. 1).

**IMPROVING CAR T-CELL THERAPY FOR THE TREATMENT OF B-CELL MALIGNANCIES**

*Improving the CAR T-cell Manufacturing*

Currently, most manufacturing protocols enrich for CD3+ T-cells to serve as substrates for engineered T-cell production (Kalos et al., 2011; Kochenderfer et al., 2010; Savoldo et al., 2011). Growing evidence suggests that selecting for specific subsets of T-cells may improve the proliferative potential and persistence of CAR T-cells in-vivo.
Advantages to studying memory T-cell populations include their ability to quickly expand in response to antigenic stimulation, and the existing tumor specificity of memory T-cells derived from cancer patients. In theory, providing the patient differentiated, effector CAR T-cells prepared to mount an immediate response against cancer cells would be the most effective way to quickly eradicate the malignancy. However, effector T-cells have inferior proliferative abilities in comparison to memory and naïve T-cell groups. Studies in primate and murine models revealed that effector cells derived from T\textsubscript{CM} populations resisted T-cell exhaustion and conferred immunologic memory in-vivo more successfully than T\textsubscript{EM} derived effector cells (C. Berger et al., 2008; Klebanoff et al., 2005). In-vitro experiments and in-vivo studies in murine models revealed that effector cells derived from naïve T-cells are less differentiated, express fewer markers of T-cell exhaustion, and have longer telomeres, all of which confer greater proliferative success than memory T-cell substrates (Hinrichs et al., 2009, 2011). Recent clinical studies confirm that naïve T\textsubscript{C} cell-derived effector cells are superior substrates for CAR T-cell therapy in their cytotoxic potency and proliferative potential (Nguyen et al., 2016). Future advancements in CAR T-cell therapy will likely select for naïve T\textsubscript{C} cell-derived effector substrates.

Common setbacks currently hindering the success of CAR T-cell therapy’s efficacy include varying rates of gene transfer, and unreliable CAR expression. While the transposon/transposase system of gene transfer is currently gaining popularity as a safer, more cost-effective alternative to retroviral transduction, messenger RNA (mRNA) transfer may prove to be a more successful method of gene transfer for CAR T-cell
therapy. In this method, mRNA is introduced into the cell by non-viral methods, most often via endocytosis, microinjection, or electroporation. Transcription can then be induced in-vitro. This method does not involve genomic integration, and results in stable, transient transgene expression. Messenger RNA transfer-mediated gene expression therefore circumvents potential genotoxicity problems associated with retroviral transduction (Rowley, Monie, Hung, & Wu, 2009). This process has successfully promoted protein expression in hematopoietic progenitor cells (Wiehe et al., 2007), and has been studied for TCR expression in TCR therapy as well (Zhao et al., 2006). It is currently being investigated in preclinical studies for CAR T-cells, as a method of examining the potential cross reactivity of CARs and healthy human tissues (Beatty et al., 2014).

**Improving CAR T-cell Efficacy**

Many scientists have asserted that the success of CAR T-cell therapy is related to the patient’s tumor burden at the time of infusion (Davila & Brentjens, 2013; Gattinoni et al., 2005; Kochenderfer et al., 2012). The extremely varied responses of patients within study groups indicates that further advancements of CAR T-cell therapy rely on a concrete understanding of how various disease factors impact a patient’s response to therapy. Such an understanding can further support the success of CAR T-cell therapy in the arena of personalized medicine, by tailoring pre-treatments, dosages of CAR T-cells, and receptor targets to each patient’s malignancy and health status.
Administering two or more sets of CAR T-cells in an infusion, each recognizing a different target, may mitigate the relapse of cancer due to downregulated CD19 variants (Sotillo et al., 2015). Anti-CD123 CAR T-cells successfully eradicated CD19- B-ALL in murine models. Additional preclinical studies show that administering a dosage of CAR T-cells composed of both anti-CD19 CAR T-cell and anti-CD123 CAR T-cell populations eradicated relapsed B-ALL, improved overall survival, and did not result in escape variants (Ruella et al., 2015). Such results indicate that multivalent targeting with combinations of CAR T-cells designed against different targets may successfully prevent the relapse of target-down-regulated escape variants.

Combining CAR T-cell therapy with other commonly administered therapies, such as the Bruton’s tyrosine kinase binding protein, ibrutinib, has been shown to increase the anti-tumor clinical efficacy of CAR T-cells in preclinical investigations (Ruella et al., 2016). Mononuclear antibodies, such as rituximab and blinatumomab, are commonly used in conjunction with chemotherapeutic agents to enhance the anti-tumor effects of both chemotherapy and immunotherapy agents (Bennett et al., 2008; Gisselbrecht et al., 2012). Combination therapy regimens to enhance the efficacy of CAR T-cells is therefore a logical next step for adoptive immunotherapy research.

**Improving the Safety of CAR T-cell Therapy**

Although second generation anti-CD19 CAR T-cell therapy is considered generally safe, and most severe adverse events have been either self-limiting or reversible, the recent fatalities of three B-ALL patients from cerebral edema in a phase II
clinical trial conducted by Juno Therapeutics reinforces the need for additional safety measures in CAR design. CAR T-cells have been shown to induce severe toxicities resulting from on-target on-tumor activity, such as CRS, tumor lysis syndrome, and encephalopathies, as well as insertional mutagenesis, and GVHD resulting from donor derived T-cell substrates, among other complications (Brentjens et al., 2011; Grupp et al., 2013, 2013; Kalos et al., 2011; Kochenderfer et al., 2012; Porter et al., 2011; Savoldo et al., 2011). While it is possible that designing CARs against targets other than CD19 may improve the safety of the engineered cells in-vivo, adding a molecular “off-switch” to the design of CAR T-cells is currently a promising strategy to avoid the onset of potentially fatal adverse events.

A number of switch-mediated CAR T-cell designs are gaining popularity as built-in safety mechanisms for T-cell immunotherapy. Administering a chemical induction of dimerization product, known as AP1903, will initiate iCasp9 mediated cellular apoptosis upon command to destroy the CAR T-cells and prevent the progression of severe adverse events. Second generation anti-CD19 and third generation anti-CD20 CAR T-cells designed with an iCasp9 “safety-switch” showed enhanced anti-tumor effects in-vivo and fewer markers of T-cell exhaustion, with the ability to be safely eliminated once apoptosis was induced in murine models (Budde et al., 2013; Hoyos et al., 2010). The safety and efficacy of the iCasp9 system has been proven in allo-HSCTs. Scientists are actively planning phase I clinical trials to evaluate the success of this system for third generation CAR T-cell therapy against relapsed, indolent B-NHL (Budde et al., 2013; Di Stasi et al., 2011).
Designing CAR T-cells to express molecular makers present on a cancer, such as CD20, would allow for the elimination of CAR T-cells in cases of post-infusion toxicities with the administration of a commonly used monoclonal antibody, such as rituximab. Such a method of CAR T-cell elimination would have the added benefit of anti-tumor cytotoxicity (Philip et al., 2014). These constructs will likely be tested in clinical trials in the near future. However, the destruction of CAR T-cells with this design, as well as molecular “off-switch” designs, will inevitably eliminate the anti-tumor effects of this therapy.

Preclinical models have shown CAR T-cells with molecular “on-switches” to be both safe and feasible alternatives to on-demand CAR T-cell destruction. This novel approach would require the administration of an activating molecule to stimulate T-cell activation in-vivo, and thus would allow physicians to tailor CAR T-cell activation to the patient’s individual condition and tolerance for the engineered T-cells (June, 2016; Rodgers et al., 2016).

CONCLUSIONS AND FUTURE DIRECTIONS

Many scientists assert that the ultimate end-goal of adoptive T-cell therapy is to shift this personalized medical therapy to the realm of commercially-produced, “off-the-shelf,” cancer curing products. This goal entails designing CAR T-cells against a variety of targets, made with donor derived T-cell substrates, that can be administered with the ease and efficiency of current monoclonal antibody immunotherapies (Campbell et al., 2015; Xiuyan Wang & Rivière, 2016). Notably, the ability of donor-derived CAR T-cells
to cause GVHD is a significant complication that can dismantle the potential success of commercially-produced CAR T-cells. Expediting the manufacturing process, decreasing the cost of production, and improving the reliability of the CAR T-cell products, in terms of their stable expression of CARs and safety in-vivo, are examples of critically important factors that must be considered before mass-produced CAR T-cells are a feasible reality.

Alternatively, the increasing popularity of personalized medicine suggests that the greatest success of CAR T-cell therapy may come from maintaining the benefits of autologous T-cell substrates. Regardless, decreasing the cost and increasing the efficiency of CAR T-cell production will likely result in significant benefits to the availability and feasibility of CAR T-cell therapy as a treatment for B-cell malignancies. Although significant variations in design, gene transfer efficiency, and safety concerns have yet to be concretely addressed in human patients, preclinical studies have shown great success in mitigating these concerns.

Second-generation anti-CD19 CAR T-cell therapy has cured patients diagnosed with B-ALL, and induced lasting complete remissions and prolonged the survival of patients with advanced B-CLL and B-NHL. Although the success of this therapy varies per the patient’s malignancy and disease status, current research suggests that reproducible, beneficial results with other CAR T-cell targets can be expected in the near future. The remarkable results of phase I and II clinical trials in high-risk, relapsed and refractory cancer indicate that CAR T-cell therapy holds a promising future as a cure for hematologic B-cell malignancies.
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