Universidade de Aveiro Secção Autónoma das Ciências da Saúde

2014

RUI PEDRO GARCIA DE OLIVEIRA BENTO

TRATAMENTO DA LEUCEMIA LINFOCÍTICA COM CARs PARA O ANTIGÉNIO CD19

CAR-MODIFIED T CELLS TARGETED TO CD19 ANTIGEN FOR LYMPHOCYTIC LEUKEMIA

Universidade de Aveiro Secção Autónoma das Ciências da Saúde

2014

RUI PEDRO GARCIA DE OLIVEIRA BENTO

TRATAMENTO DA LEUCEMIA LINFOCÍTICA COM CARs PARA O ANTIGÉNIO CD19

CAR-MODIFIED T CELLS TARGETED TO CD19 ANTIGEN FOR LYMPHOCYTIC LEUKEMIA

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Farmacêutica, realizada sob a orientação científica do Professor Doutor Miguel Forte, Professor Associado Convidado da Universidade de Aveiro e Professor Doutor Bruno Gago, Professor Associado Convidado da Universidade de Aveiro.

Dissertation presented to the University of Aveiro in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Medicine, under the supervision of Professor Miguel Forte, Invited Associate Professor at the University of Aveiro and Professor Bruno Gago, Invited Associate Professor at the University of Aveiro.

In memoriam

Susana Ferrão

(1977-2013)

Thesis Jury

President

Professor Doctor Luís Almeida

Invited Associate Professor, University of Aveiro

Professor Doctor Miguel Forte

Invited Associate Professor, University of Aveiro

Doctor Cláudia Silva

Head of Research at Bluepharma

Head of Galenical Development Unit at Luzitin

Acknowledgements

Foremost, I would like to express my sincere gratitude to both my supervisors, Professor Miguel Forte and Professor Bruno Gago for the continuous support of my MSc study and research.

My sincere thanks also goes for my fellow colleagues at Novartis Pharmaceuticals, for their encouragement, insightful comments and patience, in particular to Luís Rocha, Teresa Guerreiro, Paula Jesus and Sandra Amaral.

I am grateful to the MSc colleagues for all the support, stimulating discussions, sleepless nights before deadlines and for all the fun we have had in the last two years. In particular to Joaquim Fonseca, Márcio Barra, André Andrade and Eduardo Ribeiro.

To the three pillars of my life: God, my Family and my beloved Rita. This thesis would not have been written without their immense strength, support and consistency. Thank you for the invaluable love and understanding. We made it…

Last but not least, to Susana Ferrão - an example of hope, faith, indelible strength, courage and love – and to the warriors that do not give up their fight against cancer.

Keywords Leukemia, Chimeric Antigen Receptors, T Cells, Cell Therapy, CTL019, immunotherapy, Clinical, Cancer.

Abstract Cellular immunotherapies, or Advanced Therapy Medicinal Products (ATMPs), are emerging as novel and specific therapeutic approaches to treat diseases, such as certain types of leukemias, which are difficult or impossible to treat with today's biopharmaceutical products. Breakthroughs in basic, preclinical, and clinical science spanning cellular immunology, and cell-processing technologies has allowed clinical applications of chimeric antigen receptor–based therapies. A recent example is CTL019, a lentivirus-based gene therapy for autologous T cells, acquired by Novartis in 2012 through a global alliance with the University of Pennsylvania. Although this technology is still in its infancy, clinical trials have already shown clinically significant antitumor activity in chronic lymphocytic leukemia and acute lymphocytic leukemia. Trials targeting a variety of other adult and pediatric malignancies are under way. The potential to target essentially any tumor-associated cell-surface antigen for which a monoclonal antibody can be made opens up an entirely new arena for targeted therapy of cancer. The regulatory environment for these Advanced Therapies Medicinal Products is complex and in constant evolution. Many challenges lie ahead in terms of manufacturing process, non-conventional supply chain logistics, business models, intellectual property, funding and patient access.

Disclosure

Rui Bento is a full-time employee of Novartis Farma – Produtos Farmacêuticos SA. The contents and views expressed in this manuscript are the views of the author and not necessarily the views of Novartis. The scientific information may include data/information on investigational uses of compounds/drugs/healthcare technologies whose efficacy, safety and indications have not been established nor approved by regulatory authorities. CTL019 is an investigational compound. Efficacy and safety have not been established. There is no guarantee that CTL019 will become commercially available. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ALL	Acute Lymphocytic Leukemia
AML	Acute Myelogenous Leukemia
AMNOG	Arzneimittelmarkt-Neuordnungsgesetz
ARDS	Acute Respiratory Distress Syndrome
ATMPs	Advanced Therapy Medicinal Products
BCR	B-Cell Receptor
CACT	Center for Advanced Cellular Therapies
CAR	Chimeric Antigen Receptor
CAT	Committee for Advanced Therapies
CBER	Center for Biologics Evaluation and Research
CD19	Cluster of Differentiation 19
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
CNS	Central Nervous System
CoGS	Cost of Goods Sold
CRS	Cytokine Release Syndrome
CsA	Cyclosporine
CT	Computer Tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTL019	T Cells Engineered to Express CAR which Targets CD19
CTMP	Somatic Cell Therapies
DbD	Development by Design
DNA	Deoxyribonucleic Acid
DRGs	Diagnostic Related Groups
EBV	Epstein–Barr Virus
EC	European Commission
EGF	Epidermal Growth Factor
EMA	European Medicines Agency
EU	European Union
EudraCT	European Union Drug Regulating Authorities Clinical Trials
FcRγ	Fc-alpha Receptor
FDA	US Food and Drug Administration
FISH	Fluorescence In Situ Hybridization
FLT3	Fms-like tyrosine kinase 3
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Good Manufacturing Practice
GTMP	Gene Therapies
GVHD	Graft-Versus-Host Disease
GVL	Graft-Versus-Leukemia

HCT/Ps	Human Cells, Tissues, or Cellular or Tissue based products
HIV	Human Immunodeficiency Virus
HLH	Hemophagocytic Lympohistiocytosis
HMMR	Hyaluronan-Mediated Motility Receptor
HSCT	Hematopoietic Stem Cell Transplantation
HSV-TK	Thymidine Kinase from Herpes Simplex Virus
icasp9	inducible caspase 9
IFN-γ	Interferon Gamma
mAb	monoclonal Antibody
MDS	Myelodysplastic Syndrome
MF	Myelofibrosis
MHC	Major Histocompatibility Complex
MLL	Mixed-Lineage Leukemia gene
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MSCs	Mesenchymal Stem Cells
MSKCC	Memorial Sloan-Kettering Cancer Center
NCI	National Cancer Institute
NK	Natural Killer cells
PCR	Polymerase Chain Reaction
Ph1	Philadelphia Chromosome
PLL	Prolymphocytic Leukemia
QbD	Quality by Design
RNA	Ribonucleic Acid
ROR1	Tyrosine kinase-like Orphan Receptor 1
scFv	single-chain antibody Fragment
SLL	Small Lymphocytic Lymphoma
SME's	Small and Medium sized Enterprises
SOFA	Sequential Organ Failure Assessment
T regs	regulatory T cells
TCM	Central Memory T Cells
TCR	T Cell Receptor
TEP	Tissue Engineered Products
TLS	Tumor Lysis Syndrome
TNF-α	Tumor Necrosis Factor alpha
TPP	Target Product Profile
Tscm	memory stem T cell
WBC	White Blood Cell

1 Introduction to Leukemia

1.1 Introduction to Dysregulation of Normal Blood Cell Development

The development of leukemia is a multiple-step process that requires the normal blood cells to be susceptible at different stages. The production of normal blood cells markedly decreases, which results in varying degrees of symptoms, increased proliferation of the transformed cancer cells with reduced programmed cell death, and increased ability of these cells to proliferate [1]. There are many distinct causal mechanisms that can initiate the development of leukemia, including specific molecular abnormalities and exposures to carcinogens in the environment.

1.2 Mechanisms of Leukemia Development

Leukemia is thought to occur when precursors of mature blood cells in the bone marrow acquire mutations in their DNA [2] and as a consequence, the bone marrow makes abnormal white blood cells [3]. Changes in the DNA code can lead to rapid, aberrant growth of the cells as well as survival of the cells beyond their normal lifespan. Over time, the mutated cells can accumulate and overcome the population of normal, healthy cells in the bone marrow, enter the bloodstream, and invade other parts of the body (including lymph nodes, spleen, liver, and central nervous system), resulting in the signs and symptoms of leukemia [2].

The presence of leukemia stem cells is thought to contribute to the development and relapse of certain types of leukemia. Leukemia stem cells are a rare subset of stem cells that have specific developmental, cellular, and molecular properties that are distinct from leukemic blast cells, including the ability to self-renew. However, leukemia stem cells still retain key features of normal stem cells. They are also quiescent, meaning they are inactive or at rest. This property is thought to contribute to relapse because treatments may target and kill active leukemic blast cells but may not effectively target the leukemia stem cell population. A deeper understanding of these cells will help develop improved leukemia therapies [4].

1.3 Overview of Risk Factors

The causes of leukemia are complex and sometimes unexplainable. It does, however, usually develop from a combination of initiating factors. Certain environmental factors explain some cases, while other causes of leukemia can be traced to familial genetic abnormalities or acquired changes in genes. In studying potential causes, several leukemia risk factors have been identified.

1.4 Genetic Risk Factors

The rapid advances in human genomics and molecular techniques have helped define the role of genetic traits in the development of leukemia. Genetic factors have been shown to significantly influence the incidence of leukemia, and certain populations are known to be more susceptible to the disease.

These genetic factors include:

- Family history of leukemia and other malignant blood disorders
- Genetic chromosomal abnormalities like Down's syndrome
- Subtle genetic disorders and polymorphisms

Environmental Risk Factor	Explanation
Exposure to high levels of radiation	Prolonged exposure to radiation has been known to be linked with leukemia. Early radiologists, before the use of appropriate shielding, were found to have an increased likelihood of developing leukemia.
Smoking and second-hand smoke	Smoking is considered a risk factor for leukemia. Many people who have never smoked develop leukemia, which suggests that secondhand smoke may also play a role.
Long-term exposure to certain chemicals	Long-term exposure to chemicals such as benzene or formaldehyde has been associated with leukemia. Inhalation is the predominant route of exposure to these exogenous chemicals, and upon inhalation, these chemicals rapidly react with molecules in the body and are swiftly metabolized by erythrocytes to form adducts with DNA and proteins. - Formaldehyde is a simple one-carbon molecule found in most cells at varying concentrations as a normal product of metabolism. - materials, glues, and fabrics as well as other consumer products like medicines and beauty aids. - Benzene is a colorless and extremely flammable organic hydrocarbon that is one of the most used chemicals in the United States. Numerous studies have confirmed a link between exposure to benzene and leukemia, especially occupational exposures. Benzene is also found in car emissions and cigarette smoke and in certain foods.
Previous chemotherapy	Chemotherapeutic drugs like alkylating agents (i.e., cyclophosphamide), topoisomerase II inhibitors, and anthracyclines (i.e., doxorubicin) that are used to treat cancers in adults or children may induce secondary leukemias. These agents cause DNA damage and aberrant cell growth as a mechanism of their clinical activity, which may in turn introduce mutations and rearrangement of genes, leading to cancer development.
Certain viral infections	The human T-cell lymphoma virus type 1 (HTLV-1) was the first human retrovirus to be discovered and has been recognized as the cause of specific types of leukemia. During infection of cells with the HTLV-1, the virus incorporates its genetic information and hijacks the cellular machinery to produce an oncoprotein, Tax, which promotes hyperactivation of survival and growth pathways.

Table 1 Genetic risk factors influencing the incidence of leukemia (Adapted from **[5]**)

1.5 Leukemia Classifications

The classification of leukemia has evolved significantly over the past few decades as a result of increased understanding of the development of normal immune cells. Classifications of leukemias have developed from two distinct clinical needs—to understand the natural history of these diseases in order to predict outcomes and to make treatment decisions in a rational fashion. In these modern classifications, distinct disease entities are defined based on the combination of morphology, immunological and molecular techniques, and clinical features. Leukemias are classified two different ways—by the speed of disease progression and by the type of cells that have been transformed. Therefore, the classification of leukemias takes into consideration the aggressiveness of the cancer

and how quickly it progresses. Another factor to consider is how mature the cancer cells are compared to the stem cells from which they are derived.

1.5.1 Acute vs. Chronic

Acute

Acute refers to a disorder of rapid onset. Abnormal cells grow rapidly from their immature states, are unable to perform their functions, and do not differentiate [6]. This type of leukemia usually occurs in children and requires immediate therapeutic intervention [7].

Chronic

Chronic refers to an onset that tends to be slower. Cells in chronic disease are generally mature and functional, though abnormal, and typically accumulate in various organs gradually [6]. Conversely, chronic leukemias mostly affect adults, as it may take some time for the disease to progress to the point when effects are noted [7].

Leukemia Features	Acute	Chronic
Onset	Rapid	Slower
Cell Type	Immature	Mature
Disease Characteristics	Occurs in children, requires immediate therapy	Occurs in adults, slow progression

Table 2 Different in acute vs. chronic leukemia

1.5.2 Lymphocytic vs. Myelogenous

Another way to classify the type of leukemia is by the white blood cells from which the cancer is derived. This section will describe how cancer can occur in either the lymphoid (lymphocytic leukemia) or myeloid (myelogenous leukemia) white blood cells.

Lymphocytic

Lymphocytic leukemia arises from the transformation of normal lymphoid cells to malignant cancerous cells. Since this subgroup of leukemias is derived from cells from the lymphoid cellular pathway, lymphocytic leukemias can either be B-cell leukemias or T-cell leukemias. Lymphocytic leukemia may be distinguished from other malignant lymphoid disorders by the immunophenotype of the cells, which is similar to B- or T-precursor cells [1]. Although B-cell and T-cell leukemias may superficially resemble one another, they have distinct clinical and pathologic features and must be distinguished from one another by a pathologist. The majority of lymphocytic leukemias are B-cell type [8].

Myelogenous

Myelogenous, or myeloid, leukemia develops from the early myeloid cell pathway [9]. Depending on whether the disease is acute or chronic, this can lead to:

• An abnormal development of a type of immature white blood cell called myeloblasts, which do not differentiate into normal, healthy white blood cells [10],

• An overproduction of abnormal red blood cells or platelets from stem cells [10], or

• An increased proliferation of white bloods cells called granulocytes [11].

1.5.3 Types of Leukemia

Taking into account both the speed of disease progression and the type of cells that have been transformed leads to the division of four main types of leukemias. These common types include acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL). [7]

Acute Myelogenous Leukemia

Acute myelogenous leukemia (AML) is an aggressive disease in which the myeloid stem cells usually become a type of immature white blood cell called myeloblasts. The myeloblasts in AML are abnormal and do not differentiate into normal, healthy white blood cells [10]. Additionally, too many of these abnormal cells can be found in the bone marrow and blood and sometimes, too many stem cells become abnormal red blood cells or platelets [12].

Epidemiological and genotypic studies have demonstrated that AML cells have more than one recurring mutation that is shared within the group of diseases. Fms-like tyrosine kinase 3 (FLT3), a receptor tyrosine kinase expressed by immature hematopoietic cells and important for the normal development of stem cells, is the most commonly mutated protein in AML. FLT3 is constitutively activated by acquired mutations in approximately 30 to 35% of AML [4]. These mutations ultimately provide survival and growth advantage to the cells [4]. Because of this, the presence of the FLT3 mutation is often associated with a poorer prognosis for AML patients. Polymorphisms of NQO1 are also closely associated with increased risk of AML [13]. Most AML subtypes are distinguished from other leukemias and blood disorders by the presences of more than 20% blasts in the bone marrow [13].

Most patients who present with newly arisen AML have no identifiable risk factors [13]. There is, however, a documented progression from hematologic disorders like myelodysplastic syndrome (MDS) and myelofibrosis (MF) to AML [13]. Patients with low-risk MDS generally do not develop AML, while patients with high-risk MDS do. MDS is typically a disease that has an increased incidence with age that can contribute to and explain the high incidence of AML in the elderly [14]. AML is more common in men than in women, which especially becomes apparent with increased age and may be due to the fact that MDS, which tends to develop into AML, is also more common in men [13]. Other risk factors in developing AML include race and ethnicity, the father's age at conception (with an increased risk over age 35), and the time since the mother's last live birth (with greater risk associated with over seven years since the last childbirth). [14]

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by increased proliferation of the myeloid cell line, without the loss of the ability to differentiate [11]. The result is the overproduction of abnormal granulocytes. CML progresses through three phases:

• The chronic phase, where mature cells proliferate,

• The accelerated phase, where additional abnormal genetic events occur, and

• The blast phase, where immature cells grow rapidly.

Approximately 85% of patients are diagnosed in the chronic phase and then progress to the other phases within three to five years [11]. The hallmark of this leukemia is the presence of the Philadelphia chromosome (Ph1), a fusion chromosome that is the result of abnormal genetic translocations between chromosomes 22 and 9 resulting in a shortened chromosome 22. This translocation creates a hyperactive protein kinase product called BCR-ABL that drives the proliferation of the cancer, making growth factors often unnecessary [15]. Because BCR-ABL is the major molecular event in the development of CML, this protein is a great target for diagnosis, treatment, and disease monitoring. CML was the first cancer to be associated with a chromosomal translocation and a single, specific genetic mutation that drives the cancer. [15] [11]

Acute Lymphocytic Leukemia

Acute lymphocytic leukemia (ALL) is the most common childhood leukemia and the most common childhood cancer in developed countries [4]. ALL is thought to arise from malignant transformation of B- or T-cell progenitor cells [16]. Most adults with ALL have no identifiable risk factors [1]. However, 80% of the cases of infant ALL are associated with genetic abnormalities in the MLL gene, leading to the increase in survival factors that causes this disease to be resistant to chemotherapy and other treatments [4]. ALL also often presents in infants with features associated with poor outcome, including diseases of the central nervous system and poor response to treatment. In terms of inherited chromosomal abnormalities, children with Down's syndrome have an increased risk of developing both ALL and AML. The cumulative risk is approximately 2.1% by age 5, with most of the cases being ALL [17]. Cases of chromosomal abnormalities following treatment with topoisomerase II inhibitors have been linked with the development of ALL, but these patients are more likely to develop AML [1]. Also, the Philadelphia chromosome occurs in about 20% of adults and a small percentage of children with ALL [16]. In the majority of children and in more than 50% of adults with Ph1-positive ALL, the molecular abnormality is different from that in Ph1-positive CML.

Immunologic Subtype	Percentage of Cases	FAB Subtype	Cytogenic Abnormalities
Pre-B ALL	75%	L1, L2	t(9;22), t(4;11), t(1;19)
T-cell ALL	20%	L1, L2	14q11 or 7q34
B-cell ALL	5%	L3	t(8;14), t(8;22), t(2;8)

FAB: French American British
L1: small uniform cells
L2: large varied cells
L3: large varied cells with vacuoles

Table 3 Three Main ALL Classification Subtypes. Adapted from **[18]**

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia found in adults in Western countries [8]. The cells of origin in most patients with CLL are clonal B cells. These cells are arrested in the differentiation pathways between immature blasts and mature B cells, though they may look similar to mature lymphocytes [8]. Much progress has been made in differentiating subsets of CLL and

predicting disease progression. CLL was once grouped together as subtypes under the broad term of non-Hodgkin's lymphoma, but after extensive refinement of the classifications of blood cancers to better distinguish and group the lymphoid disorders by their clinical and biological characteristics, a separate and distinct disease group was created [19]. The exact cause of CLL is uncertain, but it is known that CLL is an acquired disorder, with familial cases being extremely rare [8]. The identification and use of molecular and cellular markers like B-cell receptor (BCR), a protein that plays a key role in signal transduction, has helped define CLL [20]. While low expression of the BCR is the hallmark of the B-CLL lymphocyte, increased expression correlates with mutated cells [20]. Another important genetic parameter in defining pathogenic and prognostic subgroups of CLL is the mutation of the VH genes, which is observed in about half of all CLL cases [21].

1.6 Epidemiology

Leukemia and the consequences of this group of diseases represent a substantial worldwide concern. This section describes the incidence and mortality of leukemia and explains the risk factors associated with this blood disorder.

1.6.1 Incidence

The American Cancer Society estimates that 31,500 individuals in the US will be diagnosed with leukemia every year [14]. From 2005 to 2009, the median age of leukemia diagnosis was 66 years of age; 10% were diagnosed under the age of 20, 4.9% between the ages of 20 and 34, 5.3% between the ages of 35 and 44, 26.7% between the ages of 45 and 64, and 52.6% 65 years of age and over [22].

Childhood Leukemias

Leukemia is the second most common malignancy in the first year of life [23]. About 85% of leukemias in children are acute [6], and ALL accounts for 65% of the acute leukemias in children [6]. Approximately 4.1 of every 100,000 young people under 20 years of age in the US are diagnosed with leukemia [24], and infant leukemia is more common in females than males, with a ratio of 1.17, female to male [23]. Leukemia is also the most common cancer diagnosis in children less than 15 years old, and ALL is approximately five times more common than AML in this age group [4].

Around 2000, the average incidence for this age group in the European Region was 46.7 cases per million per year, with a slightly lower level in eastern than in western European countries. European population-based cancer registries show an average increase in the incidence of childhood leukemia of 0.7% per year between 1970 and 1999 [25].

Adult Leukemias

As adult leukemias are more common in men than women, approximately 0.48% of men will develop leukemia between their 50th and 70th birthdays compared with 0.30% of women [22]. CLL is the most common form of leukemia in Western countries and mainly affects elderly individuals, with the median age of presentation being 72 years [8]. More than 17,000 new cases of CLL are reported each year in the US [8]. CLL is almost twice as common as CML, which makes up only 20% of all leukemias affecting adults, typically middle-aged adults [13] [6]. AML is the most common acute leukemia in adults, accounting for about 25% of all leukemias in the Western world. The incidence of acute

leukemias accounts for less than 3% of all cancers, but is still the leading cause of death to individuals under 40 who have developed the disease [14].

1.6.2 Mortality

Epidemiological studies of leukemia have focused on mortality to determine how specific populations and age groups are more susceptible to succumb to the disease. The progression of many types of leukemia is extremely variable, with survival ranging from months to decades in some cases.

Statistical reports show that the median age of death for leukemia is 75 years. Approximately 2.8% of leukemia deaths occur in patients that are under the age of 20 years. About 3.1% leukemia deaths occur in those aged 20 and 34 years, 3.1% occur between 35 and 44, 19.3% occur in those aged 45 to 64 years, and 71.8% occur in those 65 years and over. It was estimated that, while 47,150 men and women would be diagnosed with leukemia in 2012, 23,540 men and women would die from it [22].

2 The Diagnosis of Leukemia

2.1 Diagnostic Steps

Symptoms develop based on how quickly the disease progresses. As acute disease is associated with a rapid onset, symptoms develop fairly quickly when a person is inflicted with acute leukemias. Individuals are typically diagnosed right after they become ill. Conversely, for chronic disease, which has a more gradual onset, symptoms are slow to develop. It is not uncommon for some people with chronic leukemia to not even present with symptoms at diagnosis [26].

2.1.1 General Symptoms

Some of the symptoms associated with leukemia are due to the increase of abnormal blood cells that have replaced the population of normal, healthy cells. Because this is a general characteristic of all leukemias, there are a number of symptoms that can be common to all four of the major types of leukemia [26].

These symptoms include [26]: unexplained fevers, frequent infections, night sweats, fatigue, weight loss, easy bleeding or bruising, as showed below:

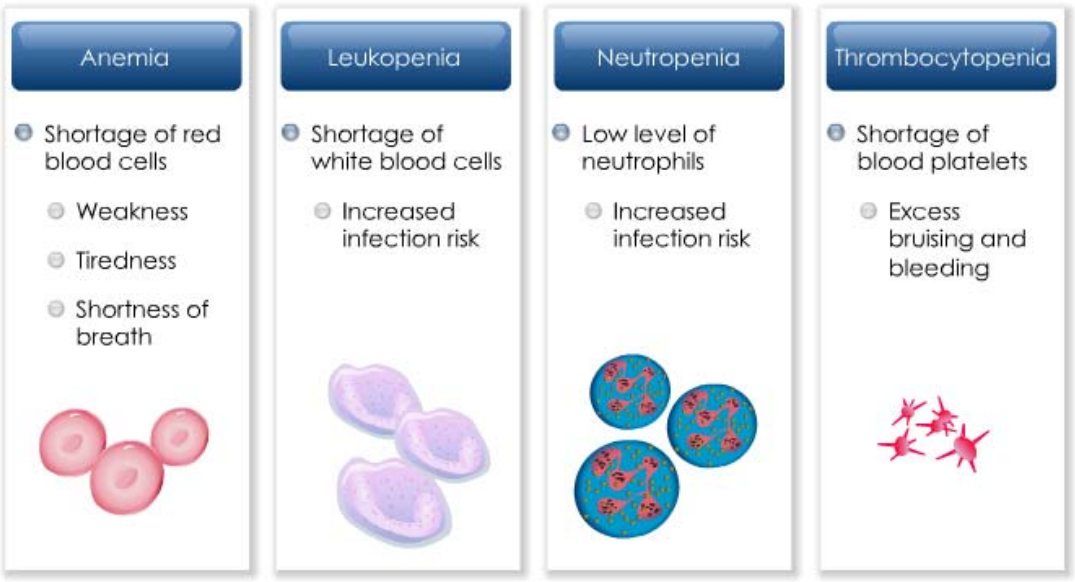

Figure 1. Cause of common symptoms associated with leukemia. Adapted from Stoppler M, 2014.

Leukemia patients are commonly referred to as having anemia, leukopenia, neutropenia, and thrombocytopenia, which cause generalized symptoms [27]. Other symptoms are due to the accumulation of leukemia cells in vital tissues and organ systems of the body. Some of the most common sites of leukemia cell accumulation are the lymph nodes, liver, spleen, kidney, lungs, and skin [26].

Many of these symptoms can also occur across the leukemia spectrum [26]: headache, confusion, balance problems, blurred vision, painful swellings in the neck, under the arms, or in the groin, shortness of breath, nausea or vomiting, abdominal pain or swelling, testicular pain or swelling, pain in the bones or joints, weakness or loss of muscle control and seizures.

2.1.2 Types of Tests and Interpretation of Results

In order to properly diagnose leukemia, healthcare professionals utilize a number of diagnostic tests. Some of these tests are not needed to establish the diagnosis of leukemia but are sometimes performed at diagnosis to predict the prognosis or to assess tumor burden.

When a clinician is confronted with an individual who is suspected of having leukemia, there are a number of tests that are available at initial diagnosis. The tests include [26] [27] [7]: blood tests, organ function tests, biopsy, genetic tests, lumbar puncture, lymph node excision and imaging techniques.

Diagnostic Tests	Description
Blood tests	• Blood is drawn from a vein and tested in order to check blood cell count, size, and maturity. • In most cases of leukemia, the overall white blood cell count will be high, while platelet, red blood cell, and neutrophil counts will be low (thrombocytopenia, anemia, and neutropenia).
Organ function tests	• Leukemia cells often accumulate in the liver and kidneys and affect normal organ function. The function of these organs may be checked by examining specific markers that may indicate damage or stress to these organs (i.e., albumin, cholesterol, creatinine).
Biopsy	• In a biopsy, a small sample of the relevant tissue from the bone marrow is taken, usually from the hip bone, and analyzed for the presence of abnormal cancerous cells under a microscope. The procedure is brief but does require a pre-injection of anesthesia. • Because leukemia may cause an increase in white blood cell count, a biopsy of the bone may be taken to examine blood cell composition.
Genetic tests	• As the presence of genetic abnormalities may be a cause of the development of leukemia, chromosomes of the cancerous cells are examined to look for potential genetic changes that also may help to classify the specific type of leukemia. • There exist a number of individual genetic tests that can be conducted: o Conventional cytogenetics: analysis of the chromosomes under a microscope to find any significant changes, a process known as karyotyping; chromosomes are best seen when the cells are undergoing division, so a sample of the blood or bone marrow is often grown in a laboratory o Fluorescent in situ hybridization (FISH): test that uses special fluorescent dyes designed to attach to specific portions of chromosomes to help identify the presence of specific changes attributed to cancer o Polymerase chain reaction (PCR): a sensitive test that amplifies DNA or RNA from blood or bone marrow to allow for the detection of even small amounts of genetic abnormalities
Lumbar puncture (Spinal tap)	• Leukemia cells can also find their way into the fluid surrounding the brain and spinal cord of the central nervous system, also known as the cerebrospinal fluid. This often causes individuals' mental processing to be affected. • In this procedure, a small injection is performed first in the site of the spinal tap to minimize the discomfort of the procedure. A hollow needle is then inserted in the back between the bones in the spine around the waist area to remove a small amount of fluid for analysis.
Lymph node	• The development of leukemia can cause the lymph nodes to enlarge as

excision	abnormal cells accumulate there, so a lymph node may be excised to check for this. • Also, a node may be biopsied if the results from the bone marrow biopsy are too difficult to interpret. This is rare but does occur.
Imaging techniques	• There are a number of individual imaging tests that can be conducted: • Chest X-rays: an imaging technique frequently used to look for signs of infection in the lungs or for lymph node involvement. • Computed tomography (CT) scans: an imaging procedure that uses a combination of X-rays and computer technology to produce cross-sectional images, both horizontally and vertically, of the body; in the diagnosis of leukemia, images of the bone and organs are especially helpful. • Magnetic resonance imaging (MRI): a diagnostic procedure that uses a combination of large magnets, radio-frequencies, and a computer to produce detailed images of organs and structures within the body. • Ultrasound: a diagnostic imaging technique that uses high-frequency sound waves and a computer to create images of blood vessels, tissues, and organs as they function to assess blood flow.

Table 4 Diagnostic Tests for Leukemia

2.1.3 Stages, Phases, and Classifications of Leukemia

With the exception of CLL, leukemia is not staged numerically (I, II, III, or IV) as many solid tumors are [28]. Instead, descriptors like acute or chronic and time-to-progression mostly indicate the severity of the disease. In addition, treatment status gives another level of disease classification.

Staging is the way cancer is classified based on certain criteria, including the size and extent of spread. Leukemias like CLL may be classified by a staging system based on the part of the body that is affected. In terms of CLL, the most commonly used staging system in the US is the Rai classification system [29] and in Europe, the Binet staging system is used.

The **Rai system** is separated into five stages [29]:

- *Stage 0*: The blood lymphocyte count is too high, usually defined as over 15,000 lymphocytes/mm3 of blood (lymphocytosis) and > 40% lymphocytes in the bone marrow. The lymph nodes, spleen, and liver are not enlarged, and the red blood cell and platelet counts are near normal. This is considered a low-risk group.
- *Stage I*: Lymphocytosis plus enlarged lymph nodes—The spleen and liver are not enlarged, and the red blood cell and platelet counts are near normal. This is considered an intermediate-risk group.
- *Stage II*: Lymphocytosis plus an enlarged spleen and possibly an enlarged liver, with or without enlarged lymph nodes—The red blood cell and platelet counts are near normal. This is considered an intermediate-risk group.
- *Stage III*: Lymphocytosis plus anemia, with or without enlarged lymph nodes, spleen, or liver—Platelet counts are near normal. This is considered a high-risk group.
- *Stage IV*: Lymphocytosis plus thrombocytopenia, with or without anemia, enlarged lymph nodes, spleen, or liver—This is considered a high-risk group.

Europe's Binet staging system can be summarized in [30]:

Stage A: Hemoglobin ≥ 10 g/dL, platelets ≥ 100,000/mm3, and < 3 enlarged areas

Stage B: Hemoglobin ≥ 10 g/dL, platelets ≥ 100,000/mm3, and ≥ 3 enlarged areas

Stage C: Hemoglobin < 10 g/dL, platelets < 100,000/mm3, and any number of enlarged areas

System	Stage	Definition	Median Survival
Rai Staging System (Common in US)			
	0	lymphocytosis (>5 G/L)	> 10 years
	I	lymphocytosis + lymphadenopathy	> 8 years
	II	lymphocytosis + splenomegaly +/-lymphadenopathy	6 years
	III	□ymphocytosis + anemia (Hb <11g%) +/-lymphadenopathy or splenomegaly	2 years
	IV	lymphocytosis + thrombocytopenia (Plt < 100 G/L) +/- anemia +/-lymphadenopathy +/- splenomegaly	< 2 years
Binet Staging (Common Ex US)			
	A	< 3 involved areas, Hb > 10g%, Plt > 100 G/L	> 10 years
	B	> 3 involved areas, Hb > 10g%, Plt > 100 G/L	7 years
	C	Any number of involved area, Hb < 10g% & Plt < 100 G/L	2 years

Table 5 Prognosis Varies Greatly by Clinical Stage **[31]**

Certain leukemias like CML are classified by phases, defined by the number of immune leukemia stem cells, or blasts, in the blood and bone marrow [27].

These phases are [32]:

- Chronic phase: fewer than 10% of blood cells are blasts.
- Accelerated phase: 10% to 19% of blood cells are blasts.
- Blast phase: 20% or more of blood cells are blasts.

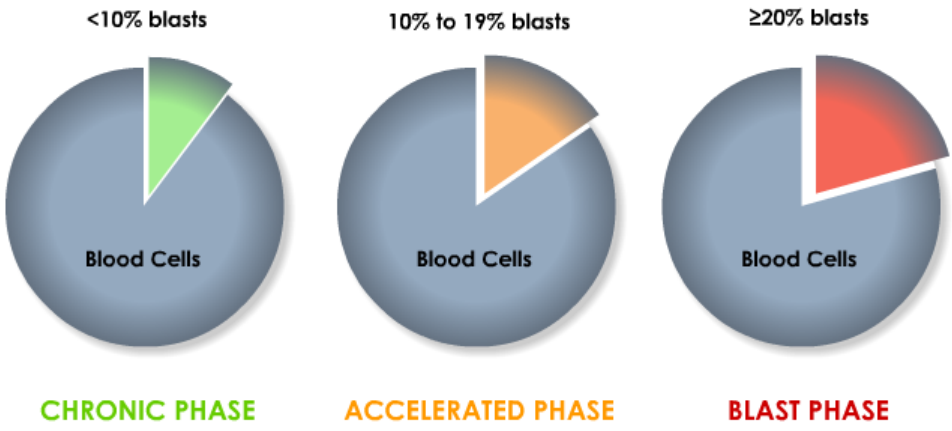

Figure 2. Phases of CML are defined by the number of blasts in the blood and bone marrow. Adapted from Duke Cancer Institute, 2011.

In general leukemias are classified rather than staged in order to determine the most appropriate therapy. Acute lymphoblastic leukemia has no standard staging system. Instead it is classified as follows [32]:

- Untreated: Leukemia was recently diagnosed and has not yet been treated.
- In remission: There exist no signs, symptoms, or medically defined presence of the disease (less than 5% leukemia cells).
- Recurrent: Signs, symptoms, and presence of the disease have returned.

2.2 Diagnosis by Leukemia Subtype – ALL and CLL

Each of the major types of leukemia (ALL, AML, CLL, and CML) may have unique signs and symptoms that help to diagnose the disease state. In addition, results of diagnostic tests and aspects of their clinical manifestations may point to a particular leukemia subtype.

2.2.1 ALL

A minority of cases of ALL are detected during blood counts and blood examinations performed for investigation of unexplained fever, pallor, and bleeding.

Symptoms

Fever is one of the most common symptoms of ALL. Patients with ALL often have fevers without any other evidence of infection [1]. ALL patients may also present with symptoms from the increased amount of abnormal, cancerous cells in the peripheral circulation (leukostasis), including respiratory distress and altered mental status. This is much less common in ALL than AML patients, however, and only occurs in patients with the highest WBC counts [1].

ALL dominates as a hematological malignancy in children, though it can present at any age. The onset may be present as fatigue, persistent fever, bleeding, and/or bone and joint pain. Young children may present with problems with walking or even the total inability to walk. In addition, 2% to 3% of children

may first have anemia and CNS system involvement in their disease [33]. A small percentage may have skeletal symptoms due to widespread osteoporosis. In children, many of these symptoms present without the presence of enlarged organs (organomegaly), swollen lymph nodes (lymphadenopathy), or leukocytosis.In adults, the symptoms of ALL are similar to those in children. Almost 50% may have fever at presentation. However, adult onset ALL is commonly associated with an enlarged liver and spleen (hepatomegaly and splenomegaly, respectively) and lymphadenopathy at diagnosis. About 10% to 20% of ALL patients present with left upper quadrant fullness and early satiety due to splenomegaly [33].

Clinical Presentation

ALL patients often have decreased neutrophil counts, regardless of whether their total white blood cell count is low, normal, or elevated, putting them at increased risk of infection. Because of this, infections are still the most common cause of death in ALL patients undergoing care for their disease [1]. Finding blasts raises the suspicion of acute leukemia, and a bone marrow exam is then suggested. A bone marrow biopsy usually confirms the diagnosis of ALL [33].

A diagnosis of ALL may be easily confused with other hematological conditions like AML, hairy cell leukemia, and malignant lymphoma. Malignant cells are often sent for conventional cytogenetic studies, such as detection of the Philadelphia chromosome and MLL gene rearrangements, to add important information for treatment decision making. Flow cytometry is usually also performed to characterize expression of lineage-defining antigens and allow determination of the specific ALL subtype [16].

2.2.2 CLL

It is not unusual for CLL to be discovered incidentally after a blood cell count is performed for another reason, as 25% to 50% of patients will be asymptomatic at the time of presentation [8]. CLL is predominately a disease of older individuals—most patients are older than 50 at the time of diagnosis. Because of this and because disease progression is often slow, patients often succumb to other medical problems rather than CLL [34].

Symptoms

Enlarged lymph nodes and node swelling are the most common presenting symptoms, seen in 87% of the patients who are symptomatic at the time of diagnosis. A smaller number of patients report less severe symptoms like fever, weight loss, and night sweats. People with CLL may also be predisposed to repeated infections, such as pneumonia. Early satiety and/or abdominal discomfort, related to an enlarged spleen, may also be noted in this patient population [8].

Clinical Presentation

The diagnosis of CLL requires the presence of at least 5x109 B lymphocytes/L (or 5,000/μL) in the peripheral blood. The tests necessary to give a diagnosis of CLL are blood count evaluations, blood smear, and the immune phenotype of the circulating lymphoid cells. Using fluorescent in situ hybridization (or FISH) during cell division, cytogenetic lesions can be identified in more than 80% of all CLL cases, with the most common being deletions in the long arm of chromosome 13 [35]. A bone marrow aspirate and biopsy generally are not required at diagnosis, but can help evaluate for factors that might contribute to symptoms like anemia and thrombocytopenia that may or may not be directly

related to organ infiltration. Bone marrow findings in CLL include normal to high cellularity, or when the bone marrow has more blood-forming cells than expected [34]. In CLL, more than 30% of the nucleated cells in the aspirate will be lymphoid [35].

Mantle cell lymphoma, hairy cell leukemia, and prolymphocytic leukemia (PLL) can have a clinical presentation very similar to CLL but are more aggressive. Additionally, CLL may also present as a lymph-node-based disease called small lymphocytic lymphoma (SLL). A combination of factors including antigen presentation, cytogenetics of chromosome translocations, intracellular mediator expression, and morphology are used to differentiate the diseases [34]. For example, CLL cells coexpress the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23. Additionally, according to the WHO classification, CLL is only distinguishable from SLL by its leukemic appearance [35].

3 The Treatment of Leukemia

3.1 Management strategies

Development of treatment strategies is driven by the need to reduce toxicities associated with current therapies, overcome the onset of drug resistance, and improve clinical efficacy. Unprecedented efforts are currently underway to define molecular mechanisms that are important in the development of leukemia and determine alternatives to conventional approaches to disease management.

3.1.1 Management Types

A Watch and Wait

In certain clinical situations, treatment is not indicated until the disease is further along. With the "watch and wait" strategy, the disease is monitored with regular physical exams and lab tests until a time when decisions can be made on the most appropriate therapy to avoid therapy-related side effects. This is more likely the case when a person has no symptoms and a disease has been diagnosed by chance [36].

Chemotherapy

Chemotherapy is treatment using cytotoxic, non-specific agents to stop the growth and spread (metastasis) of cancerous cells. Chemotherapeutic agents usually accomplish this by either inhibiting cell division or promoting cell death. Chemotherapy can be taken by mouth (oral) or is injected in the vein or muscle (intravenous or subcutaneous, respectively) in order to enter the bloodstream and reach the site of the cancer (systemic chemotherapy). Chemotherapy can also be placed directly into the cerebrospinal fluid (intrathecal chemotherapy). The main types of chemotherapy include [37]:

- Alkylating agents: drugs that inhibit DNA production by inducing DNA crosslinks
- Antimetabolites: drugs that inhibit the cell's utilization of natural metabolites like folic acid, needed for cell growth
- Plant alkaloids: drugs that block cell division, often by inhibiting the normal function of microtubules, fibrous proteins in the cells important for cell structure and mitosis
- Antibiotics: agents that interfere with cellular processes, including DNA or protein synthesis
- Purine analogs: agents that inhibit DNA synthesis by mimicking natural purines and intercalating into DNA chains
- Hypomethylators: agents that inhibit DNA methyltransferase, the cellular enzyme that methylates DNA, necessary for many cellular functions

Radiation Therapy

Radiation therapy uses high energy X-rays or other types of radiation to kill cancerous cells. There are generally two types of radiation. The choice of which is utilized depends primarily on how advanced the cancer is [37]:

- External radiation: A machine outside of the body is utilized to send radiation toward to the portion of the body where the cancer resides.
- Internal radiation: A radioactive agent is packaged in a carrier mechanism (such as a catheter) and placed directly inside the body in or near the site of the tumor.

Targeted Therapy

Targeted therapy uses agents that have been designed to specifically target cancer cells, theoretically leaving normal, non-cancerous cells unharmed. For instance, these agents may target specific proteins that are only expressed or are overexpressed on the surface of cancer cells but not healthy cells [37].

Immunotherapy

Immunotherapy uses the body's own immune system to inhibit cancer growth and disease progression by directly or indirectly boosting the normal immune response against the disease. Because of the required involvement of the immune system to the activity of immunotherapeutic agents, this type of therapy usually involves some component of the normal immune system. Though this type of therapy may carry with it its own particular side effects, immunotherapy is typically less invasive and less toxic than conventional treatment strategies like chemotherapy [38].

Surgery

Surgery as a management strategy in leukemia usually involves the removal of swollen lymph nodes (lymphadenectomy) to confirm the diagnosis of leukemia or the removal of the spleen (splenectomy) if it has begun to destroy components of the bone marrow [39].

Stem Cell Transplantation

Stem cell transplantation (SCT) is a method of getting rid of the blood-forming cells that are no longer healthy by replacing the bone marrow. To begin the process, normal stem cells are removed from the bone marrow. After specific points in the treatment plan for a patient, the stem cells are infused into the patient. There are generally two major types of SCT [39]:

- Autologous stem cell transplant (autoSCT): The non-cancerous stem cells are derived from the bone marrow of the leukemia patient.
- Allogeneic stem cell transplant (alloSCT): The non-cancerous stem cells are taken from a donor to be infused in the patient. Before referral for alloSCT, a suitable donor must be identified, ideally a fully HLA-matched sibling. If this is not possible, as is the case for many patients, alternatives for a donor include a matched unrelated person or the patient's own cord blood.

3.1.2 Treatment Phases

Induction

Induction therapy is the initial therapy given to a patient. The goals of induction therapy are to empty the bone marrow of all hematopoietic elements both healthy and cancerous and to allow the repopulation of the bone marrow with normal, functioning cells, yielding remission [40].

Postremission

Once remission is achieved, additional therapy is often required to reduce the undetectable burden of cancerous cells so that long-term disease-free survival (DFS) may be possible. Postremission therapy may involve two types of therapy:

- Consolidation therapy: therapy designed to “consolidate” the gains made with induction therapy
- Maintenance therapy: less intensive regimens used in order to “maintain” remission

Relapse/Refractory

After induction and postremission therapy, further therapy is necessary if a patient relapses or develops refractory disease. In this setting, careful analysis is conducted of a patient’s risk factors, response to prior therapy, and duration of this response.

Relapse refers to the return of cancerous cells in the bone marrow after remission is achieved, while refractory disease refers to when these cells remain even after treatment, usually a consequence of being unresponsive to therapy (drug resistance). This resistance can take place initially or over time. Often relapse and refractory disease are grouped together in one setting for the design of treatment regimens.

3.2 Treatment Recommendations – ALL and CLL

Treatment plans may incorporate multiple drugs and a number of combinations and sequences of time and dose, with an objective of restoring normal hematopoietic processes, preventing the further expansion of the abnormal, cancerous cells, and giving supportive and palliative care to relieve symptoms associated with the disease.

3.2.1 General Factors That Impact Therapy

Specific treatment for leukemia is usually determined by specific characteristics of patients [7]. These characteristics can include:

- Medical history
- Age at diagnosis
- Extent of the disease
- Tolerance for specific therapeutic agents/procedures
- Specific goals and objectives for disease management
- Pre-existing conditions (co-morbidities)
- Cytogenetics

These characteristics generally help to identify a treatment for a specific patient, with the goal of minimizing disease symptoms while avoiding excessive risk of treatment-related toxicity.

3.2.2 ALL

Specific Treatment for ALL consists of a combination of bone marrow control and systemic treatments, as well as prevention of associated involvement of the CNS [41]. The average length of treatment for ALL varies from 1.5 years to 3 years in the effort to eradicate the leukemic population [41].

Prognostic Factors

Up to 75% of adults with ALL are considered to be poor-risk with an expected Disease-Free Survival (DFS) rate of 25%. The remaining 25% are considered standard-risk, with a DFS rate greater than

50%. There are a number of factors associated in risk-adapted therapy and prognostic evaluations for ALL [41]:

- Age: In general, prognosis is better in younger patients (<25 years), possibly due to the increased incidence of Ph1-associated disease in older patients, a subgroup with poor prognosis. Children with low-risk disease have survival rates as great as 95%. Adolescent young adults have intermediate disease characteristics and prognosis.
- CNS involvement: ALL patients, regardless of age, are at risk of developing CNS involvement during the course of the disease, which can influence treatment.
- Cellular morphology: Patients with certain morphologies (such as, L3[Burkitt] morphology) may require aggressive, rapidly cycling chemotherapy.
- Chromosomal abnormalities: Patients with Ph1-positive ALL, as well as other Bcr-Abl-associated diseases have a poor prognosis, comprising over 30% of adult cases. Other chromosomal abnormalities with poor prognosis include ALL characterized by the rearrangements of the gene MLL:
    - TEL-AML1 gene fusion is associated with good outcomes
    - Hyperdiploidy associated with good outcomes
    - Evaluation of TPMT gene polymorphism – predicts patients at high risk of hematopoietic toxicity

Parameters	**Good**	**Poor**
White blood cell count	Low	High (>50 x 109 /L)
Gender	Female	Male
Age	Child	Adult or infant
Cytogenetics	Normal, hyperdiploid	Ph+, 11q23 rearrangements
Time to clear blasts from blood	< 1 week	> 1 week
Time to remission	< 4 weeks	> 4 weeks
CNS disease at presentation	Absent	Present
Minimal residual disease	Negative at 1-3m	Still positive at 3-6m

Table 6. Factors that Contribute to ALL Prognosis **[42]**

5 year event free survival		
	Child	**Adult**
Pre-B	> 80%	30-40%
T-cell	75-85%	45-55%
Ph+	20-25%	< 10%
MLL	40-50%	20%
TEL/AML1	90%	N/A

Table 7. Overall Outcomes for Adult ALL Patients Substantially Worse than for Pediatric Patients **[43]**

Treatment Strategies and Guidelines

Standard induction therapy for adults has been modeled after pediatric programs and was originally developed when supportive care options were inferior to the agents available today [44]. Most induction therapy is based on intensive approaches, including hyper-CVAD (a four-drug regimen of cyclophosphamide, vincristine, an anthracycline like doxorubicin, and dexamethasone) given over four to six weeks, based on the success achieved with short-term dose-intensive chemotherapeutic regimens in children [44]. Complete remission is obtained in 65% to 85% of patients with hyper-CVAD, with the time to reach complete remission (CR) correlated to treatment outcome [44]. In keeping with this, studies have shown that patients whose disease is in CR within four weeks of therapy have longer disease-free survival and overall survival (OR) than others who enter remission after four weeks or more of treatment.

Newer modifications to the hyper-CVAD regimens include the addition of novel agents like imatinib for patients whose leukemia is Ph1-positive (a group with poor responses to traditional chemotherapeutic regimens) and the incorporation of rituximab for patients whose leukemia is CD20-positive, with both of these approaches resulting in DFS [44]. Imatinib is an orally available tyrosine kinase inhibitor (TKI) of the Bcr-Abl kinase first investigated in CML. Imatinib has been shown to also have clinical activity as a single agent in Ph1-positive ALL, leading to 90% CR rates when included in standard induction regimens. Imatinib is now often incorporated into the therapeutic plan for ALL patients, especially the younger population. For those patients with Ph1-positive ALL who are resistant to imatinib, ponatinib, a pan Bcr-Abl tyrosine kinase inhibitor may be used [45].

Since myelosuppression in leukemia management can be both disease- and treatment-related, patients must be closely monitored during induction treatment [45]. The use of growth factors during induction may alleviate this myelosuppression and allow for timely administration of dose-intensive therapy [46].

The benefits of consolidation therapy in ALL management have been supported by a number of clinical investigations. Therapy with daunorubicin and cytosine arabinoside (Ara-C) versus no consolidation therapy has demonstrated a 38% three-year DFS rate. Because most studies have shown a benefit to consolidation therapy, regimens using the standard four- or five-drug induction regimens usually include consolidation therapy with an Ara-C-based combination with other chemotherapeutic agents [44].

The effectiveness of maintenance chemotherapy in adults has not been studied in controlled clinical trials, though several clinical studies without maintenance therapy have shown inferior results compared with controls. Although maintenance may be necessary, using a more intensive versus less intensive regimen does not appear to be beneficial. Intensification of maintenance therapy from a 12-month course of a four-drug regimen compared with a 14-month course of a seven-drug regimen did not show a difference in DFS between the two groups in clinical trials [44].

ALL patients, in contrast to patients with AML, frequently have meningeal leukemia at the time of relapse. This can present at the time of initial diagnosis, though rare (less than 10% of cases), or in the majority of cases (50% to 70%) at one year in the absence of CNS-directed therapy or at the time of relapse. For this reason, CNS prophylaxis is an important part of both induction and postremission therapy [46]. Four consecutive clinical trials have found that high-dose systemic chemotherapy reduces CNS relapse, but early intrathecal (IT) chemotherapy is necessary to achieve the lowest risk of CNS relapse [44]. This makes CNS prophylaxis with intrathecal chemotherapy essential. Current

recommendations for CNS prophylaxis therapy include cranial radiation therapy plus IT chemotherapy, a mix of high-dose systemic and IT chemotherapy or IT chemotherapy alone [45].

Few studies have compared transplantation with chemotherapy in ALL. Some studies have shown that allogeneic transplantation can also be effective therapy for patients who have experienced relapse after chemotherapy. Most suggest that allogeneic transplantation should be offered to young patients with high-risk features whose disease is in first remission. For instance, SCT seems to be a valuable option for a subgroup of infants with MLL+ ALL carrying poor prognosis. In young patients without adverse features, transplantation is reserved for relapse [44]. Older patients whose disease is in CR may be considered for investigational approaches including alloSCT. Because of the current high treatment-related morbidity and mortality associated with the use of matched unrelated donors, it is reserved for patients in second remission or beyond [47].

Patients with relapsed disease have extremely poor prognosis and are unlikely to be cured with further chemotherapy alone [45]. These patients are normally referred for investigational therapy and clinical trials or are given reinduction therapy with supportive care options, including palliative radiation, a chemotherapy combination (such as hyper-CVAD and Ara-C-based regimens), and/or novel TKI (imatinib mesylate for instance) when appropriate [45].

Disease status	Treatment Strategy
Untreated	I**nduction**: Hyper-CVAD or Ara-C-based therapy **CNS prophylaxis**: IT chemotherapy alone with systemic chemotherapy or cranial radiation
In Remission	**Postremission**: Ara-C-based therapy or other chemotherapeutic combination, imatinib or novel TKI, stem cell transplantation **CNS prophylaxis**: IT chemotherapy alone with systemic chemotherapy or cranial radiation
Recurrent/Relapse	**Reinduction**: Hyper-CVAD/Ara-C-based therapy **Palliative radiation** **Investigational agent**: Novel TKIs

Table 8. Treatment Strategies for ALL Patients

3.2.3 CLL

Treatment of CLL ranges from periodic observation with treatment of complications, like infections, to a variety of therapeutic options, including alkylating agents, purine analogs, combination chemotherapy, monoclonal antibodies, and transplantation. The disease is generally not curable, occurs primarily in an elderly patient population, and often progresses slowly. The management of CLL is typically conservative [48].

Prognostic Factors

Several factors have been associated with prognosis of CLL. Some of these include [34]:

- Lymphocyte doubling time: The doubling of peripheral blood absolute lymphocyte count in less than 12 months is associated with decreased survival.
- Expression of biologic markers: Serum beta-2 microglobulin (B2M) and CD38 expression known to be expressed on leukemia cells early in CLL progression may affect prognosis.

- Cytogenetics: The most unfavorable feature in CLL is 17p, which is present in approximately 10% of patients and is associated with a median survival time of 32 months in some clinical investigations. 17p is more likely to occur after initial diagnosis as the leukemic cell population expands. An intermediate risk is associated with the deletion of 11q, which is present in about 18% of patients and carries with it a median survival time of about 79 months. Deletion of 13q14 presents in 50% of patients and has a more favorable prognosis [34].

Treatment Strategies and Guidelines

Even with adverse prognostic factors, CLL management adopts more of an initial watch and wait strategy. Multiple studies have shown that early initiation of chemotherapy does not show clinical benefit but, rather, only increases mortality. Based on these studies, patients do not need to be treated with chemotherapy until they become symptomatic or display evidence of the rapid progression of the disease. These indicators include [49]:

- Weight loss more than 10% over six months
- Extreme fatigue
- Fever related to leukemia for longer than two weeks
- Night sweats for longer than one month
- Progressive marrow failure (anemia/thrombocytopenia)
- Progressive or symptomatic splenomegaly
- Massive or symptomatic lymphadenopathy
- Progressive lymphocytosis
- Autoimmune anemia or thrombocytopenia not responding to therapy

However, since the rate of progression may vary from patient to patient, with long periods of stability and sometimes spontaneous regression, frequent and careful observation is required to monitor the clinical course of the disease [48].

When patients become symptomatic and require treatment, a variety of treatment options are available, including chemotherapeutic agents (often used in combination) like nucleoside analogs, alkylating agents, and immunotherapy, as well as steroids and alloSCT [49].

Nucleoside analogs have shown to have major activity against CLL and are used commonly in first-line therapy. Of these, the most extensively studied is fludarabine [8], which has shown single-agent activity superior to the activities of several combination regimens, such as CVP (cyclophosphamide, vincristine, prednisone) and CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) in previously untreated disease [34]. The incorporation of fludarabine into chemotherapeutic combinations, as in the case of FCM (which is the combination of fludarabine with cyclophosphamide and mitoxantrone) has further improved response rates [49].

Patients with CLL are prone to infections, both common and unusual. This may be associated with the use of nucleoside analogs and may be severe, so these patients are carefully monitored [34]. Therefore, the early recognition of infections and a clinical response with the appropriate therapy are critical to the long-term survival of CLL patients [49].

Initial therapy often involves corticosteroids to control the autoimmune destruction before marrow-suppressive chemotherapy is utilized. Pneumococcal and influenza vaccines are often recommended for this reason [49].

Regarding the use of immunotherapy, rituximab is a monoclonal antibody [34] that is commonly used in combination with other types of chemotherapeutic agents [49]. In fact, one combination, FCR (which is fludarabine, cyclophosphamide, and rituximab) has become the most frequently chosen option for first-line therapy outside of a clinical trial [50]. Additionally, the same regimen, without cyclophosphamide (known as the FR regimen) has been shown to produce higher overall complete response rates, as well as higher complete remission rates in both untreated and relapsed CLL patients. Rituximab binds CD20 [34], which is especially important in patients with specific genetic abnormalities that lead to higher levels of CD20 expression, as the tumor cells are more susceptible to immunotherapy directed against CD20 [49].

Alemtuzumab is another monoclonal antibody approved for use as a first-line treatment or for use with fludarabine in patients with refractory disease. It is directed at CD52 and has been shown to be effective in treating CLL in patients with specific genetic abnormalities, such as p53 mutations.

In the relapsed/refractory setting, a new anti-CD20 monoclonal antibody, ofatumumab, is approved for use for patients with CLL refractory to fludarabine and alemtuzumab. Other monoclonal antibodies in development that are being evaluated in CLL include epratuzumab and lumiliximab [49].

The only known curative therapy is alloSCT, though the optimal timing of transplantation is still being investigated. It is known, however, that the delay of transplant until the development of refractory disease can result in worse patient outcomes. Younger patients or those with higher stages of disease or with adverse prognostic factors may benefit from more aggressive combination regimens and consideration for alloSCT early in their disease course [34]. Most patients are elderly and not able to be given upfront transplantation options, however [49].

Studies involving autologous transplantation and high-dose chemotherapy for CLL have failed to demonstrate a survival advantage despite initial high rates of complete molecular response, so the use of autoSCT is not recommended outside the context of a clinical trial. Alemtuzumab is being investigated for use in combination with transplantation and as consolidation treatment following initial combination therapy [34]. This agent seems to play an important role in the elimination of residual disease in patients undergoing autoSCT. The addition of alemtuzumab to regimens for alloSCT appears to also decrease the incidence of GVHD [49].

Common CLL Prognostic Markers		
Marker	Incidence %	Median Survival (years)
IgHV mutated	55	24
IgHV unmutated	45	8
CD38 > 30%	30	< 10
CD38 < 30%	70	> 15
Zap-70 > 20%	60	6-10
Zap-70 < 20%	30-40	> 15
Deletion 13q	55	17
Deletion 11q	18	6-8
Trisomy 12	16	9-11
Normal cytogenetics	18	9-11
Deletion 17p	7	2-3

Table 9 Various Prognostic Markers Complicate Staging but May Influence Patient Selection for Transplantation **[51]**

For relapsed disease, if the duration of response was greater than three to five years, consideration may be given to repeating the previous treatment. For short remissions, therapy should be modified. For younger patients with short remissions (<1 to 2 years), alloSCT may be considered following retreatment and attainment of complete remission. For older patients, palliation should be used and may include alkylating agents [34].

Disease Status	Treatment Strategy
Untreated	**Induction**: FCR regimen or other fludarabine-based combination, novel immunotherapy **Prophylaxis**: Vaccine or other preventative agent for infections
In Remission	**Postremission**: allogeneic transplantation
Recurrent/Relapse	**Reinduction**: monoclonal antibody, alloSCT **Investigational trial**: IMiDs, autoSCT **Palliative/supportive care**: alkylating agents

Table 10 Treatment Strategies for CLL Patients

4 Chimeric Antigen Receptors (CARs) for Cancer

4.1 Introduction to CARs

Several cellular therapies have been integrated into cancer treatment, including the infusion of polyclonal or antigen specific T cells, lymphokine-activated killer cells, natural killer (NK) cells, dendritic cells, and macrophages. The objective of this chapter is to describe the background, rationale, and current clinical use and experimental approaches for adoptive T cell therapies for the treatment of cancer utilizing chimeric antigen receptors (CARs).

4.1.1 Rationale for Adoptive T Cell Transfer Therapy

Over 50 years ago, Mitchison introduced the concept of adoptive cellular therapy for tumor allografts in mice [52]. Also in mice, it was suggested that allogeneic hematopoietic graft was related with eradication of leukemia cells after transplantation [53] [54]. This concept – antileukemia properties of the graft – provided the primary rationale to translate allogeneic bone marrow transplant to the clinic [55]. The first trials using autologous or allogeneic lymphocytes had disappointing results, which should not come as a surprise considering the fact that they were carried out before the principles of T cell biology and tumor antigens were properly studied. For the first 25 years, the study of adoptive cellular therapy has been Rosenberg & Terry [56].

The founding rationale for adoptive T cell therapy was defined by Weiden and colleagues [57] and stated that hematopoietic stem cell transplantation (HSCT) using syngeneic donors was less effective at preventing relapse of leukemia than use of sibling donors. Allogeneic T cells can recognize targets on leukemia cells that syngeneic T cells cannot; therefore, there may be ways to target cancer cells specifically with adoptively transferred autologous T cells.

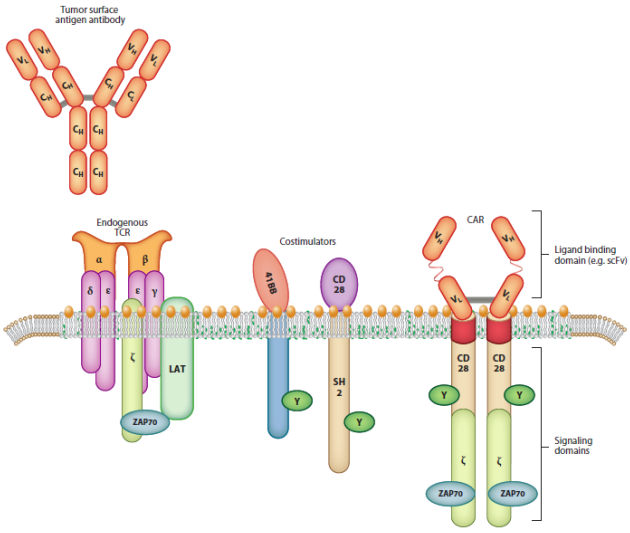

Figure 3. Antibodies can bind to surface antigens expressed on tumor cells.

Chimeric antigen receptors (CARs) have a single-chain antibody fragment (scFv), expressed in tandem with signaling elements derived from the T cell receptor (TCR) and costimulatory domains such as 4-1BB and CD28. Adapted from Barret et al.

4.1.2 Principles of T Cell Transfer

The understanding of the relevant principles of cellular and molecular immunology and cancer cell biology is crucial for successful adoptive T cell therapy. Lessons learned from the disappointing efficacy of many previous forms of adoptive cellular therapy led to insights into basic T cell function, which in turn fueled more effective translational science.

The environment of the lymphopenic host (e.g.: degree of host lymphodepletion and availability of T cell–supportive cytokines) supports homeostatic expansion, in which case adoptively transferred T cells engraft and expand more efficiently [58]. Homeostatic expansion also results in the acquisition of enhanced effector functions of the infused cells [59].

There is no such thing as optimal engineered T cells, as they will likely differ depending on the tumor and goals of the adoptive therapy. Initially, effector T cells were thought to be superior because they secreted high levels of effector cytokines and were proficient killers of tumor targets in vitro. But there is increasing evidence that infusion of naıve T cells [60], central memory T cells (TCM cells) [61], Th17 cells [62], and T stem memory cells [63] could be rewarding considering their high replicative capacity. It is not easy to choose one of these subsets for expansion and modification, as the T cell pool available for collection from a patient may be limited. Although naıve T cells or TCM cells would be expected to have excellent expansion, Th17 cells are versatile and can recruit the neutrophil compartment via their cytokine secretion profile to provide additional antitumor immune responses. On the contrary, the interfering existence of regulatory T cells (T regs) should be minimized both in the patient and in the transferred product. Preclinical models forecast high levels of T regs in the host that may block an antitumor response of transferred lymphocytes [64].

The engraftment efficiency and antitumor efficacy of adoptively transferred T cell lines in patients with melanoma is related with telomere length preservation [65]. The stimulation of CD28 maintains telomere length in T cells [66], and cultures that optimize costimulation might also improve the replicative capacity of adoptively transferred T cells.

Moreover, in order to resist cell-extrinsic forms of immunosuppression such as those mediated by TGF-β and T regs, T cells can be engineered [67]. Hence,it is probable that the eventual clinical application of adoptive T cell transfer will use combinatorial approaches of modified T cell subsets in different paths. This is especially true as one targets different tumor types, because the T cells collected from patients will have been exposed to different chemotherapy regimens (and thus may have different subsets available for collection), and the specific tumor microenvironments (e.g., lymph node, pancreas, brain) may require different approaches because tumors utilize distinct mechanisms of evasion from the immune system.

4.2 Strategies for T Cell Culture and Engineering

The first studies of T cell immunotherapy technology transferred large sums of effector T cells, which were essentially non replicative cells and therefore unable to expand in the patient to achieve an effector-to-target ratio in vivo that would be satisfactory to eliminate advanced cancers. More recently available results from trials with engineered T cells [68] have shown that the infusion of small numbers of cells may be enough considering the fact that most of the T cell expansion occur in the host instead

of ex vivo in cell culture [69]. There might be an exception to this brand new approach: in the setting of transiently engineered cells such as mRNA-transfected T cells that require large numbers of cells to be infused on multiple occasions [70] [71].

4.2.1 Approaches for T Cell Culture

The latest research indicates that, on a per cell basis, the adoptive transfer of T cells with extensive replicative capacity has greater engraftment and antitumor effects vs transfer of terminally differentiated effector cells that have a more potent cytotoxic effector function [65]. What might be seem as a paradox is probably explained by the capacity of TCM cells to self-renew and differentiate into effector T cells in vivo, while terminal effector memory T cells have lost this plasticity [72]. A possible approach could be: 1) to isolate TCM cells with the desired specificity in vitro by sorting or other means of physical separation, 2) engineer the desired specificity, 3) expand and later on infuse the TCM cells [62]. Another possible way to enrich and maintain TCM cells and thereby obviate the need for cell sorting is through the manipulation of bulk T cell-culture conditions. Cell-culture settings that augment CD28 and CD137 (4-1BB) costimulation in vitro also promote the maintenance of TCM cells in vitro [73] [74] and in vivo [75]. The use of memory stem cells, programmed for the most widespread self-renewal, has also a major potential [63] [76].

In this context, an efficient cell-culture method is to produce artificial antigen-presenting cells in one out of two ways: by coating beads with CD3-specific antibody or by transfecting cells to express CD3-engaging moieties and costimulatory molecules.

4.2.2 Approaches for T Cell Engineering

Recent breakthroughs in basic science have resulted in many approaches to engineer lymphocytes at many levels, such as genomic, RNA, epigenetic, and protein [77]. Vectors derived from gamma retroviruses or lentiviruses have been most useful for T cell-based therapies, for long term gene expression because of their ability to integrate into the host genome, with potentially permanent expression of the transgene, and for their low intrinsic immunogenicity [78] [79].

In order to achieve the best therapeutic effects, permanent transgene expression may not be mandatory. RNA-based electroporation of lymphocytes using in vitro–transcribed mRNA mediates transient expression of proteins for approximately one week and obviates the risk of integrating viral vectors. Readdressed T cells transduced with RNA encoding CARs should have the expected gains of function [70], as showed in Figure 4. Clinical trials using mRNA-transduced dendritic cells have been safely conducted [80], and trials using mRNA-electroporated T and NK lymphocytes are ongoing at several centers.

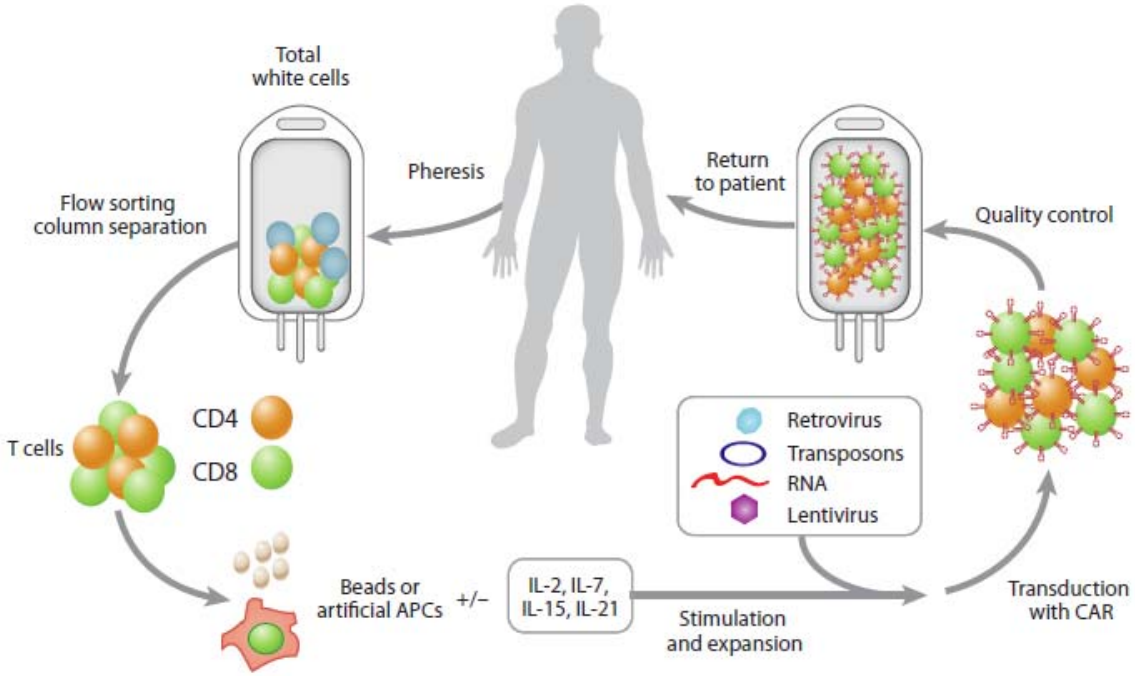

Figure 4. Chimeric antigen receptor (CAR) therapy is similar to an autologous bone marrow transplantation procedure.

T cells are collected from the patient by apheresis, and the T cells are expanded and genetically modified using several approaches before they are returned to the patient.

4.2.3 Strategies Using Synthetic Biology with Engineered T Cells

The complexity and high level of tolerance to most tumor antigens combined with immunosuppressive tumor microenvironments turns simple transfer of native isolated antitumor T cells unlikely to succeed. In order to enhance the natural function of the infused T cells, it is required the use of synthetic biology that combines elements of engineering, chemistry, computer science, and molecular biology to assemble cellular and biological tools [81].

To some extent, modification of T cells (genetic or otherwise) is a requirement in order to apply the principles of synthetic biology to tumor targeting. A potential safety concern when infusing individuals with engineered T cells is related with genetically engineered hematopoietic stem cells, when viral insertional mutagenesis was shown to cause cellular transformation [82]. Nevertheless, in patients with congenital and acquired immunodeficiency, genetically modified T cells may persist after adoptive transfer for over a decade without adverse effects [83], which might indicate that genetically modifying mature human T cells are essentially safe [84].

4.2.4 Strategies with CAR T Cells

In order to win the battle against tolerance in tumors that results from deficiencies in the TCR repertoire, T cells are genetically modified with CARs containing sequences that encode antibody-based recognition domains linked to signaling sequences (Figure 3). An advantage of CARs is that because they are specific for cell-surface molecules, they overcome the constraints of MHC-restricted

TCR recognition and avoid tumor escape through impairments in antigen presentation or human leukocyte antigen expression. Although genetic modification of T cells is not limited to conferring new antigen reactivity on recipient T cells it can also be used to insert genes that improve the efficacy of the T cells that are transduced. Such genes include those encoding molecules involved in costimulation [85], the prevention of apoptosis [86], the remodeling of the tumor microenvironment [87], and the induction of homeostatic proliferation [88], as well as CARs encoding chemokine receptors that promote directed T cell homing [89].

4.3 Current Status of Chimeric Antigen Receptor T Cell Therapy

One can roughly classify the design of CARs in clinical trials in to three different generations as demonstrated in Figure 5. The first generation CARs encode antibody-based external receptor structures and cytosolic domains that encode signal transduction modules composed of the immunoreceptor tyrosine based activation motif such as TCRζ or FcRγ [90]. The second-generation CARs also include a costimulatory signaling domain such as CD28 or 4-1BB [85] [91], and third-generation CARs include over three cytosolic domains [92]. CAR-modified T-cell potency may be further enhanced through the introduction of additional genes, including those encoding proproliferative cytokines (ie, IL-12) or costimulatory ligands (ie, 4-1BBL), thus producing “armored” fourth-generation CAR-modified T cells [93].

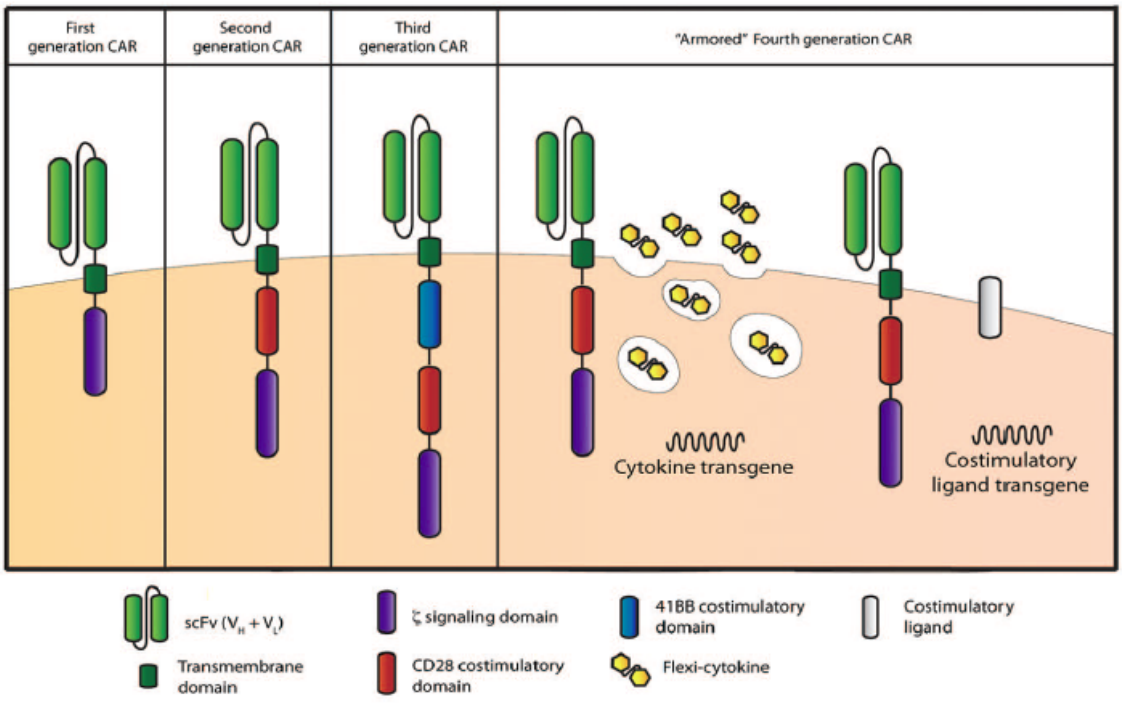

Figure 5. CAR technology evolution through the generation of more potent CARs.

First-generation CARs classically contain only one signaling domain, typically the cytoplasmic signaling domain of the CD3 TCRζ chain. Second-generation CARs containing 2 signaling domains typically include the addition of the cytoplasmic signaling domains of the costimulatory receptors CD28, 4-1BB, or OX-40, among others. Third-generation CARs attempt to harness the signaling potential of 2 costimulatory domains: classically, the CD28 domain followed by either the 4-1BB or OX-40 signaling

domains. CAR-modified T-cell potency may be further enhanced through the introduction of additional genes, including those encoding proproliferative cytokines (ie, IL-12) or costimulatory ligands (ie, 4-1BBL), thus producing “armored” fourth-generation CAR-modified T cells. Adapted from Brentjens RJ *et a*l.

4.3.1 First Generation CARs

The first CAR trials were conducted in patients with HIV, while testing a first-generationCD4ζCAR that has demonstrated modest antiviral efficacy but exceptional rates of long-term persistence that may exceed that of natural T cells [94]. Hopefully, retroviral integration site analysis showed no evidence of persistent clonal expansion or enrichment of integration sites near oncogenes or tumor suppressor genes [95].

A phase I trial testing T cells expressing a CAR specific for a folate-binding protein that is present on ovarian carcinoma cells indicated that the approach was safe, nevertheless poor expression and persistence of the transgene encoding the CAR were observed in vivo [96]. Likewise, a pilot test in children with neuroblastoma treated with autologous T cells retargeted for a tumor associated adhesion molecule (CD171) has showed that the approach is safe however was limited by poor persistence of the T cells [97]. T cells that express a CAR specific for carbonic anhydrase IX, an antigen present on the surface of clear cell renal cell carcinoma, have also been through the test [98]. Surprisingly, serious hepatic toxicity was observed in several patients within a week of T cell infusion, most likely due to carbonic anhydrase IX expression in the biliary tract. The lesson to be learned is that CAR targets must be carefully chosen to avoid off-tumor but on target adverse effects, or that extra safety features, such as suicide switches or transient expression systems [99], need to be incorporated into the vectors driving the expression of the chimeric receptor.

What one may learn from the trials testing first generation CAR T cells is that the infused product can be immunogenic. Both B cell [96] [98] and T cell responses [100] [101] have been identified in CAR trials. According to Lamers et al., the plasma from these patients neutralized target cell recognition by the CAR T cells. Furthermore, Lamers et al. [101] have shown that eight of nine evaluable patients also developed cellular immunity against their carbonic anhydrase IX–specific CAR, and that patients who developed a B cell response against the CAR also exhibited a cellular response against CAR T cells, but not necessarily the other way around.

The highest persistence of CARs reported was in the pediatric neuroblastoma trial (clinicaltrials.gov NCT00085930), where CARs were able to be detected at very low levels (0.0001% to 0.001%) up to four years after infusion [102]. Contrary to it, in the CD4ζ CAR trial (clinicaltrials.gov NCT01013415), the frequency of CAR T cells in blood was quite a few orders of magnitude higher (0.6 to 6%) five years after infusion [84].

The truth is that efficacy of first-generation CAR trials in cancer patients was disappointing. The best clinical results were reported in patients after infusion of a GD2-specific CAR, with 2 of 11 patients having long-term remissions [102].

4.3.2 Second- and Third-Generation CARs

Based on principles of T cell activation [103], it would be predicted that the first-generation CARs would become anergic except if the tumor target provided costimulation, as resting T cells with a CAR containing a TCRζ or FcRγ signaling moiety cannot be activated in the absence of costimulation [104] [105]. In 1998, two laboratories showed that the CD28 signaling domain provided costimulation when engineered in cis with the TCRζ domain into the CAR design [85] [106]. It was later shown that members of the tumor necrosis factor receptor family such as CD27, 4-1BB (CD137), and OX40

(CD134) can also provide costimulation [107] [108] [109]. Several trials are currently ongoing to test second- and third-generation CARs [110] [111].

4.3.3 CAR Trials Targeting B Cell Malignancies

Besides normal B cells, CD19 is not present on normal tissues (including pluripotent hematopoietic stem cells) and is not even shed as a soluble form into the circulation, which means that it is an excellent target, as showed in Figure 6.

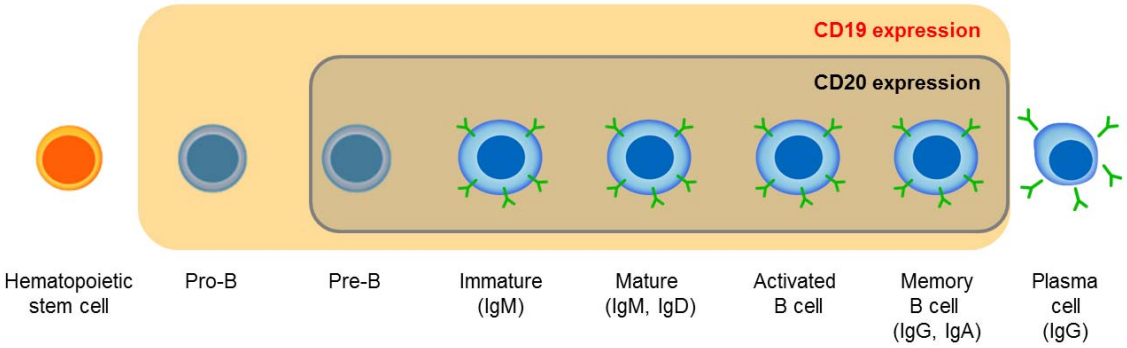

Figure 6. CD19: An Ideal Tumor Target in B-Cell Malignancies.

Promising results in chemotherapy-refractory patients have been obtained targeting the B cell lineage–restricted CD19 molecule that is expressed on B cell leukemias and lymphomas with CD19-specific CAR T cells [68] [112] [113] [114]. Durable remissions beyond two years have been observed in the initial cohort of patients with refractory and relapsed B cell chronic lymphocytic leukemia (CLL) after the infusion of autologous T cells transduced to express a CD19-specific CAR that contained a 4-1BB costimulatory domain [68] [69]. In the referred studies, the infusion of low doses of T cells led to massive in vivo expansion, subsequent tumor lysis, and a persistent aplasia of normal CD19+ B cells in most patients [68]. Considerable antitumor activity, depletion of normal B cells, and side effects related to tumor lysis and cytokine release have also been reported in patients with CLL and lymphoma by groups at the National Institutes of Health, Memorial Sloan-Kettering Cancer Center, and Baylor College of Medicine. A summary of actively recruiting clinical trials with CD19-targeted CARs, is showed in Table 11.

www.clinicaltrial.gov identifier	Center	Patient population	Age restriction	CAR construct	Gene-transfer method
Autologous T-cell trials					
NCT01044069	MSKCC	Relapsed/refractory ALL	≥ 18 y	scFv-CD28-CD3ζ	Retrovirus
NCT00466531	MSKCC	Relapsed/refractory CLL or B-NHL	≥ 18 y	scFv-CD28-CD3ζ	Retrovirus
NCT01416974	MSKCC	Residual CLL after chemotherapy	≥ 18 y	scFv-CD28-CD3ζ	Retrovirus
NCT01416974	BCM	Relapsed/refractory CLL or B-NHL	≥ 3 y	scFv-CD3ζ/scFv-CD28-CD3ζ	Retrovirus
NCT00608270	BCM	Relapsed/refractory CLL or B-NHL	≥ 3 y	scFv-CD3ζ/scFv-CD28-CD3ζ	Retrovirus
NCT00968760	MDACC	CD19+ lymphoid malignancy	18-65 y	scFv-CD29-CD3ζ	Transposon
NCT01593696	NIH	CD19+ ALL or lymphoma	1-21 y	scFv-CD28-CD3ζ	Retrovirus
NCT00924326	NIH	CD19+ B-cell malignancy	≥ 18 y	scFv-CD28-CD3ζ	Retrovirus
NCT00891215	UPenn	CD19+ B-cell malignancy	18-90 y	scFv-CD3ζ/scFv-41BB-CD3ζ	Lentivirus
NCT01029366	UPenn	CD19+ B-cell malignancy	≥ 18 y	scFv-CD3ζ/scFv-41BB-CD3ζ	Lentivirus
Allogeneic T-cell trials					
NCT01430390*	MSKCC	CD19+ leukemia	≤ 19 y	scFv-CD28-CD3ζ	Retrovirus
NCT00840853**	BCM	CD19+ ALL/CLL/NHL	≤ 80 y	scFv-CD28-CD3ζ	Retrovirus
NCT01475058***	FHCRC	CD19+ B-cell malignancy	18-75 y	scFv-CD28-CD3ζ	Lentivirus
NCT01497184	MDACC	CD19+ B-cell malignancy	18-65 y	scFv-CD18-CD3ζ	Transposon
NCT01087294	NIH	CD19+ B-cell malignancy	18-75 y	scFv-CD28-CD3ζ	Retrovirus
NCT01551043	UPenn	ALL	≥ 18 y	scFv-41BB-CD3ζ	Lentivirus
Haploidentical NK-cell trials					
NCT00995137	St Jude	Relapsed/refractory ALL	≤ 18 y	scFv-41BB-CD3ζ	Retrovirus

MDACC indicates MD Anderson Cancer Center; NIH, National Institutes of Health; FHCRC, Fred Hutchinson Cancer Research Center; NHL, non-Hodgkin lymphoma; and scFv, single-chain–variable fragment.

*Donor-derived EBV-CTLs. **Donor-derived Tri-virus CTLs. ***Donor-derived CD8+ central memory viral specific (EBV or CMV) T cells.

Table 11. Summary of actively recruiting clinical trials with CD19-targeted CARs. Adapted from Brentjens *et al*.

In clinical trials by these research groups, autologous T cells were modified to express CD19 CARs that contain a CD28 costimulatory domain [112] [113] [114] [115]. Beyond the activity in CLL and mantle cell lymphoma [68] [92], CD19:4-1BB CARs do have potent activity in pediatric acute lymphoblastic leukemia (ALL) allowing an efficient trafficking to bone marrow and cerebral spinal fluid [116]. It hasn't been properly assessed whether CARs with a CD28 and/or a 4-1BB signaling domain are preferable. It is currently under way a clinical trial led by Brentjens and collaborators infusing an equivalent number of CD19- specific CARs containing either a CD28 or 4-1BB domain to address this issue (clinicaltrials.gov NCT01044069).

These trials employed efficient retroviral or lentiviral vector transduction to introduce CARs into T cells. Whether one vector is better than the other remains unknown. A currently ongoing trial (clinicaltrials.gov NCT00968760) is testing CD19 CARs that are expressed using the nonviral-vector-mediated *sleeping beauty transposon* system [117].

Despite considerable safety data available, permanent genetic modification remains a focus of significant regulatory oversight. Some groups have integrated “suicide genes” into their T cell–engineering protocols, in which expression of a pro apoptotic gene is under the control of an inducible promoter responsive to a systemically delivered drug [99]. Though theoretically attractive, this approach does not guarantee elimination of all modified T cells, and thus may permit re-expansion of remaining CAR T cells after clearance of the activating drug.

An mRNA electroporation-based system to induce transient CAR expression results in efficient CAR delivery and expression that guarantee 100% loss of CAR-driven T cell activity within seven days without the need to administer other systemic agents [71] [70].RNA CAR T cells have demonstrated antigen-driven in vitro effector function [118] [119] and in vivo antitumor efficacy in localized models of solid and liquid tumors [70] [120] [121]. It is highly probable that several infusions of RNA-modified CAR T cells would be needed for tumor control, and the dose and T cell composition of these infusions are under investigation.

There are many other questions about the use of CARs for B cell malignancies, including major issues in clinical trial design, such as whether to provide cytokine support to the patient after CAR infusion and whether host conditioning chemotherapy is necessary or desirable, as illustrated in Figure 7.

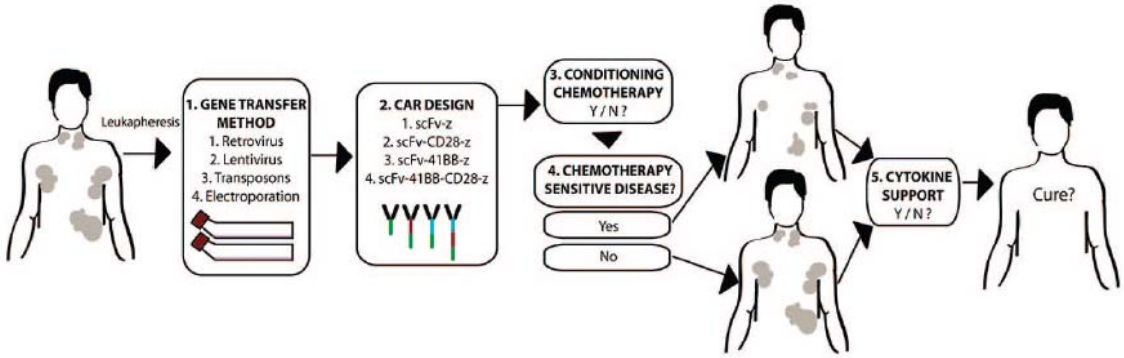

Figure 7. Variables in clinical trial design.

Multiple, potentially clinically relevant variables exist between various published clinical trial outcomes treating patients with CD19-targeted, CAR-modified T cells. There are variables in the methodology of CAR gene transfer (1), the design of the CAR (2), the inclusion or exclusion of prior conditioning chemotherapy (3), whether conditioning chemotherapy may reduce tumor burden (4), and whether additional cytokine support with IL-2 is provided exogenously after modified T-cell infusion (5). Whether one or more of these variables are indeed relevant to ultimate clinical outcomes awaits additional multicenter trials resolving these variables by direct comparison to establish the optimal conditions in which these CAR-modified T cells may induce an optimal clinical response. Adapted from Brentjens *et al*.

4.4 Issues and Future Directions for CAR T Cells

One of the major questions is whether T cell therapy can enter the routine practice of medicine. Another one is whether successful therapies can extend beyond CD19-directed CAR T cells.

4.4.1 Additional target antigens in cell therapy of leukemia

As explained earlier, the limited nature of the CD19 on regular B cells and most B-cell malignancies gave this target wider attention in both preclinical and clinical investigations for adoptive T-cell therapy

in leukemia. Nevertheless, further high-potential target antigens are under investigation in order to broaden the application of CAR technology to other hematologic malignancies. Precisely, clinical trials using CAR-modified T cells targeted to CD20 have demonstrated safety and some encouraging initial clinical outcomes [100] [122]. Other targets for CAR-modified T cells could be the receptor tyrosine kinase-like orphan receptor 1 (ROR1) or kappa-light chain of human Ig, which has the ability to expand the application of this therapy for hematologic malignancies [123] [124]. Likewise, this method with adoptive T-cell can be inferred to myeloid malignancies based on promising preclinical data using CAR-modified immune effector cells targeted to both CD33 and Lewis Y antigen, [125] [126] [127]. It may also be extrapolated as TCR-modified T cells targeted to the WT-1 antigen and the hyaluronan-mediated motility receptor (HMMR/Rhamm) [128] [129]. Excluding the anti-kappa chain CAR-modified T cells, it is uncertain if these latter antigens will be targeted through adoptive T-cell therapies in future clinical trials.

4.4.2 The Next Generation of CAR T Cells

So far, CAR-modified T cells have proven interesting initial clinical responses; nevertheless, the majority of patients treated with CD19-targeted T cells eventually develops progressive disease and succumb to their disease. There is still much to be done in terms of genetic approach to enhance the in vivo antitumor efficacy of these cells. It has been demonstrated by Brentjens *et al* that additional genetic modification of CAR-modified T cells may significantly enhance their efficacy; for example, by expressing proproliferative T cell– costimulatory ligands (4-1BBL) [130] or proinflammatory cytokines (IL-12), [131] resulting in “armored” fourth-generation CAR-modified T cells. These enhanced cells have shown additional in vivo antitumor efficacy in preclinical tumor models versus T cells modified to express the tumor-targeted CAR alone. Bottom line, there are highly promising preclinical outcomes in these studies, hence one may expect further translation from these more potent tumor-targeted T cells to the clinical setting as the next generation of CAR-modified T-cell trials.

4.4.3 Does Dose matter?

Doses of adoptively transferred cells are ordinarily described as the total number of viable cells administered, or as the total number of viable cells administered per kilogram body weight or per square meter body surface area. The optimal dose is unknown because T cells with high replicative potential will expand in the host, with the infused total dose having little relation to the steady-state number of cells that engraft and persist. Hence, dose concerns are more complex than in other areas of transfusion medicine, considering the fact that red cells or platelets do not expand after transfusion. Kalos *et al* studies regarding adoptively transferred autologous CAR T cells, frequently found that the number of cells in the host peaks two to three weeks after infusion of the cells [68].

Cytokines administrated to the host may also have a considerable impact on the persistence of adoptively transferred T cells. Others have found that the coadministration of interleukin (IL)-2 enhances the persistence of adoptively transferred human CD8+ T cells [132]. Nevertheless, Kalos *et al* have found that when autologous human CD4+ T and CD8+ T cells are given in combination, persistence is not increased by concomitant IL- 2 therapy [133]. Lastly, recent studies show that IL-2 can induce the proliferation and maintenance of effector CD8+ T cells but might actually delete memory T cells and increase the number of T regs [134]. In the opposite, IL-15 and IL-7 seem to select for the persistence of memory CD8+ T cells and might decrease the number of T regs in mice [135] and non-human primates [136].

Striking schedule-dependent increases in efficacy and the frequency of adverse effects from adoptively transferred cells have been reported when T cell infusions are given to lymphopenic hosts [137] [138]. Lymphodepleting chemotherapy is generally administered to the host several days before the adoptively transferred T cells. The drugs may have multiple effects that seem to promote the antitumor effects of the adoptively transferred T cells [58]. Cell dose, T cell replicative capacity, cytokine support, host lymphopenia, and timing of infusion are variables that require more data before optimal regimens can be identified.

4.4.4 CAR T Cells and Allogeneic Stem Cell Transplant

One of the major causes of failure after allogeneic hematopoietic cell transplant remains leukemia relapse, and the desired goal of augmenting the graft-versus-leukemia (GVL) effect without aggravating GVHD remains elusive [139]. Unmodified donor lymphocyte infusions are commonly given to treat relapse and are often complicated by GVHD. In addition, although they are dramatically effective for relapsed chronic myeloid leukemia, there is limited activity for patients with relapsed ALL. It is possible that infusion of allogeneic CAR modified T cells could enhance the efficacy of allogeneic HSCT or improve outcomes of donor lymphocyte infusions. This is supported by recent evidence that infusion of co stimulated but non-gene-modified allogeneic T cells was safe in a phase I trial [140]. In addition, a pediatric patient treated at the Children's Hospital of Philadelphia relapsed with ALL after a cord blood transplant and had T cells harvested from the patient and returned without induction of GVHD [116]. Several trials are now under way to evaluate the safety and antileukemic potential of CAR-modified allogeneic T cell infusions.

5 Toxicity with CAR T Cells

5.1 Introduction to Toxicity in T Cell Therapies

As it would be expected that all cancer therapies that are effective, there is also an emerging set of toxicities associated with T cell therapies. The toxicities can be classified in: those due to extrinsic factors present in the culture process, those due to accompanying cytokines that can be co infused with the cells, and those due to the cells themselves. Respiratory obstruction has been reported after cytotoxic T lymphocyte infusion for Epstein-Barr virus (EBV)-related lymphomas [141]. The reason behind it is probably due to a T cell–induced inflammatory response resulting in tumor edema and necrosis. Effector functions of infused T cells can be expected to include tissue damage similar to that encountered in T cell–mediated autoimmune diseases. In the case of allogeneic lymphocyte infusions, graft-versus-host disease (GVHD) and bone marrow aplasia can occur [142].

On-target toxicities were expected with CD19 CAR T cells and include B cell aplasia, tumor lysis syndrome (TLS), and cytokine release syndrome (CRS). Intravenous immunoglobulin can be used to replace quantitative antibody deficiency. TLS has been managed successfully by standard supportive therapy, including hydration, alkalinization, allopurinol, and rasburicase as required [143]. A unique feature of the TLS following CAR T cell therapy is that it may be delayed, occurring one month or more after CAR T cell infusion [69].

In patients with B cell malignancies, a delayed CRS occurs at the time of peak levels of CAR T cells in blood and bone marrow. The ideal management of CRS is still not completely understood. Corticosteroids and cytokine blockade are presently being appraised for patients with CLL (clinicaltrials.gov NCT01029366) and ALL (NCT01626495). In order to mitigate on target but off-organ toxicity to normal tissues, novel strategies such as regulating CAR expression or T cell survival are needed.

A number of off-target toxicities are theoretically possible with CAR T cells. The introduction of CARs by integrating retroviral or lentiviral vectors, transposons, and electroporation all create the risk of malignant transformation, induction of T cell lymphoproliferative disorders, or production of replication competent virus. These risks appears to be low based on the long-term follow-up data in patients treated with the CD4ζ CAR, where there have been no cases of genotoxicity in >540 patient-years of observation, and the fact that since the advent of modern packaging cell lines and plasmid designs no replication-competent virus has been observed in 297 humans enrolled on 29 different clinical protocols [83] [144].

In every CAR-based therapies that are presently being used in the clinic, cancer cells are targeted on the basis of single cancer-specific antigens and thus can essentially kill every cell that contains the targeted antigen (Figure 8). Despite the promising results, even in current therapies many 'on-target off-tumour' effects have been observed that can lead to lethal toxicity [114]. Thus, a current focus is engineering greater specificity to CAR-modified T cells.

A recent method to upsurge specificity involved the creation of a CAR-based AND logic gate that used novel CARs to target and kill cells that express two antigens but not the cells that displayed only one or none of the antigens [145] [146]. A different system focused on controlling T cell proliferation and comprised an RNA control device that allowed stabilization of interleukin-15 (IL-15; a proliferation-inducing cytokine) only in the presence of a small-molecule drug [147]. In yet another recent paper,

kinase inhibitors from human pathogens have been used to rewire the TCR signaling pathway to produce novel behaviors in T cell signaling, including a delayed TCR signaling 'pause switch' and feedback modulators in order to tune the amplitude of the T cell signaling response [148].

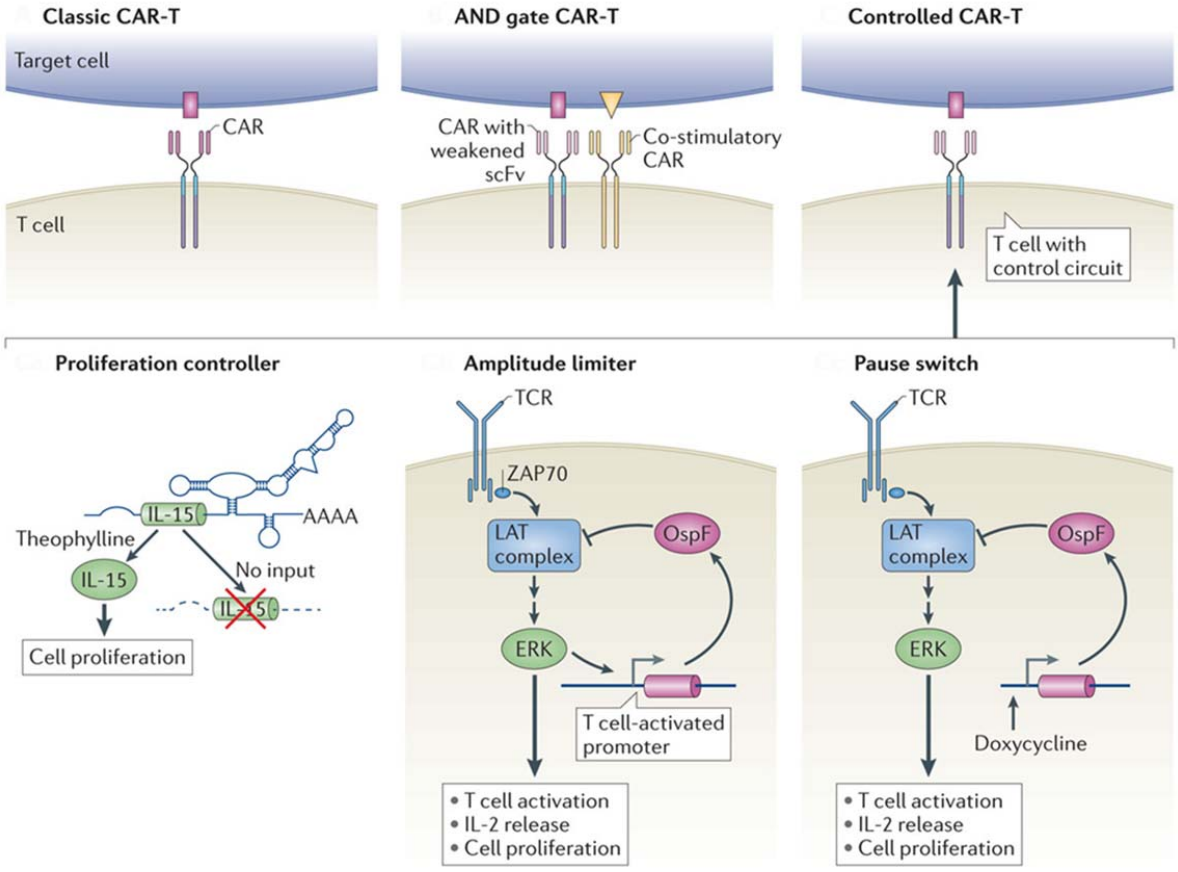

Figure 8. The classic chimeric antigen receptor (CAR) therapy (CAR-T) approach.

AND gate CAR therapy. T cells are engineered to express two CARs, one with a weakened single chain variable fragment (scFv) domain and one that contains co-stimulatory domains in its intracellular domain. Gene circuits for controlling CAR therapy activity. A RNA device enables the control of T cell proliferation. The device stabilizes the expression of secreted interleukin-15 (IL-15), a proliferation-inducing cytokine, in the presence of the small-molecule drug Theophylline. The amplitude limiter device uses a promoter that is activated upon T cell signaling and induces the expression of the bacterial virulence protein OspF, which in turn irreversibly inactivates T cell signalling. This negative feedback loop has been shown to dampen the amplitude of T cell activation. The pause switch device consists of a construct that induces OspF expression and thereby inhibits T cell activation in response to doxycycline. **[145] [147] [148]** Source: Nature Reviews, Molecular Cell Biology (2014)

Another strategy to improve the safety of this technology involves the incorporation of suicide genes into CAR-encoding vectors. Thymidine kinase from HSV (HSV-TK) has been used as an effective suicide gene in transferred T cells, but its immunogenicity could limit its future utility in adoptive T cell transfer [100] [149]. New research studies demonstrated that administration of a small molecule dimerizer (AP1903) to allogeneic HCT recipients induced rapid amelioration of acute GVHD and elimination of transplanted T cells that were engineered to express an AP1903-inducible caspase [99]. Another possible strategy that is in development involves engineering of CAR-modified T cells with a truncated human epidermal growth factor (EGF) receptor (EGFR) that lacks the EGF-binding domain

and the intracellular signaling domain, but retains the extracellular epitope to which the clinically available anti-EGFR monoclonal antibody, cetuximab, binds, potentially allowing the use of systemic administration of cetuximab as a tactic to better deplete engineered $EGFR^{+}$ T cells [150].

5.2 Cytokine Release Syndrome with CAR T Cells

In 2010, two cases of serious adverse events following the administration of CAR-T cells were reported [151] [152]. Both deaths were apparently related to a systemic cytokine release known as cytokine release syndrome (CRS). According to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAEs) Version 4.0, CRS is a disorder characterized by nausea, headache, tachycardia, hypotension, rash, and shortness of breath caused by the release of cytokines from the cells [153]. It is caused by an exaggerated systemic immune response mediated by T cells, B cells, NK cells and monocytes/macrophages which release a large amount of inflammatory mediators such as cytokines and chemokines. CRS is not a rare condition in the clinical setting. It occurs in graft-versus-host disease (GVHD) after transplantation, severe bacterial and viral infections, hemophagocytic lympohistiocytosis (HLH)/macrophage activation syndrome (MAS) and monoclonal antibody (mAb) therapy [154] [155] [156]. Cytokines trigger an acute inflammatory response and induce endothelial and organ damage, which result in microvascular leakage, heart failure and even death [157] [158] [159].Thus, it is of great importance to timely and properly manage CRS during CAR-T cell therapy.

5.2.1 Clinical Manifestations of CRS

CRS is often observed in the clinical trials to treat hematological malignancies with CD19 and CD20-specific CAR-T cells. A range of inflammatory cytokines and chemokines are intensively monitored before and after CAR-T cell infusion, including IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, sIL-2Rα, granulocyte macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)-1 [92] [68] [69] [160] [116] [113]. Considering the systemic review of six clinical trials performed in four institutions in which CRS has been reported (as showed in Table 12), one may conclude that CRS occurs in nearly two thirds of patients treated with CAR-T cells, which usually happens 6–20 days after CAR-T cell infusion. However, it could take place in a very short time after CAR-T cell infusion in some patients [151] [152] [114]. From two fatal case reports, it could be speculated that the time point of CRS may be related to baseline cytokine levels and the chance of CAR-T and cancer cell encountering [151] [152]. Once the baseline cytokine level is high at CAR-T cell infusion, or a large amount of CAR-T cells encountering with the target cells at a very short time, the CRS might be triggered earlier and with massive impact. All of the above cytokines are found elevated in part of the patients with CRS, while the cytokine profiles varies greatly among different individuals. IFN-γ, TNF-α and IL-6 are the most frequently monitored cytokines. IFN-γ and IL-6 are increased more than 10 folds in most patients with CRS when compared with the baseline, while TNF-α is rarely elevated in four of the six studies.

The cytokines and symptoms involved in CRS in the CAR-T cell clinical trials.

	MSKCC	MSKCC	Upenn	Upenn	NCI	FHCRC
Disease	CLL	ALL	CLL	ALL	CLL&lymphoma	Lymphoma
Targeted antigen	CD19	CD19	CD19	CD19	CD19	CD20
Co-stimulatory domain	CD28	CD28	4-1BB	4-1BB	CD28	CD28 + 4-1BB
Evaluable case number	8	5	3	2	8	4
Multiple cytokine elevation	5/7	2/5	2/3	2/2	4/8	1/3
Time of peak cytokines	<2 days	Day 6	Day10 and 20	Day 6 and 9	Day 7	Day 13
Individual cytokines						
IFN-γ	3/7	2/5	2/3	2/2	4/8	0/3
TNF-α	5/7[a]	1/5	0/1	0/2	4/8	0/3
IL-6	NR	2/5	2/3	2/2	NR	0/3
IL-10	NR	NR	2/3	1/2	NR	1/3
IL-2	5/7	2/5	NR	1/2	NR	0/3
IL-7	3/7	NR	NR	NR	NR	1/3
IL-8	NR	NR	2/3	NR	NR	NR
IL-12	2/7	NR	NR	NR	NR	1/3
Clinical symptoms	3/7	2/5	2/3	2/2	4/8	0/3
Fever	8/8	4/5	3/3	2/2	1/8	1/4
Rigors and chills	5/8	NR	2/3	NR	NR	1/4
Fatigue	NR	1/5	1/3	NR	3/8	2/4
Hypotension	2/8	4/5	1/3	1/2	5/8	1/4
Dyspnea	1/8	1/5	NR	NR	1/8	2/4
ARDS	NR	NR	NR	1/2	NR	NR
Encephalopathy	NR	3/8	NR	1/2	1/8	1/4
ALT/AST elevation	NR	NR	NR	1/2	2/8	1/4
Renal failure	1/8	NR	1/3	NR	3/8	NR
Cardiac disorders	1/8	1/8	1/3	NR	NR	NR

Abbreviations: MSKCC, Memorial Sloan-Kettering Cancer Center; UPenn, University of Pennsylvania; NCI, National Cancer Institute; FHCRC, Fred Hutchinson Cancer Research Center; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; NR, not reported. Cytokine elevation: 10 folds higher than the baseline level. Multiple cytokine elevation, three or more cytokines elevated with levels 10 folds higher than the baseline level. [114] [160] [68] [69] [116] [113] [92] Source: Xu, XJ *et al* (2014)

Table 12. The cytokines and symptoms involved in CRS in the CAR-T cell clinical trials.

Clinical symptoms as consequence of CRS include fever, fatigue, headache, seizure, nausea, rigors, chills, myalgias, dyspnea, acute respiratory distress syndrome (ARDS), hypotension, acute vascular leak syndrome, tachycardia, liver function impairment and renal failure (Table 12). Fever is the most frequent symptom and may be the earliest sign of CRS, it will most likely progress along with the development of CRS and can be completely resolved after the control of CRS [116]. Hypotension is relatively frequent in patients with CRS, who need immediate fluid resuscitation or vasopressor support although it can be reversed in most patients after the effective cytokine directed therapy. Grupp and Teachey *et al*., found that the manifestations of CRS in their patients is similar to HLH/MAS, with highly elevated serum ferritin, d-dimer, aminotransferases, lactate dehydrogenase and triglycerides,

hypofibrinogenemia, and hepatosplenomegaly [161] [162]. Moreover, the cytokine pattern of significant elevation of IFN-γ, IL-10 and IL-6, but not TNF-α is consistent with that of HLH as well [155].

The severity of the CRS had a good correlation with the level of cytokines. Brentjens *et al*. discovered that the degree of cytokine elevation was coincident with post-infusion fevers and episodes of relative hypotension [160]. In their study, two patients with high tumor burden presented higher cytokine levels and augmented fever severity and persistence accompanied by several organs involved, while other patients only presented mild fever or hypotension. Kochenderfer *et al*. evaluated the cytokine-associated toxicity through a score system called 'sequential organ failure assessment' (SOFA), which includes an assessment of hypotension, the platelet count, and also the respiratory, liver, renal, and central nervous system functions [113]. They discovered that patients with prominent elevations in serum IFN-γ and TNF-α after CAR-T cell infusion had a higher mean total SOFA score than those without the referred elevations.

5.2.2 Differentiation of CRS in CAR-T

Some other complications, including tumor lysis syndrome (TLS) and severe sepsis, may be similar to CAR-T cell induced CRS, which may be responsible for the elevation of cytokines and organ failure, despite the fact that management of both conditions is different. Thus, it is necessary to differentiate the above conditions and to give proper treatment. TLS is a disease-related emergency which has been described in CAR-T cell therapy. In TLS, lysed tumor cells release DNA, phosphate, potassium, and cytokines. When the accumulation of phosphate, potassium, xanthine, or uric acid is more rapid than excretion, TLS arises [163]. While cytokines may contribute to the development of inflammation, hypotension and acute kidney injury in TLS may have the same effect as well; some metabolic abnormalities which are regularly found in TLS are uncommon at early stage of CAR-T cell induced CRS, including hyperuricemia, hyperkalemia, hyperphosphatemia and hypocalcemia [164].

TLS may be diagnosed by measuring serum potassium, phosphorus, calcium, creatinine, uric acid and urine output. Allopurinol has been prescribed for the prevention and treatment of the TLS in some CAR-T cell based clinical trials [116] [151]. Another frequent complication in hematological cancer patients that present CRS is severe sepsis. Some techniques such as microbiologic culture, specific nucleic acid and antibody assay are well-established for the diagnosis of microbial infection. Also, the literature shows that the IFN-γ is seldom significantly raised, although IL-6 and IL-10 are very high in most patients with severe sepsis, which is quite different from CAR-T cell induced CRS and might be helpful for the differentiation [155] [161].

5.2.3 Precautions to avoid severe CRS

The incidence of CRS varies significantly among different clinical trials and each patient responds differently to CAR-T infusion even when a similar protocol is used. The diversity of cytokine profiles among different individuals and different clinical trials may be related to various CAR structures, underlying diseases and patients' genetic polymorphisms.

CRS induced by CAR-T cell infusion shares many common features with that caused by mAb administration. However, unlike mAb-induced side effects could be alleviated following the excretion of the drug, CRS and its related toxicity induced by CAR-T cells could be long-lasting, as proliferating T cells will increase in numbers in vivo and eventually cause CRS. As mentioned before, the 4-1BB incorporated anti-CD19 CAR modified T cells can be expanded more than 1000 folds in CLL patients [69]. Hence, the precaution of CRS in CAR-T cell therapy is considerably more important than that in mAb treatment. The following actions might be helpful to avoid severe CRS.

Inflammatory cytokine monitoring and related gene polymorphism assessment

The monitoring of inflammatory cytokines has become a standard of care in most clinical trials on CAR-T cell adoptive therapy. The baseline cytokine level is critical for the evaluation of host immune response to CAR-T cells. Alternatively, it could be an approach to screen patients who present elevated baseline cytokine level and are at high risk of severe CRS after CAR-T infusion. Likewise, the assessment of inflammatory cytokine levels at various time points after CAR-T infusion helps to diagnose CRS early, thus treat the patients timely. In Memorial Sloan-Kettering Cancer Center, the first patient who received conditioning chemotherapy (cyclophosphamide) followed by anti-CD19 CAR modified T cell infusion died 2 days later [151]. Though the authors speculated that the most likely cause of death was infection instead of the infused CAR-T cells, it is possible that hypercytokinemia before CAR-T cell infusion may initiate and aggravate the CRS in a short notice, causing a fatal outcome. Therefore, it would be vital to clinically identify which subsets of patients are more prone to developing this complication and a special cautious scheme might be established for them. Considering that polymorphisms of cytokine genes are related to the CRS severity, the assessment of inflammatory cytokine gene polymorphisms before CAR-T cell treatment could help to lower the risk for those patients.

Dose-escalation strategy for CAR-T cell infusion

In patients receiving donor lymphocyte infusion (DLI) following hematopoietic stem cell transplant, the adverse effects of DLI correlate with the infused T-cell numbers [165]. As mentioned before, the CRS caused by CAR-T cells is hard to foresee and to be controlled. Therefore, a conservative dose-escalation strategy for CAR-T cell therapy infusion is adopted by many phase I clinical trials [92] [68] [114] [166]. Today, it is not possible to provide reliable guidelines for proper starting doses of CAR-T cells. The loading dose should be adjusted depending on the type of CAR and the protocol used. For example, T cells with second- or third-generation CARs should start at a lower dose than those with a first-generation CAR. Likewise, transfer of CAR-T cells into patients receiving IL-2 administration should commence at a lower dose than transfer into patients without supplementary treatment. As a substitute, one could conduct the initial dose escalation with first generation CAR-T cells. Once the first generation CAR-T cells are shown to be safe, second-, or perhaps third-generation CAR-T cells could be explored [167]

Design of short-lived CAR-T cells

It is a major concern that the expansion and persistence of CAR-T cell in human body may increase the risk of severe long-term adverse effects, therefore short-lived CAR-T cells may be safer; because of that it has attracted great attention to genetically modify the T cells with mRNA CAR electroporation. Transgene expression of the in vitro transcribed RNA could be detected on the surface of the RNA-engineered T cells up to 7 days after RNA electroporation [70]. Multiple injections of these CAR-T cells mediate regression of tumor in different animal models [70] [168]. Although no mRNA CAR based clinical trial has been launched, this might be an important direction for CAR exploration. Alternatively, the induction of suicide genes into CARs could be another strategy. For example, inducible caspase 9 (icasp9) is remarkably effective in producing rapid (less than 120 min) apoptosis [169]. The reaction can be triggered by administrating a small molecule dimerizer that brings together two nonfunctional icasp9 molecules to form the active enzyme [170]. As a result, some investigators have incorporated icasp9 into a CAR vector targeting CD19 and showed that the activation of this suicide gene quickly induced apoptosis of CAR-modified T-cells both in vitro and in vivo [171].

Generation of less-differentiated CAR-T cells

There is increasing evidence that adoptive transfer of less-differentiated CAR-T cell subsets, memory stem T cell (Tscm) and central memory T (Tcm) cells, is associated with long persistence, strong expansion and superior antitumor immunity [172] [63] [173] . However, the Tcm and Tscm cells present less cytokine secretion and tumor-lysis ability after encountering the specific tumor antigens in vitro study [63] [174]. As a result, at least theoretically, the infusion of less-differentiated CAR-T cells may induce less quantity of cytokines in the early phase of CAR-T cell treatment. Such CAR-T cells could be generated from induced pluripotent stem cells or expanded in the presence of IL-7/IL-15 or IL-21 [174] [175]. Yet, whether the subsequent expansion of the CAR-T cells will produce delayed but milder toxicities is unknown.

5.2.4 Treatment of CRS

The clinical data of CAR-T cell therapy are limited as there are only few patients being treated up to now. Nevertheless, 2 fatalities were reported shortly after adoptive transfer of CAR-T cells, both related to CRS [151] [152]. Both patients presented respiratory distress, hypotension within several hours after CAR-T cell infusion and died several days later although intensive support had been given. The timely and active cytokine-directed therapy is critical for saving lives. Oxygen, fluid resuscitation, vasopressor and intubation supports might be required for patients with severe symptoms. Based on the available clinical trials and other related data, the following reagents could be helpful to resolve CRS.

Corticosteroids are key agents to suppress intensive inflammatory response and CRS. It has been extensively used in several kinds of CRS related diseases, including severe sepsis, GVHD, HLH/MAS, monoclonal antibody administration, etc. [156] [176] [177] The administration of methylprednisolone drops cytokine levels and relieves the correlated clinical signs in most patients with mild and moderate CRS [160]. Unlike mAb based cancer treatment, corticosteroids are seldom used as a premedication prior to CAR-T cell infusion due to the concern of affecting CAR-T cell efficacy.

Cytokines like TNF-α, IFN-γ and IL-6 play important roles in the CRS related toxicity. Several cytokine antagonists have been used as therapeutic agents to offset cytokine storm. In a phase II clinical study, the patients receiving the TNF-α inhibitor etanercept presented mild infusion reactions to rituximab and were not related to severe adverse events [178]. Teachey *et al*, successfully treated an ALL case with CRS after the blinatumomab (a CD19/CD3-bispecific T-cell receptor-engaging antibody) treatment by IL-6 receptor-directed therapy with tocilizumab [179].

Grupp *et al* reported an ALL case in which the patient developed severe and glucocorticoid-resistant CRS after receiving anti-CD19 CAR-T cell infusion. A single course of etanercept and tocilizumab combined anti-cytokine therapy had rapid clinical effects: defervescence occurred within hours, and the patient was halted from vasoactive medications and ventilator support, and the acute respiratory distress syndrome resolved [116].

The antagonists of IL-1, IL-2 and IFN-γ have not yet been reported to treat CAR-T cell related CRS. Nevertheless, they have been successfully used to treat the patients with CRS caused by other etiologies and therefore might be effective in treating CAR-T cell related CRS. The anti-IL-2 receptor antagonist antibody has been administrated with high-dose methylprednisolone to treat the CRS induced by TGN1412 [180]. The recombinant IL-1 receptor antagonist anakinra induces rapid and sustained remission of systemic juvenile idiopathic arthritis-associated MAS when combined with

corticosteroids and might successfully control intravenous steroid, immunoglobulin and cyclosporine (CsA)-resistant MAS in some specific cases [181].

5.2.5 Challenges for the management of CRS

CRS can be a double-edged sword: it is closely related to the efficacy of CAR-T cell therapy but it is likely to significantly harm the patient if the inflammatory response is devastating [182] [113]. Consequently, the balance between these two parts is an important issue in CAR-T cell therapy. The development of CRS correlates with CAR structures, underlying diseases and individual genetic background. In an attempt to improve the effectiveness of CAR-T cells by optimizing the vector structure, scientists should also focus on the safety, for example, by producing short-term CAR-T cells and incorporate the suicide gene or using mRNA approaches. For clinicians, the infusion of CAR-T cells should strictly follow the escalation scheme, initiating with low-dose or low-generation CAR-T cells. CRS related mortality must be reduced through the development of safer CARs, following strict dose-escalation scheme, intensively monitoring inflammatory cytokines and taking timely and effective measures including the administration of various antagonists of cytokines under the current situation. Optimal application of this new therapeutic approach on cancer patients is yet to be established.

6 Chimeric Antigen Receptor–Modified T Cells in Lymphocytic Leukemia

As mentioned before, by using gene-transfer techniques, one can genetically modify T cells in order to express antibodies on their surface that will confer new antigen specificity. These so called chimeric antigen receptors match an antigen recognition domain of a specific antibody with an intracellular domain of the CD3- zeta chain or FcγRI protein into a single chimeric protein [183] [184]. Despite the fact that CAR can trigger the activation of T cells in a similar way to that of endogenous T-cell receptors, the main barrier to the translation from lab bench to clinic regarding this technique, has been limited in vivo expansion of CAR T cells and poor clinical activity [111] [185]. CAR-mediated T-cell responses can be additionally improved with the addition of a costimulatory domain.

In preclinical models, Porter *et al* found that the insertion of CD137 (4-1BB) signaling domain considerably enhances antitumor activity and in vivo persistence of CAR as compared inclusion of the CD3-zeta chain alone [91] [186]. In the majority of cancers, tumor-specific antigens for targeting lack proper definition, but in the case of B-cell cancers, CD-19 is a striking target. The expression of CD19 is mostly restricted to normal and malignant B cells and B-cell predecessors [187]. In 2011 Porter *et al* initiated a pilot clinical trial of treatment with autologous T cells that express anti-CD19 CAR (CART19). A total of three patients were treated with remarkable immunologic and clinical effects of in vivo T-cell treatment with CAR receptors in one of the patients, who had advanced p53-deficient CLL.

It still remains unclear whether CAR T cells have clinical activity in Acute Lymphoblastic Leukemia (ALL). Grupp *et al* found that the infusion of T cells transduced with anti-CD19 antibody and a T-cell signaling molecule (CTL019) in two children with relapsed and refractory pre-B-cell ALL, expanded to a level above 1000 times as high as the initial engraftment level. Furthermore, CART cells were observed in the cerebrospinal fluid (CSF), where they persisted at high levels for at least 6 months. Despite the fact that eight grade 3 or 5 AE were observed (such as cytokine-release syndrome and B-cell aplasia), complete remission was acknowledged in both patients and is enduring in one patient to date [116].

CAR–modified T cells have demonstrated to be proficient in killing aggressive, treatment-refractory acute leukemia cells in vivo. Nevertheless, the rise of tumor cells that no longer express the CD19 target expresses the need to find additional targets other than CD19 in some patients with ALL [116].

6.1 Chimeric Antigen Receptor–Modified T Cells in CLL

In the previously mentioned case report, published on August 2011 in the NEJM, a patient with advanced P53-deficient CLL was administrated with autologous T cells that express anti-CD19 CAR (CART19) cell infusion, and on day 23, there was no evidence whatsoever of CLL in the bone marrow [69].

Three months after the administration of the modified T cells, computed tomography (CT) scans were performed and showed sustained remission; also, bone marrow studies at 3 and 6 months showed no evidence of CLL. According to the first author, David L. Porter, MD, professor of medicine and director of blood and marrow transplantation at the Hospital of the University of Pennsylvania, Philadelphia, the patient remained in remission after his infusions until today. This patient fully recovered from any and all side effects, has no symptoms and is fully functional. A second patient who experienced a complete response after CART19 infusions also remains in remission 1 year after therapy. The third patient had a dramatic but partial response.

In this pilot study, three patients were treated for advanced CLL with autologous T cells expressing CART19, and the study authors reported on the immunologic and clinical efficacy in one patient. That patient was diagnosed with stage I CLL in 1996, and first required treatment 6 years later. In 2002, the patient was treated with rituximab plus fludarabine, and the result was the normalization of his blood counts and partial regression of his adenopathy. The patient received additional treatment in 2006, and remained disease-free for 20 months.

In February 2009, the same patient suffered fast progressive leukocytosis and recurrent adenopathy, and its bone marrow indicated CLL. Cytogenetic testing revealed a deletion of chromosome 17p in 3 of 15 cells, and fluorescence in situ hybridization (FISH) testing showed that 170 of 200 cells had a deletion involving TP53 on chromosome 17p. Patients with TP53 deletions tend to have short remissions after standard therapies, the authors note [69].

The patient had autologous T cells collected in December 2009 and they were cryopreserved. He then received alemtuzumab for 11 weeks. This resulted in improved hematopoiesis and partially resolved adenopathy. During the next 6 months, the patient experienced stable disease with persistent and extensive bone marrow involvement and diffuse adenopathy. In July 2010, the patient was enrolled in a phase 1 clinical trial of CAR-modified T cells.

He had pentostatin and cyclophosphamide administered in order to deplete the lymphocytes; 4 days afterwards, he received 1.42_{x} 107 transduced T cells. The unselected T cells were infected with a self-inactivating lentiviral vector, designed by the authors to carry genes for the chimeric antigen receptor. No postinfusion cytokines were administered, and there were no toxic effects related to the infusion.

The patient started to experience chills and also moderate fever related with grade 2 fatigue fourteen days post infusion. Symptoms intensified during the next five days, and on day 22 the patient was diagnosed for tumor lysis syndrome following hospitalization, treatment with fluids and rasburicase.

The authors noted that on day 28, the karyotype was regular and steady in 15/15 cells. In the 200 cells examined, 198 were negative for deletion TP53 (FISH test). These values are considered among normal limits in negative control subjects.

After 3 and 6 months have passed post-infusion, the patient's physical exam keep on unremarkable. No palpable adenopathy was detected, CT scanning showed sustained remission, and bone marrow studies revealed no evidence of CLL on morphologic analysis, karyotype analysis (46,XY), or flow cytometric analysis, as shown in Figure 9 and 10.

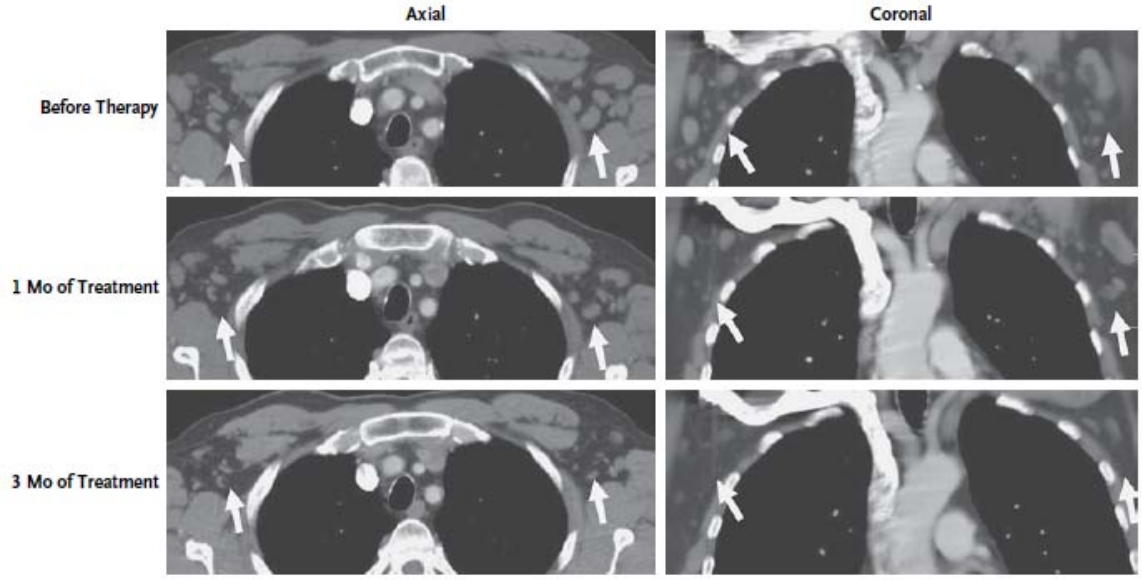

Figure 9. Contrast-enhanced CT scans obtained before the patient was enrolled in the study and 31 days and 104 days after the first infusion.

The preinfusion CT scan reveals 1-to-3-cm bilateral masses. Regression of axillary lymphadenopathy occurred within 1 month after infusion and was sustained. Arrows highlight various enlarged lymph nodes before therapy and lymphnode responses on comparable CT scans after therapy. Porter DL *et al*, 2011.

Figure 10. Bone marrow–biopsy specimens obtained 3 days after chemotherapy (day –1, before CART19-cell infusion) and 23 days and 6 months after CART19-cell infusion (hematoxylin and eosin).

The baseline specimen shows hypercellular bone marrow (60%) with trilineage hematopoiesis, infiltrated by predominantly interstitial aggregates of small, mature lymphocytes that account for 40% of total cellularity. The specimen obtained on day 23 shows residual lymphoid aggregates (10%) that were negative for chronic lymphoid leukemia (CLL), with a mixture of T cells and CD5-negative B cells. The specimen obtained 6 months after infusion shows trilineage hematopoiesis, without lymphoid aggregates and continued absence of CLL. Porter DL *et al*, 2011.

6.2 Chimeric Antigen Receptor–Modified T Cells in ALL

In April 2013, Grupp SA *et al*, published on the NEJM their experience with relapsed/refractory ALL in adults and children [116]. Twenty-two children and five adult patients with relapsed, treatment-resistant ALL have been treated so far with CTL019 at the University of Pennsylvania Perelman School of Medicine [188].

Nineteen children achieved a full response, and remission is ongoing in 14, with 5 patients experiencing relapse. The first patient ever treated with the protocol is still in remission 20 months later. All five of the adults achieved full remission, the longest of which has been 6 months. One patient later underwent bone marrow transplant and remains in remission. One patient relapsed after 3 months in complete remission, and his disease tested negative for the engineered cell target. The overall complete response rate in this group was 89% [188]. These results serve as another important milestone in order to demonstrate the potential of this treatment for patients have no other therapeutic options.

A separate report from the University of Pennsylvania discussed the quantity, lifespan, and activity of the engineered T cells once they were reinfused into the pediatric and adult patients with ALL (described above) as well as in adults with advanced relapsed/refractory CLL. Evidence suggests that patients with the greatest in vivo expansion of CART019 (to more than 5% of all CD3-positive cells) were the most likely to complete full remission. Patients with a weaker but still detectable cell expansion were considered partial responders, while those with no or minimal detectable T-cell expansion were considered non-responders. The detected CTL019 cells kept on their function as anticancer T cells for many months after infusion [189].

These expanded data provides significant proof that T cells engineered to express cancer-targeting CARs work not only dramatically but also in a sustained manner in patients with relapsed/treatment resistant leukemia. Evidence further demonstrates the potential of this immunotherapy method to help these patients achieve complete remission [190].

Furthermore, those engineered cells could be measured and tracked as a way to monitor treatment, which is an exciting finding considering that this treatment is often the last hope for these patients.

7 R&D alliances in CAR-based cellular therapies

Although adoptive T cell therapies were first developed in the 1980s, only recently entered the spotlight thanks to promising clinical results achieved with the latest generation, chimeric antigen receptor–based cell therapies. Currently one may count for at least 12 chimeric antigen receptors (CARs) projects being developed as result from multiple academic-industry collaborations, and pharma/biotech's are letting the researchers do the driving (Table 13).

Company	Academic leaders	Receptor type	Tumor-associated antigens	Date	Clinical trials
Adaptimmune	University of Pennsylvania; **University of Maryland, Baltimore**; **Roswell Park Cancer Institute**; **Washington University in St. Louis**; **Yale School of Medicine**; **City of Hope**; **The Children's Hospital of Philadelphia**; NCI; **Memorial Sloan-Kettering Cancer Center** (MSKCC)	T cell receptors (TCRs)	Cancer/testis antigen 1B (CTAG1B; NY-ESO-1) and CTAG2 (LAGE1; NY-ESO-2)	2008	Started
Celdara Medical	Geisel School of Medicine at Dartmouth College	CARs	Killer cell lectin-like receptor subfamily K member 1 (KLRK1; CD314; NKG2D)	2010	Expected late 2014
Cell Medica Ltd.	Baylor College of Medicine	TCRs	Not applicable[A]	2010	Started
Novartis (NYSE:NVS; SIX:NOVN)	University of Pennsylvania	CARs	CD19 and mesothelin	2012	Started
Kite Pharma	NCI	CAR or TCRs	CD19, VEGF receptor 2 (KDR/Flk-1; VEGFR-2), mesothelin, epidermal growth factor receptor (EGFR), NY-ESO-1 and carcinoembryonic antigen (CEA)	2012	Started
Cellectis (Euronext:ALCLS)	University College London	CARs	CD19	2012	Expected late 2014
Celgene (NASDAQ:CELG); bluebird bio (NASDAQ:BLUE)	Baylor College of Medicine	CARs	GD2, HER2 (EGFR2; ErbB2; neu) and CD19	2013	Started
Not applicable	MSKCC[B]	CARs	CD19 and prostate-specific membrane antigen (PSMA; FOLH1; GCPII)	Not applicable	Started

Table 13. Selected deals and partnerships in the adoptive T cell immunotherapeutic space from 2008 onward. Source: BCIQ: BioCentury Online Intelligence; BioCentury Archives; http://www.clinicaltrials.gov/

The August 2012 deal between Novartis AG and the University of Pennsylvania set off a wave of partnering activity in the chimeric antigen receptor (CAR)-based T cell therapeutic space, but collaborations between gene therapy company Adaptimmune Ltd. and East Coast universities and between accelerator Celdara Medical LLC and the Geisel School of Medicine at Dartmouth College had already been flying under the radar for a couple of years.

The University of Pennsylvania granted Novartis exclusive, worldwide rights to develop and commercialize CAR immunotherapies for cancer. Additionally, Novartis will provide $20 million to establish the Center for Advanced Cellular Therapies on the university's campus to co-develop CAR-based therapies to treat cancer.

In October 2012, Kite Pharma Inc. was granted exclusive access to the National Cancer Institute's (NCI) current and future engineered peripheral blood autologous T cell therapeutics to treat hematological and solid cancers. Kite has the option to an exclusive license for NCI proprietary products being developed under the Cooperative Research and Development Agreement. The company will also provide funding to the NCI.

In December 2012, Cellectis S.A. signed a broad collaboration agreement with University College London to develop CAR-expressing allogeneic T cells using Cellectis' proprietary genome engineering technologies to manufacture the T cells.

In March 2013, Bluebird bio Inc. partnered with Celgene Corp. to discover, develop and commercialize CAR immunotherapies for cancer. The partners will also work with the Baylor College of Medicine to develop new and existing CAR immunotherapy products and programs. Bluebird bio and Celgene declined to disclose details. Bluebird received an undisclosed upfront payment and is eligible for up to $225 million in option fees and milestones per product, plus royalties. Bluebird bio will be responsible for R&D through Phase I testing, after which Celgene has the option to license any products.

Therefore, one may conclude that about 20 years of cumulative work in academic centers has driven adoptive T cell therapies featuring chimeric antigen receptors (CARs) into the clinic and caught the attention of numerous biotechs and pharmas, as showed in the following diagram.

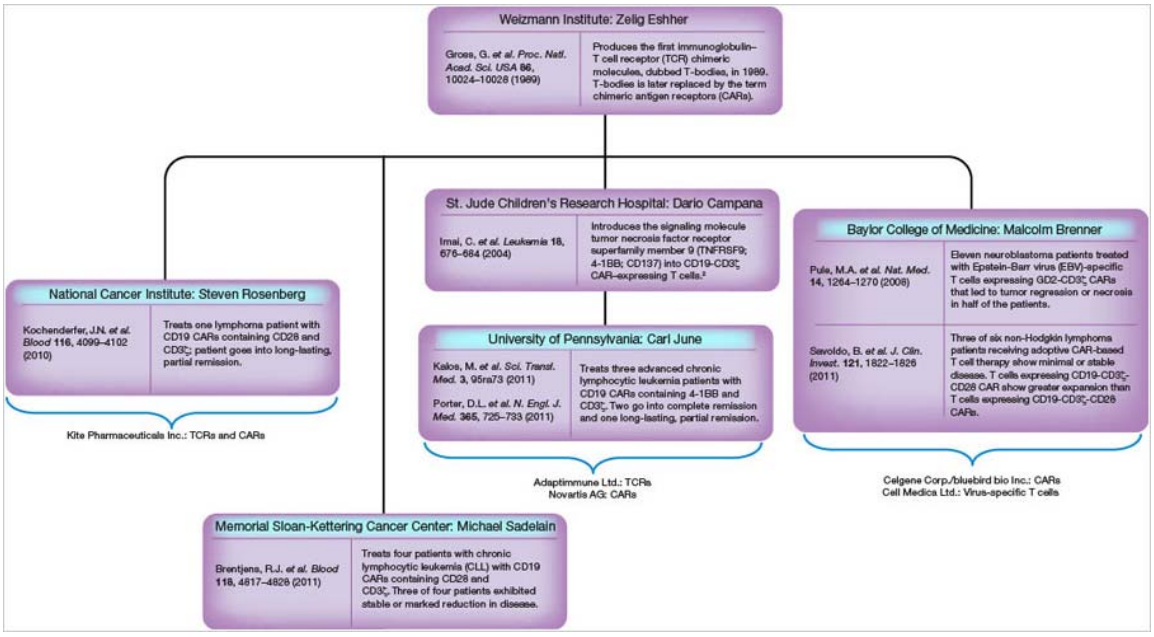

Figure 11. Preclinical and clinical studies that drove CAR-based T cell therapeutic development. Source: Baas, T. et al. **[191]**

7.1 Novartis and University of Pennsylvania broad-based R&D alliance

In an alliance aimed at bringing a new, personalized immunotherapy approach to patients with a wide variety of cancers, the University of Pennsylvania and Novartis announced in August 2012 an exclusive global research and licensing agreement to further study and commercialize novel cellular immunotherapies using chimeric antigen receptor (CAR) technologies. The agreement, which follows a Penn research team's 2011 publication of breakthrough results in several chronic lymphocytic leukemia patients treated with this personalized immunotherapy technique, paves the way for pivotal studies that have the potential to expand the use of CAR therapies for additional cancers.

The new alliance represents a marquee achievement in Penn's commitment to translational science aimed at expediting the process of bringing novel therapies to patients. Together, Penn and Novartis will build a first-of-its-kind Center for Advanced Cellular Therapies (CACT) on the Penn campus in Philadelphia -- a venture which will bring full circle the 1960 discovery of the Philadelphia chromosome, the first description of a chromosome abnormality that causes cancer. The center will be devoted to the discovery, development and manufacturing of adoptive T cell immunotherapies through a joint research and development program led by scientists and clinicians from Penn, Novartis, and the Novartis Institutes for Biomedical Research.

Penn's intellectual resources, combined with a pharmaceutical industry company like Novartis, offer a powerful symbiotic relationship in the mutual goal of finding more effective treatments for cancer. With the shared commitment to rapidly advancing new therapies and cures, this new alliance will provide the support for the essential clinical trials with engineered T cells, which could open doors for use of promising treatment options for many cancer patients who have reached the end of currently available treatments [192].

Under the terms of the agreement, Penn grants Novartis an exclusive worldwide license to the technologies used in an ongoing trial of patients with chronic lymphocytic leukemia (CLL) as well as future CAR-based therapies developed through the collaboration. Novartis will invest in the establishment of the CACT and future research of the technology. Additional milestone and royalty payments to Penn are also part of the agreement [193].

In August 2011, the Penn team detailed the results of an early trial utilizing the modified T cell approach among a small group of advanced chronic lymphocytic leukemia patients in the New England Journal of Medicine and Science Translational Medicine. The findings — including reports on two patients who remained in remission more than a year after their treatment — served as the first successful and sustained demonstration of the use of gene transfer therapy to create T cells aimed at battling cancerous tumors. The protocol involves removing a patient's cells and modifying them in Penn's cell and vaccine production facility, then infusing the new cells back into the patient's body following chemotherapy to attack their remaining tumors. Thus far, the study has involved only patients whose cancers have not responded to traditional therapy. These patients' only remaining treatment options would have been a bone marrow transplant, a procedure which carries a mortality risk of at least 20 percent.

7.2 Other partnerships

After Novartis, Kite Pharma Inc. was the next company to enter the space of cell therapy alliances, by partnering with academia and government [194]. In October 2012, Kite announced the establishment of a partnership with the NCI Surgery Branch led by Rosenberg, under a Cooperative Research and Development Agreement (CRADA) for the development of autologous T cells engineered to express TCRs or CARs directed to multiple hematological and solid tumor types. Kite has the option to an exclusive license for NCI proprietary products being developed under the CRADA. Kite is focused on advancing this NCI clinical product pipeline into multicenter studies aimed at registration and commercialization [195].

Next on the scene was Celgene Corp., who took a distinct position and aligned itself with privately held biotechnology company Bluebird bio Inc. and the Center for Cell and Gene Therapy at the Baylor College of Medicine, Texas Children's Hospital and Houston Methodist hospital. The Texas team is led by Malcolm Brenner, a professor of molecular and human genetics and the director for the Center for Cell and Gene Therapy at Baylor [196]. Celgene believes that this collaboration is uniquely positioned to advance innovative approaches to provide treatment options for patients with intractable problems in oncology [197].

Celgene has an option to license any products resulting from the collaboration after the completion of Phase I trials. Bluebird is responsible through Phase I studies. Bluebird received an undisclosed upfront payment and is eligible for up to $225 million in option fees and milestones per product, plus royalties. The company has an option for 50/50 co-development and profit-sharing rights in the U.S [197].

The deal combines Celgene's cancer drug development capabilities with Baylor's CAR T cell immunotherapy expertise and bluebird's experience using lentiviral vectors to deliver genes into target cells taken from a patient's body. The collaborators will work on CARs for liquid cancers as well as solid tumors. Tumor-associated antigens of primary interest to the partners include GD2, HER2 (EGFR2; ErbB2; neu) and CD19 [198].

In the TCR space, Baylor College of Medicine has a deal with Cell Medica Ltd., which in-licensed T cell isolation technology in 2010. Baylor and Cell Medica are running a 40-patient, open-label Phase II trial of Cytorex EBV, an autologous cell therapy involving Epstein-Barr virus (EBV)-specific cytotoxic T cells for lymphoma and nasopharyngeal carcinoma [199].

Cell Medica CEO, Gregg Sando, claims that efforts are to develop cell therapeutics with Baylor which uses TCRs that are specific for viruses associated with cancer. For these cells genetic, engineering steps are not needed because the T cells are targeting virus antigens expressed by the malignant cells and not a variation of self. One can use the naturally occurring sequences from TCRs produced by the body in response to viral infection. There are also second efforts already underway that focus on developing T cells that will be re-engineered to express TCRs for tumor antigens, such as Wilms tumor 1. Cell Medica claims not to be interested in going after CD19 with CARs since that area is saturated, but is open to other targets [199].

Other CAR deals include a partnership between the Geisel School of Medicine at Dartmouth College and Celdara Medical LLC, which is headquartered in the Dartmouth Regional Technology Center, which is a private, not-for-profit technology incubator [191].

Both partners are developing autologous and allogeneic CAR T cells based on killer cell lectin-like receptor subfamily K member 1 (KLRK1; CD314; NKG2D), which is an NK cell receptor. NKG2D recognizes several ligands that are found on the tumors of about 90% of patients with cancer. It has shown efficacy and immune system engagement in multiple murine models of both solid and liquid tumors. By May 2014, the enrollment of patients with cancer in a Phase I trial will start in order to determine maximum tolerated dose and gain insight into mechanism of action [191].

In December 2012, University College London and Cellectis S.A. partnered to develop CARs and 7 months later reported that their allogeneic, CD19-specific, CAR-based T cells were curative in mice with human leukemia cells [200].

The lone unpartnered academic player with a significant presence in the CAR space is MSKCC, which is finishing up Phase I trials, but in order to continue to further trials, MSKCC research team will need help from industry.

MSKCC is composed of Memorial Hospital, the world's largest cancer hospital, and Sloan-Kettering Institute. MSKCC has also created its own Cell Therapy and Cell Engineering Facility that meets FDA's GMP requirements. The facility is undergoing upgrades and is being expanded to 6,000 square feet.

CAR-based T cells are designed and preclinically tested at Sloan-Kettering Institute. T lymphocytes can be collected from and re-infused into patients at Memorial Hospital, where patients also can receive preconditioning regimens prior to re-infusion and supportive care after the procedure. The therapeutic T cells can be engineered directly at the Cell Therapy and Cell Engineering Facility [201].

8 Regulatory Environment of Advanced Therapy Medicinal Products in the EU and US

8.1 Introduction to R&D Activities on ATMPs in the EU: current landscape

The cell and gene therapy regulatory environment is complex and in constant evolution, owing to the wide variety of technology platforms and their early state of maturity compared to pharmaceuticals (small molecule drugs), biopharmaceuticals and medical devices. As long as the science of cell and gene therapies continues to advance, regulations will continue to evolve and adapt, yet at a slower pace. In addition, due to the differences in processing technologies between different gene/cell therapy approaches, regulatory strategies are likely to differ from product to product. Early and frequent interactions with health authorities are therefore strongly recommended throughout product development and launch.

In order to provide for a common framework for the marketing of so-called advanced therapy medicinal products (ATMPs), Regulation (EC) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products was adopted in 2007 [202].

According to the report from the Commission to the European Parliament and the Council, the ATMP Regulation was designed to ensure a high level of human health protection as well as the free movement of ATMPs in the EU. The cornerstone of the Regulation is that a marketing authorization must be obtained prior to the marketing of ATMPs. In turn, the marketing authorization can only be granted if, after a scientific assessment of the quality, efficacy and safety profile, it is demonstrated that the benefits outweigh the risks. The application for a marketing authorization must be submitted to the European Medicines Agency and the final decision is taken by the Commission. This procedure ensures that these products are assessed by a specialized body (the Committee for Advanced Therapies, CAT) and that the marketing authorization is valid in all the EU Member States [203].

The ATMP Regulation permitted EMA to make scientific recommendations as to whether a given product should be considered an ATMP. The ATMP Regulation applies since 30 December 2008. However, a transitional period was foreseen for ATMPs that were already in the EU market when the Regulation was adopted. Specifically, gene therapy and somatic cell therapy were required to comply with the Regulation by 30 December 2011, while tissue engineered products were required to comply with the new requirements by 30 December 2012 [204].

Up to 250 distinct ATMPs were reported in the EudraCT during the period 2004-2010, considering the database of all clinical trials that have started in the EU after 1st May 2004. The majority of research in advanced therapies is conducted by small companies and entities that operate on a non-for-profit basis. Thus, almost 70% of sponsors for clinical trials on ATMPs reported in EudraCT are non-for-profit organizations or small and medium sized enterprises (SME's); big pharmaceutical companies accounting for less than 2% of all sponsorships [203].

8.2 Market Authorizations and Overview of the ATMP Regulation

The ATMP Regulation has created a common framework for the assessment of advanced therapies in the EU. We are still at the early days of the development of advanced therapies and only four ATMPs

have been granted a marketing authorization. However, the much higher activity of the CAT in the area of scientific advice and classification, as well as the high number of clinical trials involving ATMPs, is a signal of a dynamic research sector.

8.2.1 Marketing Authorizations

Ten marketing authorization applications for ATMPs had been submitted to the EMA by 30 June 2013. Five of them concerned products that were previously on the EU market. Out of the ten marketing authorization applications, four have successfully completed the procedure and have been granted a marketing authorization by the Commission:

- ChondroCelect, a tissue engineered product indicated for repairing single symptomatic cartilage defects of the femoral condyle of the knee in adults [205];

- Glybera, a gene therapy medicinal product indicated for adult patients diagnosed with familial lipoprotein lipase deficiency (LPLD) and suffering from severe or multiple pancreatitis attacks despite dietary fat restrictions [206];

- MACI, a combined ATMP indicated for the repair of symptomatic, full thickness cartilage defects of the knee (grade III and IV of the Modified Outerbridge Scale) of 3-20 cm2 in skeletally mature adult patients [207];

- Provenge, a somatic cell therapy medicinal product indicated for the treatment of asymptomatic or minimally symptomatic metastatic (non-visceral) castrate resistant prostate cancer in male adults in whom chemotherapy is not yet clinically indicated [208].

In contrast, four marketing authorization applications have failed. One of these applications corresponded to a product that was on the market prior to the entry into force of the ATMP Regulation. Two marketing authorization applications were under assessment by the CAT on 30 June 2013 [203].

8.2.2 Scope of ATMP Regulation

Three types of medicinal products are considered ATMPs: gene therapies (GTMP), somatic cell therapies (CTMP), and tissue engineered products (TEP), as showed in Table 14. The assessment whether a product falls under any of these categories may involve complex scientific judgments. Specifically, the question whether a manipulation of a living material is to be considered as substantial may be difficult to answer. Even the question whether the cells or tissues are intended to fulfil the same function in the donor and in the recipient can be challenging in some cases (e.g. bone marrow material).

	GTMP	CTMP	TEP
Active substance	Recombinant nucleic acid, including recombinant vector, virus, naked or complex plasmids, virus producing cells, in vitro genetically modified cells	• Cells/tissues that have been *substantially modified*[1] so that their characteristics / functions / properties have been altered to achieve the intended function • Cells/tissues intended for non-homologous use	• Engineered cells or tissues
Therapeutic effect	Mode of action is directly related to nucleic acid sequence (or product of that sequence)	Intended to *treat, prevent or diagnose* a disease via a pharmacological / immunological / metabolic mode of action	Regenerates, repairs or replaces a human tissue

Table 14. The European Commission's regulation 1394/2007 establishes the legal and regulatory framework for ATMPs in Europe.

ATMPs include tissue engineered products (TEP), gene therapy medicinal products (GTMP) and cell therapy medicinal products (CTMP).

If a product falls within the definition of a CTMP and a GTMP (as is the case with **CTL019**), it is considered a GTMP. In case of doubt, the Committee for Advanced Therapies (CAT) offers a free classification procedure [209]. All ATMPs must be assessed centrally by the CAT at the EMA. The CAT liaises with the Committee for Medicinal Products for Human use (CHMP) to issue the final approval, and both must agree that the benefit/risk ratio is positive. Rapporteur and Co-Rapporteur are both from CAT, and each has a CHMP coordinator assigned.

8.3 Requirements for the marketing authorization of ATMPs

Commission Directive 2009/120/EC provides for adapted requirements in terms of the information that applicants must provide when applying for a marketing authorization of an ATMP. However, it is widely felt that additional flexibility should be applied, particularly in the area of quality, with a view to ensure that the marketing authorization application requirements take due consideration of scientific progress and specific characteristics of ATMPs. This view has been shared by representatives from industry, patients, hospitals, academia and non-for-profit organizations [203].

In addition to possible specific adaptations of quality or efficacy/safety data requirements, it has been suggested that, to allow advanced therapies to kick off, alternative approaches to reduce regulatory costs should also be explored. Thus, representatives from industry, patients, hospitals, academia and non-for-profit organizations suggested the introduction of a marketing authorization granted on the basis of limited data to be used in a restricted setting, particularly in cases of unmet medical needs. The data collected on the uses in the restrictive settings could be subsequently used to expand the marketing authorization up to the point of becoming a standard authorization [203].

8.3.1 The case of autologous ATMPs

In the case of autologous products the cells/tissues are harvested from a patient, then treated or expanded, and finally they are introduced back into the same patient (as is the case with **CTL019**). The

starting material (i.e. the cells/tissues) is different for each patient and, as a consequence, the manufacturing process of these products has specific features as compared with other medicinal products.

Nevertheless, not all autologous products face the same manufacturing challenges. In this regard, it is appropriate to distinguish two different scenarios:

> 1) Autologous products where the patient's cells/tissues are transported to a pharmaceutical company and the final medicinal product is delivered back to the hospital for implantation/injection in the same patient. ChondroCelect, MACI and Provenge, which received a centralized marketing authorization, are examples of such autologous ATMPs. Other example is CTL019, as the first step involves the manufacture of recombinant lentiviral vectors carrying the CAR construct. In a second step, T cells obtained from a cancer patient through leukophoresis are transduced with the CAR lentiviral vector, selected and expanded in vitro, and re-infused into the same patient.
>
> 2) Cases where the patient's cells/tissues are manipulated in the hospital (e.g. by means of medical devices that are developed for cell separation and manipulation) prior to re-administration to the same patient.

EMA consultants generally agree that autologous ATMPs should not be regulated as medicines. While this approach would reduce the developmental costs associated with the use of these products, in the Commission's view, the need to ensure an adequate level of public health protection should prevail over economic considerations.

However, it is important that the requirements that apply to autologous products are proportionate and adapted to the specific characteristics. Requiring autologous products that are manufactured at the hospital prior to the administration to the patient to comply with the quality controls and manufacturing requirements of standardized chemical-based medicinal products would prevent the development of these treatments in practice as a batch release certification would be required per treatment and a manufacturing license would be required per hospital [203].

8.4 EU versus US Regulations

In the US, cell and gene therapies are grouped under the term Human cells, tissues, or cellular or tissue based products (HCT/Ps). HCT/Ps are human cells or tissues that are intended for implantation, transfusion, infusion, or transfer into a human recipient. HCT/Ps are currently governed by section 362 of the Public Health Services Act and regulated by the Center for Biologics Evaluation and Research (CBER). Regulations are outlined in 21 CFR Parts 1270 (human tissues intended for transplantation) and 1271 (human cells, tissues, and cellular and tissue based products). Parts 1270 and 1271 require tissue establishments to screen and test donors, to prepare and follow written procedures for the prevention of the spread of communicable disease, and to maintain records. They also outline current good tissue practices for HCT/Ps. If cells are for homologous use or only minimally manipulated, they require no pre-market approval as defined in the tissue regulation 21 CFR 1270.10 [210].

FDA has approved a number of cell therapies so far, in earlier years under the medical devices regulations, and more recently under the current HCT/P regulations. These products are listed on the

CBER website. FDA has not yet approved any human gene therapy product for sale. However, the amount of gene-related research and development occurring in the United States continues to grow at a fast rate and FDA is actively involved in overseeing this activity [210].

Regulatory requirements between EU and US are broadly similar. The main differences are summarized in the table below.

	EU	US
Main legislation	ATMP regulation (EC) 1394/2007 GMP: Eudralex Vol 4, Annex 2	Public Health Act, Section 351 – Biologics; 21 CFR 1270 and 1271
Responsible agency for licensing	European Commission (EC)	FDA
Responsible agency for clinical trials	National competent authorities and local ethics committees	FDA, Institutional Review Board, NIH (if federally funded)
Other responsible bodies	European Medicines Agency (EMA), Committee for Advanced Therapies (CAT)	Center for Biologics Evaluation and Research (CBER), Office of cellular, tissue and gene therapies (OCTGT)
Classification	Tissue Engineered Products (TEP) distinguished from Cell Therapy Medicinal Product (CTMP) Free classification procedure	No distinction between TEP and CTMP (all are considered HCT/Ps) No classification procedure
Data storage	30 years	10 years

Table 15. Regulatory requirements for ATMPs in EU and US.

8.5 Intellectual Property for ATMPs

Despite the regulatory hurdles, orphan drug status and various trade secrets may help to keep exclusivity for ATMPs such as CTL019, these will not be absolute bars to competition. In addition it is unclear at present how important the trade secrets and allegedly essential know-how is, given that there is no evidence of certain measures in the process are indeed required or not for efficacy of the product.

The patent aims to protect the therapeutic cellular compositions and their production and applications. Infringers of the patent could in principle be other pharmaceutical or health care companies working on similar cell based therapies. But more likely infringers of the patent would be academic hospitals that are using the technology, perhaps not commercially but by-passing an approved ATMP, copying (at least parts of) the procedure and using a very similar cellular composition.

It is questionable whether a manufacturer, such as Novartis, could successfully enforce their IP against such academic and clinical (non-profit, academic or health care providing) infringers, carrying out medical treatment of individual patients. In most countries physicians and pharmacists treating individual patients are immune from patent infringement suits. In addition enforcing patents against academic hospitals and transplant centers might generate bad publicity and/or repercussions from clients, payors, patient and governmental organizations, and there may be no viable option to enforce IP against above mentioned potential infringers.

The fact that a ATMP like CTL019 cannot be narrowly defined hampers meaningful searching. Stem cells and cell based therapies is a densely patented field with many pending applications. The process of obtaining cell products like CTL019 involves standard techniques with standard products such as leukapheresis from the patient's blood and associated lab reagents; therefore, unlikely to require licenses from third parties. Also, many steps are on human subjects, hence are not considered patentable in most jurisdictions.

8.5.1 Several trade secrets may help protect ATMPs

The manufacturers have most likely identified several trade secrets and essential know how that would protect the process for obtaining the cell product according to specification. Trade secrets would provide exclusivity for an almost indefinite period; until the secret is broken, or the know-how is independently developed by competitors or has become moot due to better designs and alternative routes.

Without disclosing the identity of the trade secrets orally disclosed at the due diligence, many optimizations in the ATMPs procedures that are classified as a trade secret are empirical in nature, rather than based in science and from controlled experimentation. Based on very few observations and it is difficult to establish whether the trade secret / know how is an essential part of the ATMP product and it's process or not.

Hence the value of at least some of the trade secrets cannot be clearly established, as long as it is not clear whether it can be designed around whilst staying within the ATMP approved label or not. The method to obtain the therapeutic cellular compositions comprises several optimizations and in addition avoids several standard steps that allegedly would render the product inferior or even completely ineffective. If some or all of these trade secrets and essential know how indeed turn out to be essential for the efficacy of the cell product, trade secrets could contribute significantly to the exclusivity of the product. However, it may turn out that some of these trade secrets / essential know-how is not essential or can be circumvented by other measures.

9 Pricing and Patient Access to Cellular Therapies

In order to define the pricing strategy of ATMP, one must secure the classification as such. It is fundamental whether cell therapies have been subject to substantial manipulation. There are quite a few manipulations that are noted as being non-substantial (e.g., cutting, grinding, shaping, concentrating and purifying) but the list is non-exhaustive and the Committee for Advanced Therapies can also consider any other manipulation as non-substantial. Hence, there are likely to be cellular therapies that do not accomplish ATMP classification.

9.1 Reimbursement and Funding of ATMPs

Presently, there are numerous factors that determine the type of reimbursement evaluation that a drug will undergo: is it eligible for orphan status? Will it be used exclusively in specialist hospitals? Is it in-patient only? It is yet to be known whether cellular therapies will be treated differently or if ATMP status itself has any impact.

There is a considerable spectrum of ATMPs: from those covering an in vivo course of treatment and those that encompass an ex vivo procedure, as showed in Figure 12. It is acceptable that an ATMP consisting of a single course of in vivo treatment (such as Glybera) could be evaluated by HTA bodies in the same way as a traditional drug. On the other hand, an ex vivo procedure which implicates the manipulation of cells (such as ChondroCelect or CTL019) may not fit a conventional HTA evaluation [211].

Besides the differences across the spectrum of ATMPs, there will be variation across EU markets regarding how an individual ATMP is treated by payers (for example, so far Glybera hasn't been considered to the short list of products that will undergo an Early Benefit Assessment in Germany, suggesting that gene therapies may not be mandated by AMNOG) [212] [213].

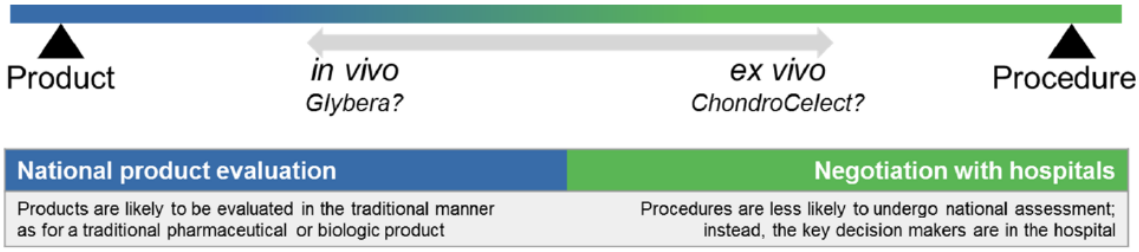

Figure 12. Spectrum of ATMPs and likely evaluations. Source: PriceSpective, 2013

One should bear in mind that many ATMPs are likely to involve multiple steps in hospital and ex hospital procedure [214] for example, CAR modified-T cells such as CTL019 (Figure 13):

1) Blood draw and leukapheresis: White blood cells including T cells are separated from the patient's blood (leukapheresis);
2) Viral vector: Genes encoded to recognise cancer cells are transferred into the patient's T cells using an inactive virus called a viral vector;
3) Modified T cell: The modified T cells are grown in the laboratory;

4) Chemotherapy: The patient receives chemotherapy to reduce the level of white blood cells and help the body accept the modified T cells;
5) T-cell infusion: The modified T cells are re-infused into the patient's blood, where they seek cancers cells and destroy them;

For the biotech's and pharma companies, it is critical to consider how each step will be funded once the ATMP is available: are diagnostic related groups (DRGs) used for the pre-existing steps? Is there headroom in the current DRG for the new product? If not, what is the process for creating a new DRG? Lastly, for each of these scenarios, who are the decision-makers?

EU payers anticipate very high costs for this type of treatment, and want to manage access tightly, such as restricting these technologies to a small number of centers. Inter-regional funding mechanisms exist in all markets, and is not considered a hurdle. However, there may be inequalities in access based on medical policy by insurer / sickfund/ region. As the competency and infrastructure to facilitate these ATMPs treatment is centralized in select centers, meaning that even "broad" access is relatively limited.

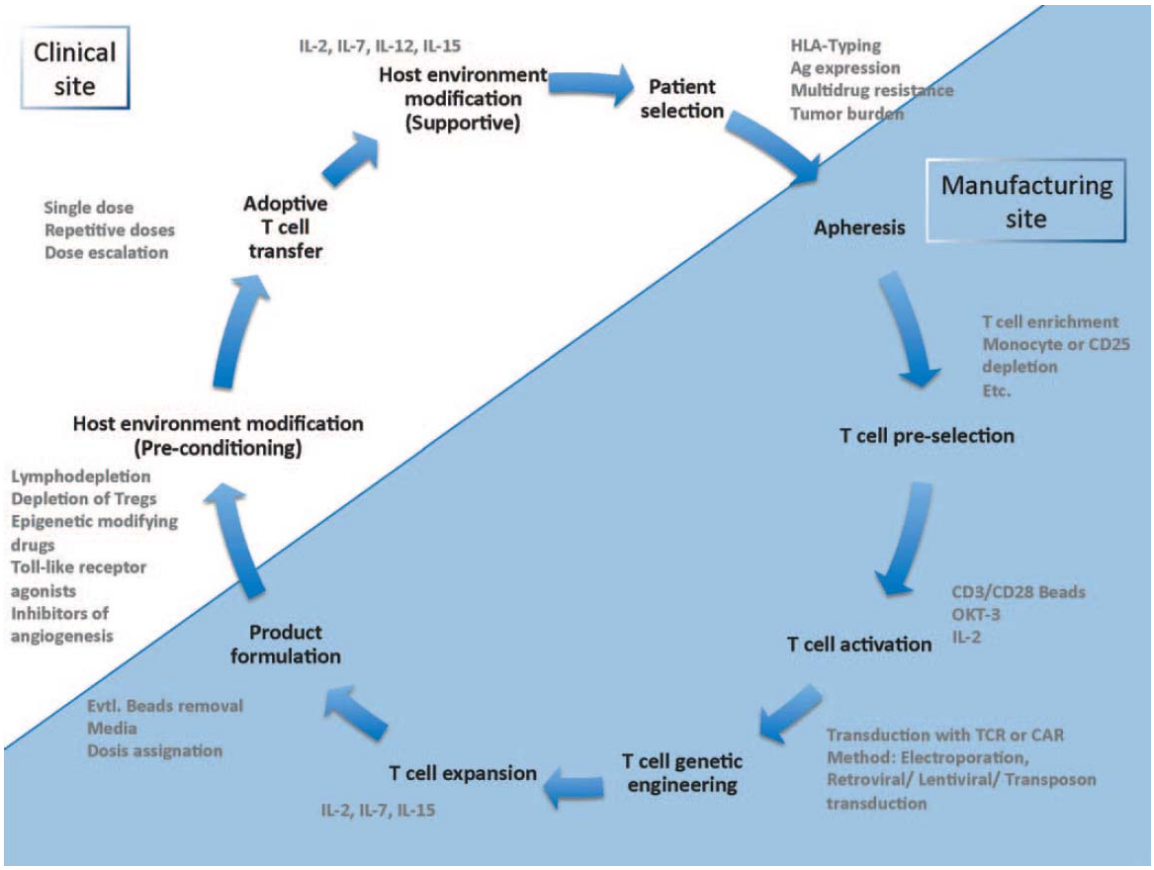

Figure 13. The life cycle of a T-cell-based process. Source: Wieczorek A *et al, 2013*.

9.2 Value Demonstration and Pricing

As with many therapeutic interventions, the ultimate goal for an ATMP is to achieve access to as many eligible patients as possible at an appropriate, value-based price. The key success factors are: national, regional and local formulary access as well as time to patient access in a commercially-viable volume of patients. Building on EMA marketing authorization, there are quite a few steps that a new medicinal product must go through so it gains reimbursement and, therefore, patients may access the technology.

During the HTA, while defining the value and reimbursed price of a new health technology, payers will most likely evaluate a new compound against a comparator. In order to do so, they will analyze the data package of a new compound and decide whether it is positively differentiated from that which is currently available. At that point, they will look for cost-efficacy and decide whether the positive differentiation is worth paying for. As a result, clear evidence of the magnitude of benefit is the key driver of value. In theory, this will be no different for ATMPs; however, technologies like CTL019 or ChondroCelect are not compounds, but complex procedures; hence, it is most likely that in many cases there will be no comparator or that there are insufficient data to make a meaningful comparison.

For medicinal products (ATMPs included), the evidence requirements for P&R and patient access decisions are different from those for regulatory approval. A regulatory license from the EMA is not sufficient to secure reimbursement, as seen by ChondroCelect which was denied reimbursement in France [215], not recommended for use in Spain [216], available only via private insurers in the UK [217] and reimbursed in Germany on a case-by-case basis [218].

Possibly the most challenging assessment for payers is for products that claim to cure a patient of a disease, such as leukemia. In theory, payers would be willing to pay millions of Euros for an intervention that were to cure a patient that otherwise would die, particularly if it resulted in no other healthcare costs related to that disease over the patient's life. However, are there any payers willing to take that gamble with only a few years' data? Payers are risk averse and will expect the realized benefit in some patients to fall short of the best expected benefit, as showed below [211].

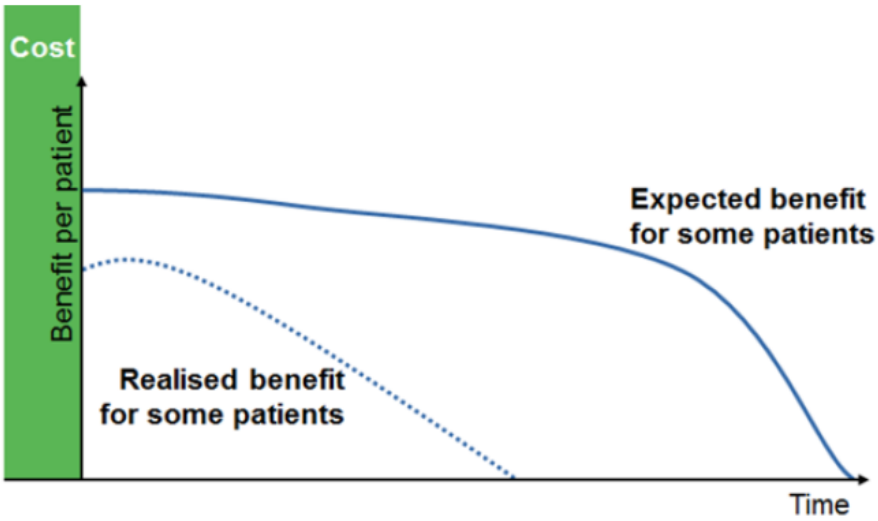

Figure 14. The payer dilemma when funding products that purport to cure disease. Source: PriceSpective, 2013

Companies should, for that reason, be willing to consider innovative approaches to pricing ATMPs in order to accommodate payer skepticism and caution. One approach could be an annuity-based payment scheme (theoretically, a risk sharing agreement) under which payers spread the payment across years, only paying if the patient remains disease free as seen in Figure 15. To make a more compelling agreement to the payer, it is likely that the onus would be on the company to confirm that the patient remains disease free, perhaps via a patient registry and monitoring program. There are further complications that would need to be assessed before a scheme like this could be implemented: for example, what would happen if a patient moved countries or died from another cause?

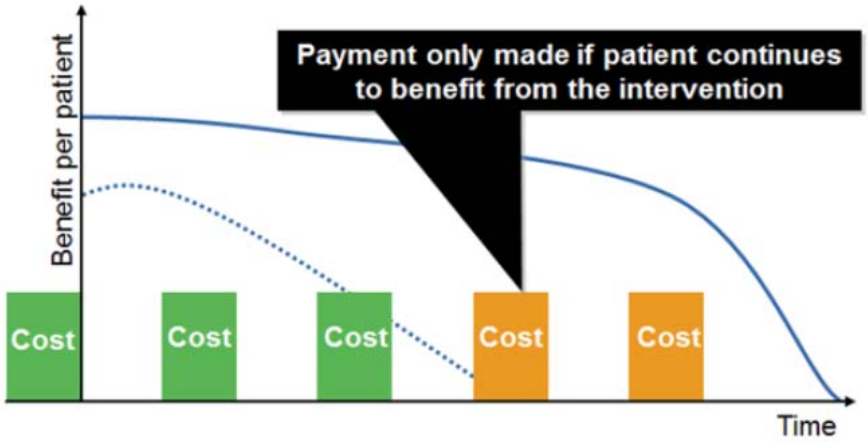

Figure 15. Staggering payment such that the intervention is paid only for patients continuing to benefit. Source: PriceSpective, 2013

Despite the issues linked to innovative pricing schemes, companies should do an early assessment of payer reactions to the anticipated value proposition, in order to determine: likely payer objections, data gaps, and possible mitigation strategies (follow-up studies, risk sharing schemes, etc).

10 Business Model Considerations for Development of Cell Therapies

10.1 Introductory Development and Commercialization of Cell Therapies

The cell therapy industry continues to grow, if one measures the increasing numbers of clinical trials and patients treated [219]. Several discussions regarding the differences between "off-the-shelf" (allogeneic) and "patient-specific" (autologous and matched allogeneic) therapies continue, and it is most likely that both will find success.

According to Shaw R. *et al*, the best way to approach development for a cell therapy product is to consider three fundamental drivers that guide development [220]:

1) Speed to market (Which pathway will allow for fastest access to the commercial market?)
2) Operational efficiency (Which option provides the most efficient use of operational and financial resources?)
3) Reduction of risk (How can a company reduce the many risks involved in development of its new cell therapy?)

The clinical trial process is intended to provide a means by which candidate therapies can be proven to be safe and efficacious. Once a technology (e.g., chimeric antigen receptor-transduction, CAR-T) or cell type (e.g., mesenchymal stem cells, MSCs) has been targeted for development, a company wants to generate data as quickly as possible to support its safety and efficacy profiles. Figure 16 illustrates a typical development and commercialization pathway for a product candidate.

Figure 16. Development and commercialization path for cell therapies. Source: Shaw R *et al*, 2014

Cell therapies attributes are highly dependent on the manufacturing processes; therefore commercialization is unlikely to be successful without an effective development process. Companies use a number of approaches to optimize process and product development.

Figure 17 not only outlines some mechanisms recognized by the FDA, EMA and other regulatory agencies concerning Target Product Profiles (TPP) and quality by design (QbD), but even goes a step further to define development by design (DbD), whereby critical aspects of quality, cost of goods sold (CoGS), scale, and sustainability are each addressed. Most companies are formally adopting this DbD approach, as many are starting to gain experience with cell therapy development.

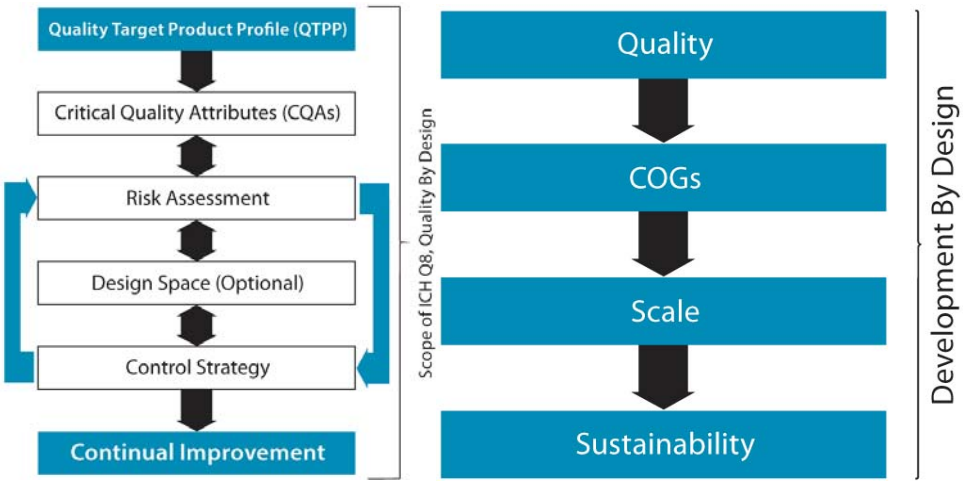

Figure 17. Target product profile (TPP) and commercial manufacturing vision. Source: Shaw R *et al*, 2014

10.1.1 Attributes and Challenges of Development by Design

Most 'quality' is foundational, as recognized by QbD. For cell therapies the manual, open, and human-dependent nature of many process steps presents an even bigger and substantial risk, because these technologies rely heavily on their manufacturing processes to meet final-product critical quality attributes. A manufacturing process is only as strong as its weakest link. Hence, in the example of a patient-specific product, the strength of the process is directly related to reducing the risk of failure to treat the patient. Automation, integration, and closed-system designs are key tactics to elevate process robustness. Therefore, the main development challenges, according to Progenitor Cell Therapy Services (a NeoStem group company), are [221]:

CoGS: The current high CoGS for cell-therapy products — typically driven by labor and testing costs for patient-specific products— demands a sizable commercial value proposition. As processes mature, the focus on CoGS for commercial viability becomes critical. DbD allows for prospective approaches to address CoGS as appropriate for a given scale and stage of development.

Scale: Migrating from a clinical-scale process capable of making tens to hundreds of patient doses per year to a commercial-scale process with the capacity to make thousands to tens of thousands of patient doses can present significant comparability risk. In particular, cell-therapy products inherently possess high complexity, with one or more mechanisms of action that are often incompletely understood. In addition, there is currently a lack of analytical tools or in-vivo models for judging product comparability.

Sustainability: Even when quality, CoGs, and scale objectives are met, there can be a very real risk that manufacturing cannot be sustained over a full product life cycle. For example, a key risk is disruption of the relatively fragile and immature supply chain currently supporting the cell therapy industry. A disruption could halt manufacturing for an extended period. In the worst-case scenario, one process step relying on supply chain elements that become unavailable could require changes to be developed, tested, and comparability demonstrated. To mitigate risks to business sustainability, companies need to assess the full range of supply chain inputs to their manufacturing processes: reagents, consumables, equipment, and human resources. Furthermore, such assessments should include every unit operation methodically, both process and testing.

A significant challenge in every development program is that, while quality realization must occur early in development and be well-established by phase 2 trials, realization of the other aspects listed above is not required until much closer to commercialization, as illustrated by Figure 18. This puts significant pressure on cell-therapy developers to defer their investments in CoGS, scale, and business sustainability — until, in some sense, it may be too late. Developers often defer such investment until the comparability risks of making changes to address related concerns become substantial. In addition, extreme changes in process scale becoming necessary as a developer moves toward commercialization can quickly create quality risks that were not yet encountered.

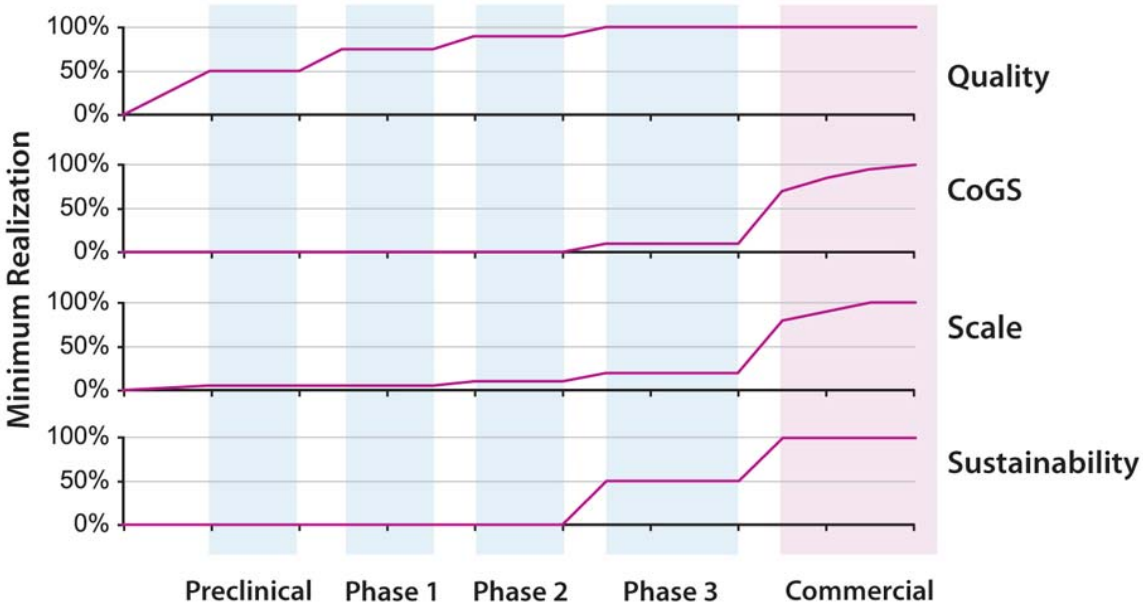

Figure 18. Early development quality realization. Source: Shaw R *et al*, 2014

DbD aims to guide strategy, provide structure and discipline to plan for, and address commercialization risks while there is still time to address them. A successful DbD program is implemented in the framework of three drivers: speed to market, operational efficiency, and reduction of risk. It can be done in a way that is appropriate to each development stage. The goal should be to meet an ideal commercial manufacturing vision of providing consistently high product quality at reasonable cost that meets demand over the commercial life of the product.

10.1.2 Contract Manufacturing Option

Once a company has licensed or discovered a technology or cell type, it becomes a primary asset for the organization. Many companies choose to maintain their research internally so they can develop the best understanding of their technologies or cell types. Such knowledge is considered to be essential for development, troubleshooting, and fundamental scientific understanding. However, contracting for process development and manufacturing expertise may be a preferred option that can effectively support the drivers discussed above.

A recent industry report from Pharma IQ described the most important factors that developers consider when choosing a contract development and manufacturing organization (CDMO), illustrated in Figure 19. Remarkably, cost was not the most common reason for choosing one contract manufacturer over

another. The report showed that “fit with long-term strategy” was far more important in making the choice. The same report identified the following as top concerns when working with a contract manufacturer: communication, teamwork between client and CMO, compliance with all emerging regulations, an ability to work within restrictive time frames, compliance with due diligence, adequate technology and experience, and an ability to protect intellectual property [222].

As the cell therapy industry grows and matures, many companies seek the most efficient model by which to develop their products. Speed to market, operational efficiency, and reduction of risk are three main drivers for business model choices. If companies choose to outsource development work to a CMO, “strategic fit” will be a main factor in how they make their selection among the pool of available partners.

10.2 Assessing Commercial Opportunities for Autologous and Allogeneic Cell Therapies

There are two primary cell sources used to produce cell-based therapies: autologous (self-derived) and allogeneic (derived from a donor). It is interesting to compare and contrast the two approaches in order to understand whether there is an emerging preference in the market. While the current clinical trials underway are slightly biased to autologous cell-based therapies, it is clear that both approaches are being actively pursued. Although allogeneic therapies have significant advantages over autologous therapies, they do have a distinct disadvantage regarding potential immunogenicity. New hybrid autologous business model provides the ability for autologous-based therapies to mitigate some of the advantages that allogeneic cell-based therapies enjoy, including cost of goods [223].

10.2.1 Autologous versus allogeneic business models

Autologous cell-based therapies are derived from a patient who is both the donor and the recipient. The cells are often harvested from the patient, sent away to a facility for manufacturing and then returned to the physician for delivery into the patient (there are variations in this model depending on the product and indication, among other factors). Allogeneic cell-based therapies are derived from a healthy donor, expanded, a master cell bank is created, and aliquots of cells are manufactured and shipped to the physician for subsequent delivery into the patient (there are variations in this model depending on the product and indication, among other factors), as showed in Figure 19. This scheme demonstrates the typical high-level business models for autologous-based therapy versus an allogeneic model with a particular focus on their differences. The primary differences between the two models are centered on the extra procedure and lag time for an autologous therapy that is not present with allogeneic therapies. Nevertheless, allogeneic therapies may require some type of immunosuppression, which can complicate the model. Furthermore, the potential immunogenicity of allogeneic cells could hinder efforts to re dose the patient.

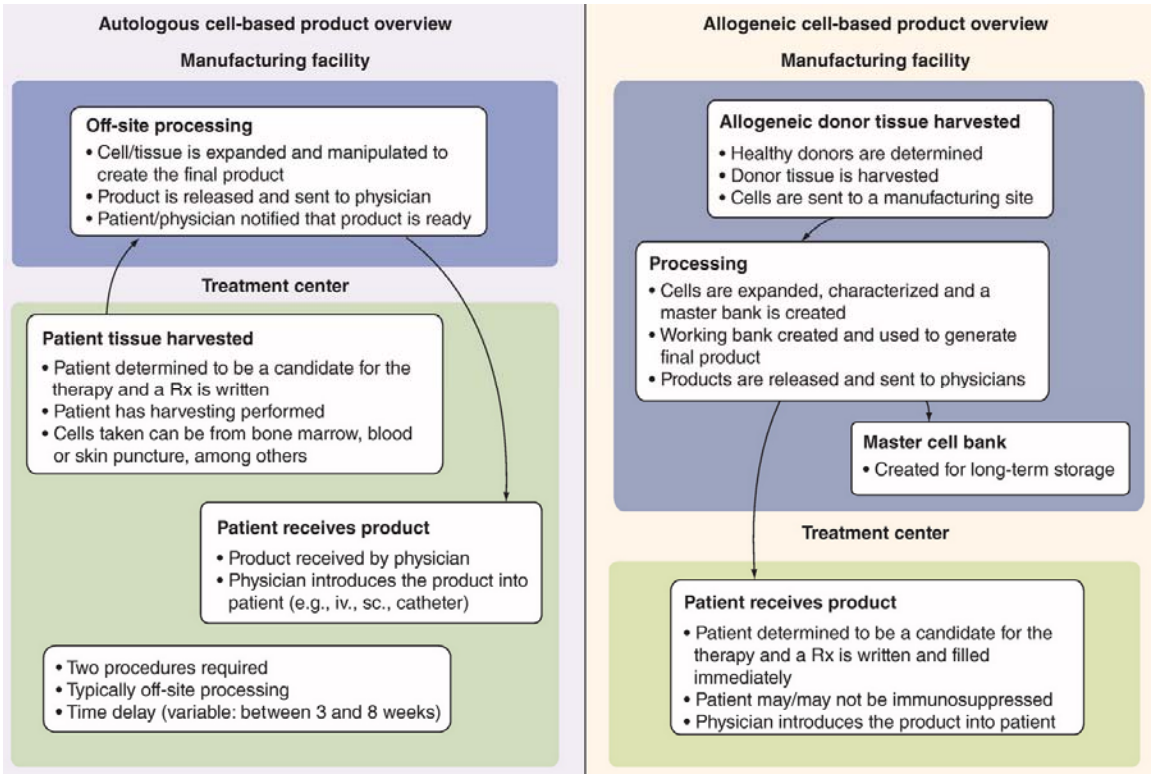

Figure 19. Autologous and allogeneic cell-based business models compared. Source: Smith DM, 2012.

Lastly, there are autologous-based products that can be 'manufactured' in a point-of-care device at the treatment center. The business model for these types of products is a 'device-based razor and razor-blade business model'. This model involves a company offering a one-time product (e.g., razor or inkjet printer or cell separation device) at a reduced cost or even a loss. The company then makes its profit by selling a complementary product that is required to operate the first product (e.g., the razor blades, ink or disposables) [224]. The second product must be bought repeatedly as it is consumable.

10.2.2 A hybrid Autologous Model

Considering some of the challenges associated with autologous cell-based approaches, there has been the emergence of a hybrid autologous business model that has advantages that overcome some of the disadvantages of the traditional autologous business model (Figure 20). The primary difference in this model is that the patient only has to undergo a single cell-isolation procedure even if multiple doses or treatments are required. After harvest, the cells are shipped to the manufacturing site, expanded, a master cell bank is created and any re dosing that is required occurs using the master cell bank. The first dose the patient receives resembles the traditional autologous business model, but all subsequent doses are readily available in the way that an allogeneic therapy would be. This should have significant benefits to the patient and providers, as well as to the company that owns the product. These benefits include: reduction in cost of goods (COGs), uniform and consistent product for all doses, more control over when the patient is re dosed, and simplification of the product for the healthcare provider, all offset by slightly increased costs to store the master cell bank.

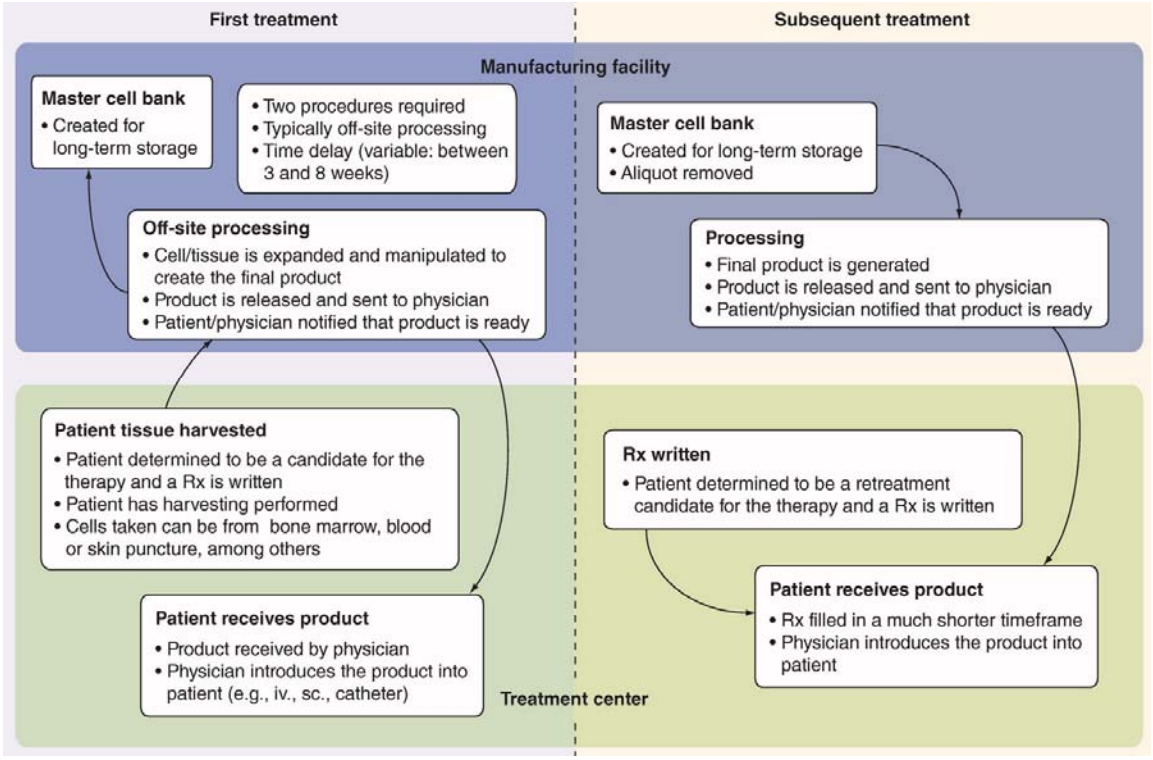

Figure 20. Hybrid autologous cell-based business model. Source: Smith DM, 2012.

10.2.3 Which Cell-based Approach is More Likely to Succeed?

A comparison of autologous versus allogeneic cell-based products has been carried out, particularly relating to scientific aspects, which nicely highlights some of the benefits and disadvantages of each cell-based approach [225]. Therefore, a key question emerges: 'is either approach preferred in the market today?'

One can examine ongoing clinical trials for some clues to see if there is already a preference for one cell-based approach over another. According to one source, 52% of Phase II/III industry-sponsored trials (n = 23) are autologous [223], while another source counted 47 industry sponsored Phase I–III trials, with 58% being autologous [226]. These sources confirm that the industry is mixed (with a slight bias towards autologous-based therapies) as to which approach is the preferred method. In fact, one could argue that the industry/market/investors are currently betting that both approaches have potential in the market.

Looking at key comparative parameters from a business model perspective, allogeneic-based therapies have significant benefits versus autologous cell-based therapies (Figure 21) [227]. As mentioned above, the potential issue of allogeneic therapies is immunogenicity, which could be a large hurdle. Yet, if this hurdle can be overcome, the benefits of allogeneic therapy are superior to autologous therapy from a commercial perspective, primarily in the scalability and reduced COG benefits that allogeneic therapies enjoy. In addition, older or extremely sick patients may not have suitable numbers or quality of stem cells for an autologous treatment. Though, the hybrid autologous model closes the gap significantly with allogeneic therapy, with the added benefit that these products are unlikely to have immunogenicity issues. Additionally, each successive hybrid autologous dose

should lower the COG, given that cells only need to be sourced from the patient, once spreading the initial harvesting costs across multiple doses.

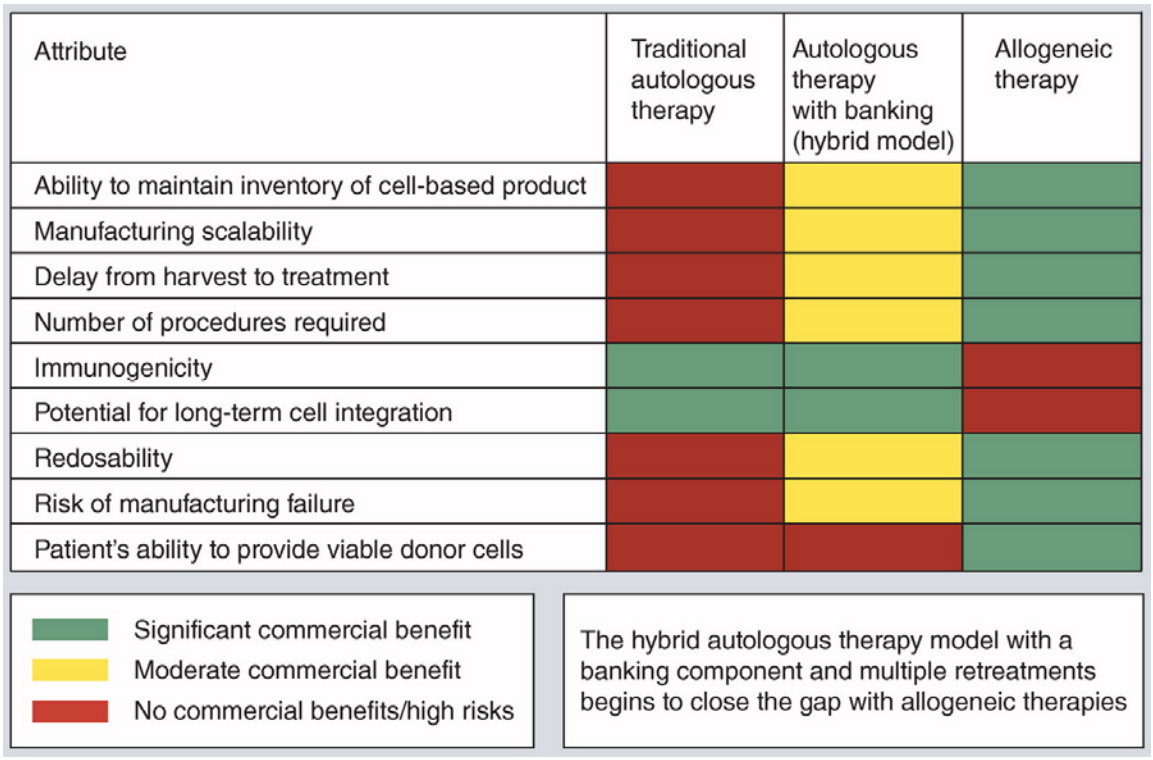

Figure 21. Comparison of autologous versus allogeneic therapies and potential commercial advantages/disadvantages. Source: Smith DM, 2012

10.2.4 Cost of Goods Comparison

The major benefit of allogeneic cell-based therapies is that they are likely to result in a reduced COG. There are not many published comparisons of COG across different therapeutic classes. A thorough search was undertaken to find data to support the potential gross margins (defined as total revenue minus COG divided by total revenue) across different technology modalities (Table 16). Small-molecule gross margins tend to be in the 80–95% range (based on gross margins of companies that sell exclusively small molecules), depicting the economies of scale benefits that can occur within the manufacturing process. Large molecules (recombinant proteins and monoclonal antibodies) have gross margins in the 60–85% range based on the gross margins of companies dominated by these types of therapeutics [228]. The gross margins for biologics have improved considerably over the last 10 years as manufacturing yields have consistently improved [229]. In addition, while small-molecule drugs typically sell for US$1000–3000 per year, large molecules can sell for $10,000 or more per year [230].

Therapy	Gross margin range	Gross margins for representative companies
Small molecule	80–95%	Acorda (81.5%), AVANIR (94.3%), Vertex (91.6%)
Large molecule	60–85%	Human Genome Sciences (60%), Amgen (85%), Novo Nordisk (78%)
Allogeneic cell therapy	60–80%	Advanced Biohealing (60–80%)
Hybrid autologous cell therapy	60–70%?	Is the range 60–70% or higher?
Autologous cell therapy	50–60%	Provenge (50–60%), Carticel (52%†)
Medical device industry	50–60%	Medical appliances and equipment gross margin (58%)

Table 16. Cost of goods comparisons. Source: Smith DM, 2012

One of the challenges with the autologous business model is the lack of scale benefits, but the hybrid autologous business model described above could bring some COG benefits. Could these benefits bring the gross margins up to the 60–70% range that is more in line with biologics? This would make these therapies more attractive to large biopharmaceutical companies that are used to higher margin products.

In the following example, one can compare the revenue and COG for a hypothetical autologous product versus a hypothetical allogeneic product (Figure 22) in a single indication.

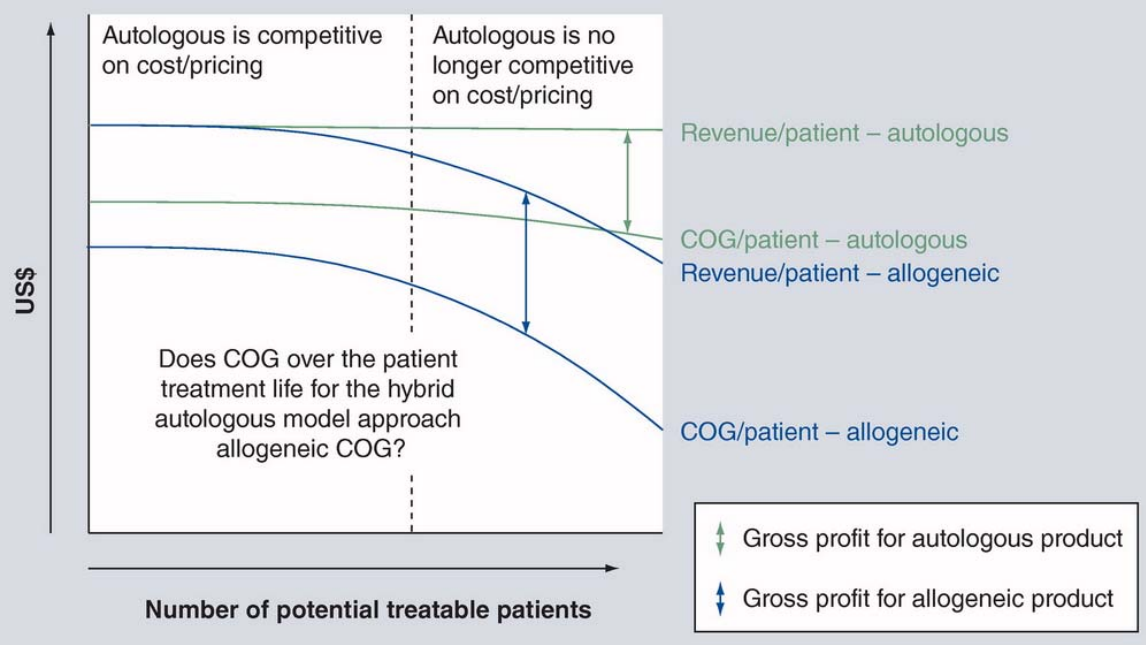

Figure 22. Relative cost of goods versus profitability by type of therapy. Source: Smith DM, 2012

10.2.5 Finding the 'sweet spot' for each cell-based approach

One may ask if there is a 'sweet spot' for each cell-based approach? Figure 23 graphically depicts the opportunity space. Current autologous cell-based therapies in development are likely limited to orphan diseases (less than 200,000 patients) initially, in which pricing for therapies could be similar to biologics (up to $100,000/year). However, this could change if an autologous product could demonstrate significant efficacy and safety in a serious disease affecting a large patient population. The hybrid autologous cell-based therapies would probably be more competitive with allogeneic-based therapies. A key for the hybrid autologous therapies is the number of dosings – more doses would drive down the COGs, while fewer doses would keep the COGs closer to the autologous model. Still, it is important to note that it is unlikely that a product using the hybrid autologous model would ever have equal COGs to an allogeneic cell-based product.

Furthermore, it is critical to acknowledge that the value proposition of any product is based on a host of factors, including: efficacy, safety, cost and supply chain. Thus, while the analysis here places all

allogeneic- and autologous-based approaches into respective buckets, individual products could differ markedly from these ‘generic’ products discussed here. For example, if an autologous therapy did demonstrate curative abilities combined with strong safety in a large proportion of treated diabetics (the value proposition), the market would react favorably to this value proposition and many patients would be treated. This would make the company that developed this technology very successful financially, if they could scale up manufacturing and distribution while making a reasonable gross margin on the product. In fact, this autologous therapy could easily out-compete an allogeneic therapy that was lacking the same robust value proposition.

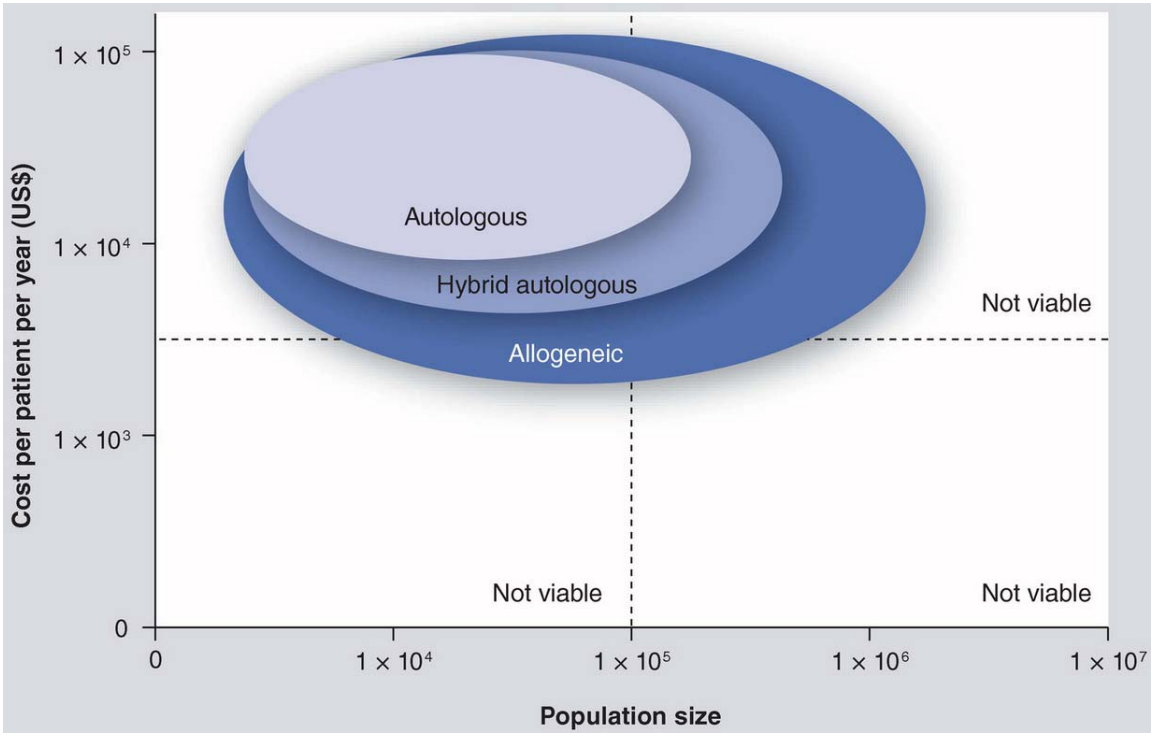

Figure 23. Finding the ‘sweet spot’ for cell-based therapies. Source: Smith DM, 2012

10.3 Summary of Challenges for Commercial Manufacturing of Cell Therapies

Most cell therapies are currently developed by universities, small and medium sized companies, or charitable organizations. They use technologies that are readily available and not necessarily fit for commercial use. Manufacturing processes are mostly manual and still at laboratory scale. These therapies pose some unique challenges to commercialization that need to be addressed:

	Challenges
Maturity of the Technology	Cell therapies are innovative, and not a lot of products have reached the market yet. A lot of the necessary infrastructures, including regulatory infrastructures, commercial models, and reimbursement infrastructures, still need to be implemented. Non-clinical and clinical trial designs may pose challenges.
Complexity and Diversity of Therapies	The unique diversity and inherent complexity of different therapies bear unknown risks regarding safety (especially long-term), quality, and efficacy of the products. Long-term follow-up of patients might be required.
Business Models	In the traditional business model for pharmaceuticals, commercial viability is based on economies of scale, and a significant population is served with a defined product for a given therapy. Centralized production ensures global supply. For Cell Therapies, different business models need to be developed for different therapies, ranging from a “Service” (autologous treatments), over a “Product” (allogeneic treatments; gene therapies), to a “Point of care” type model typical for devices (autologous therapies, immediate use therapies, bed-side therapies involving minimal manipulation). Many therapies will serve small patient populations, and the conventional batch production philosophy with robust segregation/changeover procedures becomes very expensive. Decentralized if not local production (for products with extremely short shelf life) and significant investments into manufacturing facilities might be needed (existing biotech facilities are not adequate). For autologous therapies, the criticality of batch failures needs to be addressed: manufacturing failures directly result in failed patient treatments.
Intellectual property	Manufacturing processes involve the use of technologies (e.g. cell separation by magnetic beads) that are protected by intellectual property (IP). A thorough IP evaluation is needed to ensure freedom to operate in a commercial setting.
Cost of Goods	Whether a therapy is commercially successful will be heavily influenced by its manufacturing cost. Cell therapies are likely to be significantly more expensive to produce than small molecule and biological drugs due to the complexity of their manufacturing process, and cost can become an issue during commercial production. High cost is associated with small batch production, manual operations, required extensive QC testing (QC samples likely represent a significant volume of product and total cost), and expensive, labor-intensive technologies and procedures.
Reimbursement	Commercial success of a therapy is also tied to its reimbursement. Reimbursement routes for innovative therapies need to be considered early on.
Non-conventional Supply Chains	Autologous therapies will require non-conventional supply chains, from the patient to the manufacturing site and back to the same patient again. It is critical to ensure the logistics of the supply since a lost product directly results in a lost

	treatment. Traceability and product identity must be ensured, especially for autologous products.
Scale up versus "Scale out"	Gene therapies are amenable to *scale up* and large scale batch processing, similar to traditional biotech products. Autologous processes need to be *scaled out* for commercial manufacturing, enabling parallel processing of multiple, separate patient-specific products. Allogeneic processes can be scaled up or scaled out, depending on the resulting product. Development of scalable processes will require significant investments over a considerable period of time. Many processes will need to be re-engineered.
Manual Labor	Current cell therapy manufacturing technologies are mostly manual, requiring highly skilled technicians. Skilled manual labor difficult to hire and expensive to train, especially to work in an aseptic environment under GMP conditions. Manual operations also increase the risk of batch failures due to operator errors.

10.4 Points to Consider for a Successful Commercialization

Successful commercialization of cell and gene therapies requires more than providing its efficacy and safety to regulators. Therapies must be commercially viable, which implies cost-effectiveness and scalability. In addition, a product must be affordable and accessible to a significant patient population. Particular attention should be given to the following topics:

Manufacturing **process development**	The variability in the manufacturing process needs to be limited to ensure product consistency, quality and purity. This includes control of raw material, process understanding/controls, and the use of closed systems for every step, and automation. As for biologics, the final process should be established before entering pivotal clinical trials. **Control source/nature of raw materials** Ensure raw materials are available in adequate quality and sufficient quantity for commercial supply. For critical raw materials, establish a second supplier. **Understand the process** Controls (if possible in real-time) and operating limits need to be established using the QbD approach based on risk management. Critical process parameters affecting critical quality attributes need to be identified and appropriate controls established. In some cases, one might consider to remove high risk operations, e.g. sterile connections that are established manually. These connections are high risk, the ideal process should have no manually established sterile connections. **Closed systems** Closed systems, single use/consumable systems for all primary contact surfaces have the following advantages: • limit risk of contamination by operators • processing can be performed in lower-grade cleanrooms (large clean rooms are very costly to build and operate)

	Automation Automated, customized cGMP systems need to be developed to reduce dependability on manual labor, cost and the risk of production failures.
Analytics	Final product release testing is likely to represent a significant part of the product, in terms of volume (number of samples) and cost. The goal should be to strive for final product release testing that is: • rapid (to account for the short shelf life of many products, in hours or days), • relatively inexpensive (especially if performed for every dose), • limited in necessary sample volume (to leave product for patient), • able to test complex product composition
Scale	Develop scalable manufacturing processes. Owing to the complexity and diversity of therapeutic approaches, processes need to be customized to each therapeutic approach. **Allogeneic cell therapies** and **gene therapies** reflect the current large-batch pharmaceutical investment model. Most of them can be scaled up. However, a number of allogeneic therapies will require small batch production. **Autologous cell therapies**, **patient-specific gene therapies** have a significant therapeutic advantage, however, one batch is produced for one patient; the batch size is 1 vial. These small batch sizes require an approach called "Scale-out", whereby increasing numbers of patients are served by increasing the number of manufacturing units or manufacturing suites. Decentralized production & supply (depending on product stability). Sample identity must be ensured for autologous treatments.

10.4.1 Summary of Technical Challenges for CTL019

1) It is a new experience and technology for Novartis due to very limited in-house experience in cell therapy. Dedicated teams & workstreams will be needed.
2) Cell Processing involves human serum & other derived raws; a defined medium and better defined processes will be required. Current T-cell expansion processes are manual, therefore automation ensuring robust & reproducible processes will be critical. Also, the process of freezing initial cells and final product needs careful attention & refinement.
3) Regarding the lentivirus technology, current lentivirus processes are not scalable for commercial manufacturing and contain animal derived raw materials. The bulk production of infectious lentiviruses including batch release with major adaptations may face quality and IP issues.
4) Other challenges: the scalability of a 100% individualized therapy (1 treatment = 1 batch = 1 release). How will the handling/transport of infectious materials and genetically modified human cells work? The regulatory environment is highly heterogeneous and specially challenging in the EU.

11 Future Directions and Conclusion

As T cell therapy advances beyond early phase clinical trials, the one of the next challenges will be to formulate strategies that improve cell isolation and culture in order to allow large scale automation of CAR-modified T cell production, reduce the expense of engineered T cell therapies, and facilitate their delivery beyond the academic research environment. Despite the early success of therapy of B cell malignancies with T cells modified to target CD19, it remains unknown whether similar results will be achieved by targeting other antigens expressed by B cell malignancies or antigens expressed by other tumors; careful research from lab bench to clinic to define the characteristics of effective CAR-modified T cell preparations and facilitate the design and clinical application of CAR-modified T cell therapies will ensure that at least some of these questions are addressed.

The field of adoptive therapy with engineered T cells is poised for substantial clinical advances that are now possible because of improved cell-culture and gene-transfer methods. In some cases, engineered autologous T cells may obviate the need for allogeneic HSCT, so that it is conceivable that autologous HSCT with T cell infusions could reach or exceed the efficacy of allogeneic HSCT but without the risk of GVHD. A major challenge will be to identify unique tumor antigens that can be targeted with selective T cell therapy. However, the major challenge currently facing the field is to conduct randomized clinical trials demonstrating sufficient clinical benefit to justify the logistics and expense of customized cellular therapies.

12 Bibliography

[1] K. Seiker, "Acute lymphoblastic leukemia," 5 February 2014. [Online]. Available: http://emedicine.medscape.com/article/207631-overview#showall. [Accessed 27 February 2014].

[2] Mayo Clinic, "Leukemia," 2013. [Online]. Available: http://www.mayoclinic.org/diseases-conditions/leukemia/basics/definition/CON-20024914?p=1&METHOD=print. [Accessed 27 February 2014].

[3] NCI, "What you need to know about leukemia," September 2013. [Online]. Available: http://www.cancer.gov/cancertopics/wyntk/leukemia.pdf. [Accessed 27 February 2014].

[4] D. Gilliland, C. Jordan and C. Felix, "The molecular basis of leukemia," *Hematology Am Soc Hematol Educ Program,* pp. 80-97, 2004.

[5] P. Kwan and P. Urayama, "Environmental and Genetic Risk Factors for Childhood Leukemia: Appraising the Evidence," *Cancer Investigation,* vol. 1, pp. 60-75, 2005.

[6] M. Stoppler, "Types of leukemia," 2014. [Online]. Available: http://www.medicinenet.com/leukemia/page3.htm#types_of_leukemia. [Accessed 01 March 2014].

[7] Stanford Cancer Institute, "Overview of Leukemia - Blood Diseases," 2014. [Online]. Available: http://cancer.stanford.edu/blood/leukemias/. [Accessed 1 March 2014].

[8] M. Mir, "Chronic Lymphocytic Leukemia," 2014. [Online]. Available: http://emedicine.medscape.com/article/199313-overview#showall. [Accessed 1 March 2014].

[9] American Cancer Society, "What is chronic myeloid leukemia?," 2013. [Online]. Available: http://www.cancer.org/cancer/leukemia-chronicmyeloidcml/detailedguide/leukemia-chronic-myeloid-myelogenous-what-is-c-m-l. [Accessed 1 March 2014].

[10] NCI, "General Information About Adult Acute Myeloid Leukemia (Health Professionals Version)," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/adultAML/healthprofessional. [Accessed 1 March 2014].

[11] E. Besa, "Chronic myelogenous leukemia," 2014. [Online]. Available: http://emedicine.medscape.com/article/199425-overview#showall. [Accessed 1 March 2014].

[12] NCI Dictionary of Cancer Terms, "AML," 2014. [Online]. Available: http://www.cancer.gov/dictionary?cdrid=44363. [Accessed 1 March 2014].

[13] K. Seiter, "Acute Myelogenous Leukemia," 2012. [Online]. Available: http://emedicine.medscape.com/article/197802-overview#showall. [Accessed 1 March 2014].

[14] B. Deschler and M. Lübbert, "Acute myeloid leukemia: epidemiology and etiology," *Cancer,* vol. 9, no. 107, pp. 2099-107, 2006.

[15] C. Jamieson, "Chronic myeloid leukemia stem cells," *Hematology Am Soc Hematol Educ Program,* pp. 436-42, 2008.

[16] NCI, "General Information About Adult Acute Lymphoblastic Leukemia (ALL) (Health Professional Version)," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/adultALL/healthprofessional. [Accessed 1 March 2014].

[17] National Cancer Institute, "Childhood Acute Lymphoblastic Leukemia (ALL)," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/childALL/HealthProfessional. [Accessed 1 March 2014].

[18] D. Longo, A. Fauci and D. Kasper, Harrison's Principles of Internal Medicine, 18th ed., McGraw-Hill Professional, 2011.

[19] T. Shanafelt, S. Geyer and N. Kay, "Prognosis at diagnosis: integrating molecular biologic insights into clinical practice for patients with CLL," *Blood,* vol. 15, no. 103(4), pp. 1202-10, 2004.

[20] G. Dighiero, "CLL biology and prognosis," *Hematology Am Soc Hematol Educ Program,* pp. 278-84, 2005.

[21] J. Byrd, S. Stilgenbauer and I. Flinn, "Chronic lymphocytic leukemia," *Hematology Am Soc Hematol Educ Program,* pp. 163-83, 2004.

[22] National Cancer Institute, "Leukemia - SEER Stat Fact Sheets," 2014. [Online]. Available: http://seer.cancer.gov/statfacts/html/leuks.html. [Accessed 1 March 2014].

[23] L. Ries, M. Smith, J. Gurney, M. Linet and T. Tamra, "Cancer Incidence and Survival among

Children and Adolescents: United States SEER Program 1975-1995," *National Cancer Institute, SEER Program,* 1999.

[24] CDC, "Cancer Among Children," 2013. [Online]. Available: http://www.cdc.gov/cancer/dcpc/data/children.htm. [Accessed 1 March 2014].

[25] World Health Organization, "Incidence of Childhood Leukemia," December 1999. [Online]. Available: http://www.euro.who.int/__data/assets/pdf_file/0005/97016/4.1.-Incidence-of-childhood-leukaemia-EDITED_layouted.pdf. [Accessed 2 March 2014].

[26] M. Stoppler, "Leukemia Symptoms," 2014. [Online]. Available: http://www.medicinenet.com/leukemia/page5.htm#symptoms. [Accessed 2 March 2014].

[27] American Cancer Society, "Chronic Myeloid Leukemia - Early Detection, Diagnosis and Staging Topics," 2013. [Online]. Available: http://www.cancer.org/cancer/leukemia-chronicmyeloidcml/detailedguide/leukemia-chronic-myeloid-myelogenous-detection. [Accessed 2 March 2014].

[28] Cleveland Clinic, "Leukemia," 2013. [Online]. Available: http://my.clevelandclinic.org/disorders/diseases/leukemia/can_overview.aspx. [Accessed 4 March 2014].

[29] American Cancer Society, "How is chronic lymphocytic leukemia staged?," 2013. [Online]. Available: http://www.cancer.org/cancer/leukemia-chroniclymphocyticcll/detailedguide/leukemia-chronic-lymphocytic-staging. [Accessed 3 March 2014].

[30] NCCN, "NCCN Clinical Practice Guidelines in Oncology: Non-Hodgkin's Lymphoma," 2013. [Online]. Available: http://www.nccn.org/professionals/physician_gls/pdf/nhl.pdf. [Accessed 3 March 2014].

[31] A. Seung, "Standard of care and novel treatments for chronic lymphocytic leukemia," *Am J Health Syst Pharm,* vol. 67, no. 21, pp. 1813-24, 2010.

[32] Duke Cancer Institute, "Stages of Leukemias," 2011. [Online]. Available: http://www.dukehealth.org/cancer/patient-care-services/leukemias-and-lymphomas/about/care_guides/stages-of-leukemias. [Accessed 3 March 2014].

[33] N. Ali, "Acute Lymphoblastic Leukemia," *Hematology Updates,* pp. 41-44, 2010.

[34] B. Abbott, "Chronic lymphocytic leukemia: recent advances in diagnosis and treatment,"

Oncologist, vol. 11, pp. 21-30, 2006.

[35] M. Hallek, B. Cheson and D. Catovsky, "Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute–Working Group 1996 guidelines," *Blood,* vol. 111, no. 12, p. 5446–5456, 2008.

[36] Leukaemia & Lymphoma Research, "Watch and Wait - Monitoring while treatment is not necessary," December 2011. [Online]. Available: http://leukaemialymphomaresearch.org.uk/sites/default/files/watch_and_wait_dec_2011.pdf. [Accessed 3 March 2014].

[37] National Cancer Institute, "Adult Acute Myeloid Leukemia Treatment (PDQ®)," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/adultAML/healthprofessional/page4. [Accessed 3 March 2014].

[38] American Cancer Society, "What is immunotherapy?," 2013. [Online]. Available: http://www.cancer.org/treatment/treatmentsandsideeffects/treatmenttypes/immunotherapy/immunotherapy-what-is-immunotherapy. [Accessed 3 March 2014].

[39] eMedicine Health, "Leukemia," 2014. [Online]. Available: http://www.emedicinehealth.com/leukemia-health/page9_em.htm. [Accessed 3 March 2014].

[40] The Leukemia and Lymphoma Society, "Chemotherapy and Drug Therapy," 2012. [Online]. Available: http://www.lls.org/diseaseinformation/leukemia/acutemyeloidleukemia/treatment/chemotherapydrugtherapy/. [Accessed 3 March 2014].

[41] National Cancer Institute, "Adult Acute Lymphoblastic Leukemia Treatment (PDQ®)," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/adultALL/HealthProfessional/page4. [Accessed 04 March 2014].

[42] S. Faderl and S. O'Brien , "Adult acute lymphoblastic leukemia: concepts and strategies," *Cancer,* vol. 116, no. 5, pp. 1165-76, 2010.

[43] S. Sallan, "Myths and lessons from the adult/pediatric interface in acute lymphoblastic leukemia," *Hematology Am Soc Hematol Educ Program,* pp. 128-32, 2006.

[44] K. Seiter and E. Besa, "Acute Lymphoblastic Leukemia Treatment & Management," 2014.

[Online]. Available: http://emedicine.medscape.com/article/207631-treatment#showall. [Accessed 4 March 2014].

[45] National Cancer Institute, "Treatment for Untreated Adult ALL," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/adultALL/HealthProfessional/Page5#Section_87. [Accessed 4 March 2014].

[46] S. Faderl , S. Jeha and H. Kantarjian , "The biology and therapy of adult acute lymphoblastic leukemia," *Cancer,* vol. 98, no. 7, pp. 1337-54, 2003.

[47] National Cancer Institute, "Treatment for Adult ALL in Remission," 2014. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/adultALL/HealthProfessional/Page6#Section_111. [Accessed 4 March 2014].

[48] National Cancer Institute, "Chronic Lymphocytic Leukemia Treatment (PDQ®)," 2013. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/CLL/healthprofessional/page3. [Accessed 5 March 2014].

[49] M. Mir and E. Besa, "Chronic Lymphocytic Leukemia Treatment & Management," 2014. [Online]. Available: http://emedicine.medscape.com/article/199313-treatment#showall. [Accessed 5 March 2014].

[50] National Cancer Institute, "Stage I, II, III, and IV Chronic Lymphocytic Leukemia," 2013. [Online]. Available: http://www.cancer.gov/cancertopics/pdq/treatment/CLL/healthprofessional/page5. [Accessed 5 March 2014].

[51] T. Parker and M. Strout, "Chronic lymphocytic leukemia: prognostic factors and impact on treatment," *Discov Med,* vol. 57, no. 11, pp. 115-23, 2011.

[52] N. Mitchison, "Studies on the immunological response to foreign tumor transplants in the mouse.The role of lymph node cells in conferring immunity by adoptive transfer.," *J. Exp. Med,* vol. 102, pp. 155-157, 1955.

[53] D. Barnes, C. Ford and P. Ilbery, "Tissue transplantation in the radiation chimera," *J. Cell Physiol. Suppl,* vol. 50, p. 123–38, 1957.

[54] D. Barnes and J. Loutit , "Treatment of murine leukaemia with x-rays and homologous bone marrow," *Br. J. Haematol.,* vol. 3, p. 241–52, 1957.

[55] G. Mathe, J. Amiel, L. Schwarzenberg and e. al., "Adoptive immunotherapy of acute leukemia: experimental and clinical results," *Cancer Res.,* vol. 25, p. 1525–31, 1965.

[56] S. Rosenberg and W. Terry , "Passive immunotherapy of cancer in animals and man," *Adv. Cancer Res.,* vol. 25, p. 323–88, 1977.

[57] P. Weiden , N. Flournoy and E. Thomas , "Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts," *N. Engl. J. Med.,* vol. 300, p. 1068–73, 1979.

[58] C. Klebanoff, H. Khong, P. Antony and e. al, "Sinks, suppressors and antigen presenters: how lymphodepletionenhances T cell-mediated tumor immunotherapy," *Trends Immunol.,* vol. 26, p. 111–17, 2005.

[59] W. Dummer, A. Niethammer, R. Baccala and e. al, "T cell homeostatic proliferation elicits effective antitumor autoimmunity," *J. Clin. Invest.,* vol. 110, p. 185–92, 2002.

[60] C. Hinrichs, Z. Borman, L. Cassard and e. al., "Adoptively transferred effector cells derived from naıve rather than central memory CD8 T cells mediate superior antitumor immunity," *Proc. Natl. Acad. Sci. USA,* vol. 106, p. 17469–74, 2009.

[61] C. Berger , M. Jensen and P. Lansdorp , "Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates," *J. Clin. Invest.,* vol. 118, p. 294–305, 2008.

[62] C. Paulos, C. Carpenito, G. Plesa and e. al, "The inducible costimulator ICOS is critical for the development of human TH17 cells," *Sci. Transl. Med.,* vol. 2, p. 55–78, 2010.

[63] L. Gattinoni , E. Lugli and Y. Ji , "A human memory T cell subset with stem cell-like properties," *Nat. Med.,* vol. 17, p. 1290–97, 2011.

[64] J. Lee, E. Hayman, H. Pegram and e. al., "In vivo inhibition of human CD19-targeted effector T cells by natural T regulatory cells in a xenotransplant murine model of B cell malignancy," *Cancer Res.,* vol. 71, p. 2871–81, 2011.

[65] J. Zhou, X. Shen, J. Huang and e. al, "Telomere length of transferred lymphocytes correlates with in vivo persistence and tumor regression in melanoma patients receiving cell transfer therapy," *J. Immunol.,* vol. 175, p. 7046–52, 2005.

[66] N. Weng, B. Levine, C. June and e. al., "Regulated expression of telomerase activity in human T lymphocyte development and activation," *J. Exp. Med.,* vol. 183, p. 2471–80, 1996.

[67] M. Chen, M. Pittet, L. Gorelik and e. al, "Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo," *Proc. Natl. Acad. Sci. USA,* vol. 102, p. 419–24, 2005.

[68] M. Kalos , B. Levine and D. Porter , "T cells expressing chimeric receptors establish memory and potent antitumor effects in patients with advanced leukemia," *Sci. Transl. Med.,* vol. 3, pp. 73-95, 2011.

[69] D. Porter , B. Levine and M. Kalos , "Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia," *N. Engl. J. Med.,* vol. 365, p. 725–33, 2011.

[70] Y. Zhao, E. Moon and C. Carpenito , "Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor," *Cancer Res.,* vol. 70, p. 9062–72, 2010.

[71] D. Barrett , Y. Zhao and X. Liu , "Treatment of advanced leukemia in mice with mRNA engineered T cells," *Hum. Gene Ther.,* vol. 22, p. 1575–86, 2011.

[72] R. Seder , P. Darrah and M. Roederer, "T-cell quality in memory and protection: implications for vaccine design," *Nat. Rev. Immunol.,* vol. 211, p. 58–66, 2008.

[73] B. Levine , W. Bernstein and N. Craighead , "Effects of CD28 costimulation on long term proliferation of CD4+ T cells in the absence of exogenous feeder cells," *J. Immunol.,* vol. 159, p. 5921–30, 1997.

[74] M. Maus , B. Kovacs and W. Kwok , "Extensive replicative capacity of human central memory T cells," *J. Immunol.,* vol. 172, p. 6675–83, 2004.

[75] H. Zhang , K. Snyder and M. Suhoski , "4-1BB is superior to CD28 costimulation for generating CD8+ cytotoxic lymphocytes for adoptive immunotherapy," *J. Immunol.,* vol. 179, p. 4910–18, 2007.

[76] C. Turtle , H. Swanson and N. Fujii , "A distinct subset of self-renewing human memory CD8+ T cells survives cytotoxic chemotherapy," *Immunity,* vol. 31, p. 834–44, 2009.

[77] C. June , B. Blazar and J. Riley, "Engineering lymphocyte subsets: tools, trials and tribulations," *Nat. Rev. Immunol.,* vol. 9, p. 704–16, 2009.

[78] A. Miller and G. Rosman , "Improved retroviral vectors for gene transfer and expression," *Biotechniques,* vol. 7, p. 980–90, 1989.

[79] L. Naldini, U. Blomer and P. Gallay , "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector," *Science,* vol. 272, p. 263–67, 1996.

[80] Z. Su , J. Dannull and B. Yang , "Telomerase mRNA-transfected dendritic cells stimulate

antigenspecific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer," *J. Immunol.,* vol. 174, p. 3798–807, 2005.

[81] Y. Chen , K. Galloway and C. Smolke , "Synthetic biology: advancing biological frontiers by building synthetic systems," *Genome Biol.,* vol. 12, p. 240, 2012.

[82] S. Hacein-Bey-Abina , A. Garrigue and G. Wang , "Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1," *J. Clin. Invest.,* vol. 118, p. 3132–42, 2008.

[83] L. Muul , L. Tuschong and S. Soenen , "Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial," *Blood,* vol. 101, p. 2563–69, 2003.

[84] J. Scholler , T. Brady and G. Binder-Scholl , "Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells," *Sci. Transl. Med.,* vol. 4, pp. 132-53, 2012.

[85] A. Krause , H. Guo and J. Latouche , "Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes," *J. Exp. Med.,* vol. 188, p. 619–26, 1998.

[86] J. Charo , S. Finkelstein and N. Grewal , "Bcl-2 overexpression enhances tumor-specific T-cell survival," *Cancer Res.,* vol. 65, p. 2001–8, 2005.

[87] S. Kerkar , R. Goldszmid and P. Muranski , "IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors," *J. Clin. Invest.,* vol. 121, p. 4746–57, 2011.

[88] L. Cheng , C. Ohlen and B. Nelson , "Enhanced signaling through the IL-2 receptor in CD8+ T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8+ T cells rather than promotion of cell death," *Proc. Natl. Acad. Sci. USA,* vol. 99, p. 3001–6, 2002.

[89] E. Moon , C. Carpenito and J. Sun , "Functional CCR2 receptor enhances tumor localization and eradication by human T cells expressing a mesothelin-specific chimeric antibody receptor," *Clin. Cancer Res.,* vol. 17, p. 4719–30, 2011.

[90] Z. Eshhar , T. Waks and A. Bendavid , "Functional expression of chimeric receptor genes in human T cells," *J. Immunol. Methods,* vol. 248, p. 67–76, 2001.

[91] M. Milone , J. Fish and C. Carpenito , "Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo," *Mol.*

Ther., vol. 17, p. 1453–64, 2009.

[92] B. Till , M. Jensen and J. Wang , "CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results," *Blood,* vol. 119, p. 3940–50, 2012.

[93] R. Brentjens and K. Curran, "Novel cellular therapies for leukemia: CAR-modified T cells targeted to the CD19 antigen," *Hematology Am Soc Hematol Educ Program,* pp. 143-151, 2012.

[94] S. Deeks , B. Wagner and P. Anton, "A phase II randomized study of HIV-specific T-cell gene therapy in subjects with undetectable plasma viremia on combination anti-retroviral therapy," *Mol. Ther.,* vol. 5, p. 788–97, 2002.

[95] M. Dudley , J. Yang and R. Sherry , "Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens," *J. Clin. Oncol.,* vol. 26, p. 5233–39, 2008.

[96] M. Kershaw , J. Westwood and L. Parker , "A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer," *Clin. Cancer Res.,* vol. 12, p. 6106–15, 2006.

[97] J. Park , D. DiGiusto and M. Slovak , "Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma," *Mol. Ther.,* vol. 15, p. 825–33, 2007.

[98] C. Lamers , S. Sleijfer and A. Vulto , "Treatment ofmetastatic renal cell carcinomawith autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience," *J. Clin.Oncol.,* vol. 24, pp. 20-22, 2006.

[99] A. Di Stasi , S. Tey and G. Dotti , "Inducible apoptosis as a safety switch for adoptive cell therapy.," *N. Engl. J. Med.,* vol. 365, p. 1673–83, 2011.

[100] M. Jensen, L. Popplewell and L. Cooper, "Anti-transgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor re-directed T cells in humans," *Biol. Blood Marrow Transplant.,* vol. 16, p. 1245–56, 2010.

[101] C. Lamers , R. Willemsen and P. van Elzakker, "Immune responses to transgene and retroviral vector in patients treated with ex vivo engineered T cells," *Blood,* vol. 117, p. 72–82, 2011.

[102] C. Louis , B. Savoldo and G. Dotti , "Anti-tumor activity and long-term fate of chimeric antigen receptor positive T-cells in patients with neuroblastoma," *Blood,* vol. 118, p. 6050–56, 2011.

[103] P. Bretscher and M. Cohn , "A theory of self-nonself discrimination," *Science,* vol. 169, p. 1042–49, 1970.

[104] A. Loskog , V. Giandomenico and C. Rossig , "Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells," *Leukemia,* vol. 20, p. 1819–28, 2006.

[105] T. Brocker, "Chimeric Fv-xi or Fv-epsilon receptors are not sufficient to induce activation or cytokine production in peripheral T cells," *Blood,* vol. 96, p. 1999–2001, 2000.

[106] H. Finney , A. Lawson and C. Bebbington, "Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product," *J. Immunol.,* vol. 161, p. 2791–97, 1998 .

[107] C. Imai , K. Mihara and M. Andreansky, "Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia," *Leukemia,* vol. 18, p. 676–84, 2004.

[108] H. Finney , A. Akbar and A. Lawson , "Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain," *J. Immunol.,* vol. 172, p. 104–13, 2004.

[109] D. Song , Q. Ye and M. Poussin, "CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo," *Blood,* vol. 119, p. 696–706, 2012.

[110] D. Kohn , G. Dotti and R. Brentjens , "CARS on track in the clinic: report of a meeting organized by the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) sub-committee on cell and gene therapy," *Mol. Ther.,* vol. 19, p. 432–38, 2011.

[111] B. Jena , G. Dotti and L. Cooper , "Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor," *Blood,* vol. 116, p. 1035–44, 2010.

[112] J. Kochenderfer , W. Wilson and J. Janik , "Eradication of B-lineage cells and regression of lymphoma in a patient treatedwith autologousTcells genetically-engineered to recognize CD19," *Blood,* vol. 116, p. 4099–102 , 2010.

[113] J. Kochenderfer , M. Dudley and S. Feldman , "B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptortransduced T cells," *Blood,* vol. 119, p. 2709–20, 2012.

[114] R. Brentjens , I. Riviere and J. Park , "Safety and persistence of adoptively transferred

autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias," *Blood,* vol. 118, p. 4817–28, 2011.

[115] B. Savoldo , C. Ramos and E. Liu , "CD28 costimulation improves expansion and persistence of chimeric antigen receptor–modified T cells in lymphoma patients," *J. Clin. Invest.,* vol. 121, p. 1822–25, 2011.

[116] S. Grupp , M. Kalos and D. Barrett , "Chimeric antigen receptor-modifiedTcells for acute lymphoid leukemia," *N. Engl. J. Med.,* vol. 368, p. 1509–18, 2013.

[117] P. Kebriaei , H. Huls and B. Jena , "Infusing CD19-directed T cells to augment disease control in patients undergoing autologous hematopoietic stem-cell transplantation for advanced B-lymphoid malignancies," *Hum. Gene Ther.,* vol. 23, pp. 444-50, 2012.

[118] K. Birkholz , A. Hombach and C. Krug , "Transfer ofmRNAencoding recombinant immunoreceptors reprograms CD4+ and CD8+ T cells for use in the adoptive immunotherapy of cancer," *Gene Ther.,* vol. 16, p. 596–604, 2009.

[119] H. Almasbak , E. Rian and H. Hoel , "Transiently redirected T cells for adoptive transfer," *Cytotherapy,* vol. 13, p. 629–40, 2011.

[120] S. Yoon , J. Lee and H. Cho , "Adoptive immunotherapy using human peripheral blood lymphocytes transferred with RNA encoding Her-2/neu-specific chimeric immune receptor in ovarian cancer xenograft model," *Cancer Gene Ther.,* vol. 16, p. 489–97, 2009.

[121] P. Rabinovich , M. Komarovskaya and S. Wrzesinski , "Chimeric receptor mRNA transfection as a tool to generate antineoplastic lymphocytes," *Hum. Gene Ther.,* vol. 20, p. 51–61, 2009.

[122] Z. Jin , S. Maiti and H. Huls , "The hyperactive Sleeping Beauty transposase SB100X improves the genetic modification of T cells to express a chimeric antigen receptor," *Gene Ther.,* vol. 18, pp. 849-856, 2011.

[123] M. Hudecek , T. Schmitt and S. Baskar , "The B-cell tumor associated antigen ROR1 can be targeted with T cells modified to express a ROR1-specific chimeric antigen receptor," *Blood,* vol. 116, pp. 4532-4541, 2010.

[124] J. Vera , B. Savoldo and S. Vigouroux , "T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells," *Blood,* vol. 108, pp. 3890-3897, 2006.

[125] A. Dutour , V. Marin and I. Pizzitola , "In vitro and in vivo antitumor effect of anti-CD33 chimeric

receptor-expressing EBV-CTL against CD33 Acute myeloid leukemia," *Adv Hematol.,* pp. 6830-65, 2012.

[126] S. Peinert , H. Prince and P. Guru , "Gene-modified T cells as immunotherapy for multiple myeloma and acute myeloid leukemia expressing the Lewis Y antigen," *Gene Ther.,* vol. 17, pp. 678-686, 2010.

[127] T. Schirrmann and G. Pecher , "Specific targeting of CD33(+) leukemia cells by a natural killer cell line modified with a chimeric receptor," *Leuk Res.,* vol. 29, pp. 301-306, 2005.

[128] S. Spranger , I. Jeremias and S. Wilde , "TCR-transgenic lymphocytes specific for HMMR/Rhamm limit tumor outgrowth in vivo," *Blood,* vol. 119, pp. 3440-3449, 2012.

[129] H. Stauss , S. Thomas and M. Cesco-Gaspere , "WT1-specific T cell receptor gene therapy: improving TCR function in transduced T cells," *Blood Cells Mol Dis.,* vol. 40, pp. 113-116, 2008.

[130] M. Stephan , V. Ponomarev and R. Brentjens , "T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection," *Nat Med.,* vol. 13, pp. 1440-1449, 2007.

[131] H. Pegram , J. Lee and E. Hayman , "Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning," *Blood,* vol. 119, pp. 4133-4141, 2012.

[132] C. Yee , J. Thompson and D. Byrd , "Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells," *Proc. Natl. Acad. Sci. USA,* vol. 99, p. 16168–73, 2002.

[133] R. Mitsuyasu , P. Anton and S. Deeks , "Prolonged survival and tissue trafficking following adoptive transfer of CD4 zeta gene-modified autologous CD4+ and CD8+T cells in human immunodeficiency virus–infected subjects," *Blood,* vol. 96, p. 785–93, 2000.

[134] H. Zhang , K. Chua and M. Guimond , "Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells," *Nat. Med.,* vol. 11, p. 1238–43, 2005.

[135] C. Ku , M. Murakami and A. Sakamoto , "Control of homeostasis of CD8+ memory T cells by opposing cytokines," *Science,* vol. 288, p. 675–78, 2000.

[136] C. Berger , M. Berger and R. Hackman , "Safety and immunologic effects of IL-15 administration in non human primates," *Blood,* vol. 114, p. 2417–26, 2009.

[137] M. Dudley , J. Wunderlich and P. Robbins , "Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes," *Science,* vol. 298, p. 850–54, 2002.

[138] A. Rapoport , E. Stadtmauer and N. Aqui , "Rapid immune recovery and graft-versus-host disease like engraftment syndrome following adoptive transfer of costimulated autologous T cells," *Clin. Cancer Res.,* vol. 15, p. 4499–507, 2009.

[139] E. Alyea , D. DeAngelo and J. Moldrem , "NCI First International Workshop on the Biology, Prevention and Treatment of Relapse after Allogeneic Hematopoietic Cell Transplantation: report from the committee on prevention of relapse following allogeneic cell transplantation for hematologic malignan," *Biol. Blood Marrow Transplant.,* vol. 16, p. 1037–69, 2010.

[140] D. Porter , B. Levine and N. Bunin , "A phase I trial of donor lymphocyte infusions expanded and activated ex-vivo via CD3/CD28 co-stimulation," *Blood,* vol. 107, p. 1325–31, 2006.

[141] H. Heslop and C. Rooney , "Adoptive cellular immunotherapy for EBV lymphoproliferative disease," *Immunol. Rev.,* vol. 157, p. 217–22, 1997.

[142] N. Kernan , N. Collins and L. Juliano , "Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease," *Blood,* vol. 68, p. 770–73, 1986.

[143] M. Cairo , B. Coiffier and A. Reiter , "Recommendations for the evaluation of risk and prophylaxis of tumour lysis syndrome (TLS) in adults and children with malignant diseases: an expert TLS panel consensus," *Br. J. Haematol.,* vol. 149, p. 578–86, 2010.

[144] A. Bear , R. Morgan and K. Cornetta , "Replication-competent retroviruses in gene-modified T cells used in clinical trials: Is it time to revise the testing requirements?," *Mol. Ther.,* vol. 20, p. 246–49, 2012.

[145] C. Kloss, M. Condomines and M. Cartellieri, " Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells," *Nature Biotech,* vol. 31, pp. 71-75, 2013.

[146] E. Lantis , "Chimeric antigen receptor T cells with dissociated signaling domains exhibit focused antitumor activity with reduced potential for toxicity in vivo," *Cancer Immunol. Res.,* 2013.

[147] Y. Chen and M. Jensen, "Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems," *Proc. Natl Acad. Sci. USA,* vol. 107, p. 8531–8536, 2010.

[148] P. Wei, "Bacterial virulence proteins as tools to rewire kinase pathways in yeast and immune

cells," *Nature,* vol. 488, p. 384–388, 2012.

[149] C. Berger, M. Flowers and E. Warren , "Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation," *Blood,* vol. 107, p. 2294–302, 2006.

[150] X. Wang, W. Chang and C. Wong, "A transgene-encoded cell surface polypeptide for selection, in vivo tracking, and ablation of engineered cells," *Blood,* vol. 118, p. 1255–63, 2011.

[151] R. Brentjens, R. Yeh and Y. Bernal, "Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial," *Mol. Ther.,* vol. 18, p. 666–668, 2010.

[152] R. Morgan, J. Yang and M. Kitano, "Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2," *Mol. Ther.,* vol. 18, p. 843–851, 2010.

[153] NCI-CTC, "Common Terminology Criteria for Adverse Events (CTCAE) v4.0," 20 March 2013. [Online]. Available: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm. [Accessed 05 June 2014].

[154] J. Ferrara, S. Abhyankar and D. Gilliland, "Cytokine storm of graft-versus-host disease: a critical effector role for interleukin-1," *Transplant Proc.,* vol. 25, p. 1216–1217, 1993.

[155] X. Xu, Y. Tang and C. Liao, "Inflammatory cytokine measurement quickly discriminates gram-negative from gram-positive bacteremia in pediatric hematology/oncology patients with septic shock," *Intens. Care Med.,* vol. 39, p. 319–326, 2013.

[156] P. Bugelski, R. Achuthanandam and R. Capocasale, "Monoclonal antibody-induced cytokine-release syndrome," *Exp. Rev. Clin. Immunol.,* vol. 5, p. 499–521, 2009.

[157] H. Wang and S. Ma, "The cytokine storm and factors determining the sequence and severity of organ dysfunction in multiple organ dysfunction syndrome," *Am. J. Emerg. Med.,* vol. 26, p. 711–715, 2008.

[158] W. Lee and A. Slutsky, "Sepsis and endothelial permeability," *N. Engl. J. Med.,* vol. 363, p. 689–691, 2010.

[159] A. Rudiger and M. Singer, "Mechanisms of sepsis-induced cardiac dysfunction," *Crit. Care Med.,* vol. 35, p. 1599–1608, 2007.

[160] R. Brentjens, M. Davila and I. Riviere, "CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia," *Sci. Trans. Med.,* Vols. 5177-138, 201.

[161] X. Xu, Y. Tang and H. Song, "Diagnostic accuracy of a specific cytokine pattern in hemophagocytic lymphohistiocytosis in children," *J. Pediatr.,* vol. 160, p. 984–990 , 2012.

[162] Y. Tang, X. Xu and H. Song, "Early diagnostic and prognostic significance of a specific Th1/Th2 cytokine pattern in children with haemophagocytic syndrome," *Br. J. Haematol.,* vol. 143, p. 84–91, 2008.

[163] S. Howard, D. Jones and C. Pui, "The tumor lysis syndrome," *N. Engl. J. Med.,* vol. 364, p. 1844–1854, 2011.

[164] M. Cairo and M. Bishop, "Tumour lysis syndrome: new therapeutic strategies and classification," *Br. J. Haematol.,* vol. 127, p. 3–11, 2004.

[165] H. Kolb, "Graft-versus-leukemia effects of transplantation and donor lymphocytes," *Blood,* vol. 112, p. 4371–4383, 2008.

[166] L. Kandalaft, D. Powell Jr. and G. Coukos, "A phase I clinical trial of adoptive transfer of folate receptor-alpha redirected autologous T cells for recurrent ovarian cancer," *J. Trans. Med.,* vol. 10, p. 157, 2012.

[167] H. Ertl, J. Zaia and S. Rosenberg, "Considerations for the clinical application of chimeric antigen receptor T cells: observations from a recombinant DNA Advisory Committee Symposium held June 15," *Cancer Res.,* vol. 71, p. 3175–3181, 2011.

[168] D. Barrett, X. Liu and S. Jiang, "Regimen-specific effects of RNA-modified chimeric antigen receptor T cells in mice with advanced leukemia," *Hum. Gene Ther.,* vol. 24, p. 717–727, 2013.

[169] S. Tey, G. Dotti and C. Rooney, "Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation," *Biol. Blood Marrow Transplant,* vol. 13, p. 913–924, 2007.

[170] K. Straathof, M. Pule and P. Yotnda, "An inducible caspase 9 safety switch for T-cell therapy," *Blood,* vol. 105, p. 4247–4254, 2005.

[171] V. Hoyos, B. Savoldo and C. Quintarelli, "Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety," *Leukemia,* vol. 24, p. 1160–1170, 2010.

[172] E. Han, X. Li and C. Wang, "Chimeric antigen receptor-engineered T cells for cancer immunotherapy: progress and challenges," *J. Hematol. Oncol.,* vol. 6, p. 47, 2013.

[173] C. Klebanoff, L. Gattinoni and N. Restifo, "Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy?," *J. Immunother.,* vol. 35, p. 651–660, 2012.

[174] N. Cieri, B. Camisa and F. Cocchiarella, "IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors," *Blood,* vol. 121, p. 573–584, 2013.

[175] M. Themeli, C. Kloss and G. Ciriello, "Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy," *Nat. Biotechnol.,* 2013.

[176] M. Oppert, R. Schindler and Husung, "Low-dose hydrocortisone improves shock reversal and reduces cytokine levels in early hyperdynamic septic shock," *Crit. Care Med.,* vol. 33, p. 2457–2464, 2005.

[177] P. Martin, J. Rizzo and J. Wingard, "First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation," *Biol. Blood Marrow Transplant,* vol. 18, p. 1150–1163, 2012.

[178] T. Lin, M. Lucas and N. Heerema, "A phase II study of the TNF-alpha inhibitor etanercept and thrice weekly rituximab in relapsed CLL/SLL: Clinical activity in the absence of del(17p13) genomic abnormalities," *Blood,* p. 2841, 2006.

[179] D. Teachey, S. DRheingold and S. Maude, "Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy," *Blood,* vol. 121, p. 5154–5157, 2013.

[180] G. Suntharalingam, M. Perry and S. Ward, "Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412," *N. Engl. J. Med.,* vol. 355, p. 1018–1028, 2006.

[181] A. Kelly and A. Ramanan, "A case of macrophage activation syndrome successfully treated with anakinra," *Nat. Clin. Pract. Rheumatol.,* vol. 4, p. 615–620, 2008.

[182] X. Xu, H. Zhao and Y. Tang, "Efficacy and safety of adoptive immunotherapy using anti-CD19 chimeric antigen receptor transduced T-cells: a systematic review of phase I clinical trials," *Leukemia Lymphoma,* vol. 54, p. 255–260, 2013.

[183] G. Gross , T. Waks and Z. Eshhar , "Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity," *Proc Natl Acad Sci USA,* vol.

86, pp. 10024-8, 1989.

[184] B. Irving and A. Weiss , "The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways," *Cell,* vol. 64, pp. 891-901, 1991.

[185] M. Sadelain , R. Brentjens and I. Rivière, "The promise and potential pitfalls of chimeric antigen receptors," *Curr Opin Immunol,* vol. 21, pp. 215-23, 2009.

[186] C. Carpenito , M. Milone and R. Hassan , "Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains," *Proc Natl Acad Sci USA,* vol. 106, pp. 3360-5, 2009.

[187] F. Uckun , W. Jaszcz and J. Ambrus , "Detailed studies on expression and function of CD19 surface determinant by using B43 monoclonal antibody and the clinical potential of anti-CD19 immunotoxins," *Blood,* vol. 71, pp. 13-29, 1988.

[188] S. Grupp , N. Frey and R. Aplenc, "T Cells Engineered With a Chimeric Antigen Receptor (CAR) Targeting CD19 (CTL019) Produce Significant In Vivo Proliferation, Complete Responses and Long-Term Persistence Without Gvhd In Children and Adults With Relapsed, Refractory ALL," in *ASH Annual Meeting. Abstract 168.* , New Orleans, LA, 2013.

[189] M. Kalos , F. Nazimuddin and J. Finklestein, "Long-term functional persistence, B cell aplasia, and anti-leukemia efficacy in refractory B cell malignancies following T cell immunotherapy using CAR-redirected T cells targeting CD19.," in *ASH Annual Meeting*, New Orleans, LA, 2013.

[190] A. Goodman, "Mounting Success in Trials of Genetically Engineered T Cells to Treat Leukemias and Lymphomas," *The ASCO Post,* 15 January 2014.

[191] T. Baas, "Driving CAR-based cellular therapies," *SciBX,* vol. 6, p. 41, 2013.

[192] Perelman School of Medicine University of Pennsylvania, "University of Pennsylvania and Novartis Form Alliance to Expand Use of Personalized T Cell Therapy for Cancer Patients," University of Pennsylvania, 6 August 2012. [Online]. Available: http://www.uphs.upenn.edu/news/News_Releases/2012/08/novartis/. [Accessed 13 May 2014].

[193] Novartis, "Novartis Media Releases," Novartis, 6 August 2012. [Online]. Available: http://www.novartis.com/newsroom/media-releases/en/2012/1631944.shtml. [Accessed 13 May 2014].

[194] BioCentury BCIQ, "Kite Pharma Inc.," 5 May 2014. [Online]. Available:

http://www.biocentury.com/companies/kite_pharma_inc?utm_source=nature. [Accessed 14 May 2014].

[195] Kite Pharma Inc., "Kit Pharma Partners with the National Cancer Institute to Develop Novel Cellular Immunotherapy Clinical Products," 16 October 2012. [Online]. Available: http://www.kitepharma.com/c/news/releases/101612.php. [Accessed 14 May 2014].

[196] Bluebird bio, "bluebird bio Announces Global Strategic Collaboration with Celgene to Advance Gene Therapy in Oncology," 21 March 2013. [Online]. Available: http://investor.bluebirdbio.com/phoenix.zhtml?c=251820&p=irol-newsArticle&ID=1817134&highlight=. [Accessed 14 May 2014].

[197] R. McBride, "Celgene makes big splash in gene therapy with two key cancer collaborations," Fierce Biotech, 21 March 2013. [Online]. Available: http://www.fiercebiotech.com/story/celgene-makes-big-splash-gene-therapy-two-cancer-collaborations/2013-03-21. [Accessed 14 May 2014].

[198] C. Moore, "Bluebird Bio Collaborates With Celgene And Houston's Center for Cell and Gene Therapy to Advance Gene Therapy in Oncology," Bio News Texas, 21 March 2013. [Online]. Available: http://bionews-tx.com/news/2013/03/21/bluebird-bio-collaborates-with-celgene-and-houstons-center-for-cell-and-gene-therapy-to-advance-gene-therapy-in-oncology/. [Accessed 14 May 2014].

[199] Cell Medica, "Cell Medica secures £17 million ($26.5 million) equity investment to establish US operations and fund cell therapy development," 23 July 2012. [Online]. Available: http://www.cellmedica.co.uk/news/cell-medica-secures-17-million-265-million-equity-investment/. [Accessed 14 May 2014].

[200] Cellectis, "Cellectis Group announces groundbreaking in vivo proof of concept testing of their flagship UCART19 product for curative therapy of leukemia," 4 June 2013. [Online]. Available: http://www.cellectis.com/en/content/cellectis-group-announces-groundbreaking-vivo-proof-concept-testing-their-flagship-ucart19-0. [Accessed 14 May 2014].

[201] J. Grisham, "New Trial Advances Cell-Based Immune Therapy for Certain Leukemias," Memorial Sloan Kettering , 20 February 2014. [Online]. Available: http://www.mskcc.org/blog/new-trial-advances-cell-based-immune-therapy-certain-leukemias. [Accessed 14 May 2014].

[202] European Commission, " Regulation (EC) No 1394/2007 on advanced therapy medicinal products," 10 December 2007. [Online]. Available: http://ec.europa.eu/health/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf.

[Accessed 12 May 2014].

[203] European Commission, "Report from the commission to the European Parliament and the Council in according with article 25 of Regulation (EC) N.º 1394/2007," European Commission, Brussels, 2014.

[204] European Commission Health and Consumers Directorate-General, "Summary of the responses to the public consultation - Regulation n.º 1394/2007 on ATMPs," 03 04 2013. [Online]. Available: http://ec.europa.eu/health/files/advtherapies/2013_05_pc_atmp/2013_04_03_pc_summary.pdf. [Accessed 13 05 2014].

[205] European Comission, "Marketing authorisation granted by Commission Decision C (2009) 7726 of 5 October 2009.," European Comission, Brussels, 2009.

[206] European Comission, "Marketing authorisation granted by Commission Decision C (2012) 7708 of 25 October 2012," European Comission, Brussels, 2012.

[207] European Comission, "Marketing authorisation granted by Commission Decision C (2013) 4190 of 27 June 2013.," European Comission, Brussels, 2013.

[208] European Comission, "Marketing authorisation granted by Commission Decision C (2013) 5841 of 6 September 2013," European Comission, Brussels, 2013.

[209] European Medicines Agency, "Advanced therapy medicinal product classification," 19 12 2013. [Online]. Available: http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000296.jsp&mid=WC0b01ac058007f4bc. [Accessed 13 05 2014].

[210] US Food and Drug Administration, "Cellular & Gene Therapy Guidances," FDA, March 2014. [Online]. Available: http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm. [Accessed 13 May 2014].

[211] R. Walker, "Pricing Cellular Therapeutics - Considerations for Advanced Therapy Medicinal Products in the EU," PriceSpective, London, 2013.

[212] J. Ruof, F. Schwartz and J. Schulenburg, "Early benefit assessment (EBA) in Germany: analysing decisions 18 months after introducing the new AMNOG legislation," *The European Journal of Health Economics,* no. June, 2013.

[213] Gemeinsame Bundesausschuss, "The Federal Joint Committee," 2013. [Online]. Available: https://www.g-ba.de/informationen/nutzenbewertung/. [Accessed 14 May 2014].

[214] A. Wieczorek and L. Uharek, "Genetically Modified T Cells for the Treatment of Malignant Disease," *Transfus Med Hemother,* vol. 40, p. 388–402, 2013.

[215] Haute Autorité de Santé , " Synthèse d'avis ChondroCelect – CT-8020," HAS, Paris, 2010.

[216] Grupo GENESIS de la SEFH, "Informe Técnico de Evaluación Condrocitos Humanos Autólogos," SEFH, Madrid, 2012.

[217] E. Bravo and C. D'Augusta, "Tigenix - Living Medicines," Kempen HealthCare Conference, Amsterdam, 2012.

[218] InEK, "Informationen nach § 6 Abs. 2 KHEntgG für 2013: Neue Untersuchungs- und Behandlungsmethoden," Die InEK GmbH, Siegburg, 2013.

[219] Alliance for Regenerative Medicine, "Regenerative Medicine Annual Report," ARM, Washington, DC, 2013.

[220] R. Shaw, B. Hampson and S. Zvonic, "Business Model Considerations for Development of Cell Therapies," *BioProcess International,* vol. 12, no. 4, pp. 10-14, 2014.

[221] Progenitor Cell Therapy, [Online]. Available: http://pctcelltherapy.com/. [Accessed 05 June 2014].

[222] Pharma IQ, "How pharmaceutical companies will pick their contract manufacturing organizations in 2012," [Online]. Available: http://www.pharma-iq.com/ShowLargeImageWindow.cfm?image=/article_images/large/pharma-industry-infonuggets.jpg. [Accessed 05 June 2014].

[223] D. Smith, "Assessing commercial opportunities for autologous and allogeneic cell-based products," *Regen. Med.,* vol. 7, no. 5, pp. 721-732, 2012.

[224] S. Tobak, "The versatility and longevity of the razor and blades business model.," 11 December 2008. [Online]. Available: http://www.cbsnews.com/news/the-versatility-and-longevity-of-the-razor-and-blades-business-model/. [Accessed 05 June 2014].

[225] C. Mason and P. Dunnill, "Assessing the value of autologous and allogeneic cells for regenerative medicine," *Regen. Med.,* vol. 4, p. 835–853, 2009.

[226] R. Buckler , "Opportunities in regenerative medicine – the global industry and market trends," *Bioprocess Int.,* vol. 9, pp. 14-19, 2011.

[227] D. Driscoll , "Addressing business models, reimbursement, and cost of goods," *Bioprocess Int.,* vol. 9, p. 46–49, 2011.

[228] S. Finnegan and K. Pinto, "Globalisation of biotech offshoring," 21 December 2006. [Online]. Available: http://saffron.pharmabiz.com/article/detnews.asp?articleid=36781&sectionid=50. [Accessed 05 June 2014].

[229] S. Farid , "Process economics of industrial monoclonal antibody manufacture," *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.,* vol. 15, p. 8–18, 2007.

[230] M. Herper, "The world's most expensive drugs," *Forbes,* 19 02 2010.

[231] K. Nguyen , M. Devidas and S. Cheng , "Factors influencing survival after relapse from acute lymphoblastic leukemia: a Children's Oncology Group study," *Leukemia,* vol. 22, no. 12, pp. 2142-2150, 2008.

[232] H. Einsiedel , A. von Stackelberg and R. Hartmann, "Long-term outcome in children with relapsed ALL by risk-stratified salvage therapy: results of trial against lymphoblastic leikemia-relapse study of the Berlin-Frankfurt-Munster Group 87.," *J Clin Oncol,* vol. 23, no. 31, pp. 7942-7950, 2005.

[233] A. Roy, A. Cargill and S. Love , "Outcome after first relapse in childhood acute lymphoblastic leukaemia - lessons from the United Kingdom R2 trial," *Br J Haematol,* vol. 130, no. 1, pp. 67-75, 2005.

[234] A. Sander , M. Zimmermann and M. Dworzak, "Consequent and intensified relapse therapy improved survival in pediatric AML: results of relapse treatment in 379 patients of three consecutive AML-BFM trials," *Leukemia,* vol. 24, no. 8, pp. 1422-1428, 2010.