TÉCNICO LISBOA

UNIVERSIDADE DE LISBOA
INSTITUTO SUPERIOR TÉCNICO

ENGINEERING OF T CELLS WITH CHIMERIC ANTIGEN RECEPTORS AS A THERAPY FOR HEMATOLOGIC CANCERS

Diogo Gomes da Silva

Supervisors: Doctor Joaquim Manuel Sampaio Cabral
Doctor Carlos Alexandre de Almeida Ramos
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Thesis approved in public session to obtain the PhD Degree in Bioengineering

Jury final classification: Pass with distinction

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Abstract

Surgery, chemotherapy, and radiation therapy, alone or in combination, have been the mainstay of cancer treatment. Together with newer and more accurate diagnostic tools, these approaches have contributed to substantially improved outcomes. However, the prognosis of most malignancies remains poor. Given their complexity, most cancers will ultimately require a more personalized management to achieve cure or control. Recently, the focus of cancer treatment has shifted toward targeted therapies, including immunotherapy. Adoptive T-cell therapy is a form of immunotherapy that involves the isolation of lymphocytes with the intent to stimulate and expand ex vivo potent antigen-specific T cells that are subsequently infused into a patient to treat a disease. T cells can recognize tumor cells through their mutated proteins when presented by diseased cells. However, despite some recent evidence suggesting a positive correlation between T cell tumor infiltration and better prognosis, tumors employ several clinically relevant mechanisms to avoid detection by T cells. Chimeric antigen receptors (CARs) were developed to solve some of the challenges of adoptive cell therapy. CARs transform T cells into tumor-specific killer cells capable of recognizing any molecule associated with particular malignancies. The activity of CAR-T cells against B cell malignancies (targeting CD19) was recently demonstrated in several clinical trials, in which the majority of patients with refractory or relapsed disease achieved complete remissions. Extending the success of CAR-T cells to other types of tumors will only be possible by generating and testing CARs against other tumor associated antigens.

The work described here demonstrates that CD7, a lymphocytic marker, is a potential target for the majority of patients with T-cell malignancies and 30% of patients with acute myeloid leukemia. Nonetheless, since not only malignant but also normal T cells express CD7, use of the gene editing tool CRISPR/cas9 is required for removal of CD7 so as to avoid fratricide among CAR-T cells. CD7 CAR-T cells have a protective role in preclinical models for both forms of leukemia and limited off targeting of normal T and NK cells. In contrast, a CAR generated against CD3, a marker expressed in the majority of patients with T-cell lymphomas and leukemias, demonstrated unexpected toxicity because of CD3 CAR-T cell elimination by normal T cells. Thus, while CD7 CAR showed promising pre-clinical results and will be tested in clinical trials, results with CD3 CAR in vitro were not compelling enough for further clinical studies.

This work also highlights a toxic effect that can be seen with one of the endodomains commonly used in clinically available CARs – CD137 or 4-1BB. Although co-stimulatory domains are required to achieve optimal in vivo expansion and persistence of CAR-T cells, both characteristics associated with better outcomes, their role it is not fully understood. This work demonstrates that the inclusion of 4-1BB as an intracellular domain into a variety of CARs increases the number of CAR molecules on the cell surface, through a positive feedback loop, which leads to undesired constitutive activation and cell death that perturbs CAR-T cell expansion and killing activity. Changes in CAR sequence and viral vector can restore normal expression of CARs, leading to increased CAR-T cell performance in preclinical models. These models ensure 4-1BB-containing CAR are optimized before being tested in the future in the clinic.

Keywords: Immunotherapy; CAR; Cancer; Hematology; T cells.

Resumo

Cirurgia, quimioterapia e radioterapia têm sido a base do tratamento oncológico. Estas abordagens quando combinadas com novas e precisas formas de diagnostico contribuíram para um aumento do seu sucesso. No entanto o prognóstico para a maioria dos cancros é ainda desfavorável. Dada a sua complexidade, a maioria dos cancros necessita de uma gestão mais personalizada. Recentemente o foco da oncologia tem privilegiado terapias dirigidas como por exemplo a imunoterapia. A transferência adoptiva de células T é um tipo de imunoterapia que envolve o isolamento de células T com a intenção de as estimular e expandir ex vivo, antes de serem administradas no doente. As células T podem eliminar células tumorais reconhecendo proteínas mutadas quando estas são apresentadas pelas células malignas. No entanto, apesar de evidências mais recentes sugerirem uma correlação positiva entre a infiltração de células T nos tumores e um prognóstico mais favorável, os tumores têm ao seu dispor mecanismos para evitar serem reconhecidos por células T. Receptores quiméricos de antigénio (CARs em inglês) foram desenvolvidos com o intuito de solucionar algumas das dificuldades observadas na terapia celular adoptiva. Os CAR transformam as células T em células capazes de eliminar especificamente células tumorais através do reconhecimento de qualquer molécula cuja expressão se encontre associada a um cancro em particular. A actividade de células T com CARs em cancros hematologicos de células B (através do reconhecimento do CD19) foi recentemente demostrada em ensaios clínicos em que a maioria dos doentes com tumores refractários atingiram remissão completa. Estender o sucesso desta terapia a outros tumores só será possível através da criação e desenvolvimento de CARs que reconhecem outros antigénios associados a tumores.

O trabalho aqui descrito demostra que o CD7, um marcador linfocítico, é um interessante alvo para a maioria dos casos de cancros hematológicos de células T e 30% de doentes com leucemia mieloide aguda. No entanto, visto que para além das células malignas, também células T normais expressam CD7, o uso de CRISPR é necessário para a remoção do CD7 de forma a evitar o fratricídio entre células T com CAR. Células T CD7 CAR têm um papel protector em modelos pré-clínicos de ambos os tipos de leucemia e uma toxicidade limitada às células NK e T. No entanto, um outro CAR criado para reconhecer a proteína CD3- um marcador expresso na maioria dos doentes com linfomas e leucemias de células T- demostrou uma inesperada toxicidade visto que células T CD3 CAR são eliminadas por células T normais. Assim, enquanto o CD7 CAR demostrou resultados pré-clínicos promissores de forma a ser testado em ensaios clínicos, os resultados aqui obtidos in vitro com o CD3 CAR não são convincentes o suficiente para serem transportados para estudos clínicos.

Este trabalho também realça um efeito tóxico que pode ser observado com um dos endodomínios mais usados em CARs já disponíveis clinicamente- CD137 ou 4-1BB. Embora domínios co-estimulatórios sejam necessários de forma a permitir a expansão e persistência de células T in vivo, o seu papel ainda não é completamente conhecido. Neste trabalho a inclusão do 4-1BB como domínio intracelular em vários CARs aumenta o numero de moléculas CAR na membrana celular levando a uma ativação constitutiva indesejável e à morte celular, afectando a expansão e actividade citotóxica. Alterações na sequencia do CAR ou vector viral podem normalizar a sua expressão, levando ao aumento do desempenho das células T CAR em modelos pré-clínicos. Estes modelos asseguram que CARs com 4-1BB são optimizados antes de serem testados na clinica no futuro.

Palavras-chave: Imunoterapia; CAR; Cancro; Hematologia; Células T

Agradecimentos

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Não só por questão protocolares mas essencialmente por querer dedicar um espaço especial à minha família gostaria por ultimo de dedicar algumas palavras à minha irmã e aos meus Pais. Independentemente dos objectivos (dos só propostos aos heroicamente atingidos) a família tem um lugar fulcral por vezes invisível, muitas vezes injusto. Algures entre o desejo involuntário de querer partilhar o máximo da vida com os entes queridos e a intencionalidade forçada de lhes permitir as melhores condições reside um estado de alma muitas vezes difícil de atingir. Dentro do valor que meras palavras têm, considero importante enfatizar que tenho tido uma família que tem atingido esse equilíbrio, permitindo-me voar para lá do horizonte mais perto sem me deixar perder o norte e muito menos o ponto de partida. As palavras dificilmente fazem jus ao sentimento mas se o suor demonstrativo do nosso esforço se correlaciona diretamente com nosso próprio trabalho a coragem de enfrentar o que a vida tem para nos dar parece ser, aos meus olhos, directamente proporcional ao ambiente que nos rodeia. O que atingi e mais importante o que ainda atingirei, não pode portanto, ser destilado do amor, da ajuda e da oportunidade concedida para perseguir os meus sonhos que a minha família me tem dado ao longo destes anos que vai muito para além dos quatro que demoraram a realização deste trabalho.

A escrita de uma tese de Doutoramento peca, bastante, na tentativa de demonstrar as dificuldades que, estou seguro, todos os seus executantes enfrentam. Peca também na maioria das vezes em demonstrar o sentimento do aluno perante a tese e o trabalho aqui explanado. Nos vários anos que levam ao culminar de um exercício académico como este ficam muitas lágrimas mas ficam também muitos sorrisos e até sentimentos de orgulho de fora. Tais sentimentos embora inegavelmente importantes ganham outra importância quando temos com quem os partilhar. Porque não escrevo agradecimentos apenas na esperança que sejam lidos dedico por ultimo mais do que agradecimentos o trabalho em si a minha Mãe a quem o tempo num jogo de roleta russa resolveu tirar-me a oportunidade de agora findado ter a oportunidade de ler e partilhar tais momentos. A sua partida prematura em nada belisca o efeito determinante na minha pessoa não só durante a realização deste trabalho como no que ainda ai vem.

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List of abbreviations

AICD- Activation-Induced Cell Death

ALL- Acute Lymphoblastic Leukemia

AML- Acute Myeloid Leukemia

APC- Antigen Presenting Cells

BCG- Bacillus Calmette-Guérin

BCR- B Cell Receptor

CAIX- Carbonic Anhydrase IX

CAR- Chimeric Antigen Receptor

CLL- Chronic Lymphoblastic Leukemia

CML- Chronic Myeloid Leukemia

CMV- Cytomegalovirus

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeat

CRS- Cytokine Release Syndrome

EBV- Epstein-Barr Virus

FACS- Fluorescence-activated cell sorting

FDA- Food and Drug Administration

FFluc- Firefly Luciferase

GLP- Good Laboratory Practice

GMP- Good Manufacturing Practices

gRNA- guide RNA

GvHD - Graft-vs-Host Disease

HIV- Human Immunodeficiency Virus

HLA- Human Leucocyte Antigen

HSCT- Hematopoietic Stem Cell Transplant

IFN-γ- Interferon-gamma

ITAM- Immunoreceptor tyrosine-based Activation Motif

IRES- Internal ribosome entry site

KO- Knockout

LSC- Leukemic Stem Cells

LTR- Long Terminal Repeat

LV- Lentivirus

mAb- Monoclonal Antibody

MHC- Major Histocompatibility Complex

MRD - Minimal Residual Disease

NGFR- Nerve Growth Factor Receptor

NK- Natural Killer

PBMC- Peripheral Blood Mononuclear Cell

RV- Retrovirus

scFv- Single-chain Fragment Variable

TALEN- Transcription activator-like effector nuclease

TAP- Transporter Associated with Antigen Processing Protein

TILs- Tumor Infiltrating Lymphocytes

TCR- T Cell Receptor

TNF- Tumor Necrosis Factor

VSTs- Virus-specific T cells

WT1- Wilms Tumor 1

Thesis Outline

Persistence, expansion, trafficking and strong immune responses are key characteristics of T cells. However, use of T cells as a therapy for cancer has until now collided with the similarities between tumor and normal cells. Synthetic biology opened the adoptive cell therapy field to a new world where T cells are no longer limited to TCR-recognizable antigens. Self-antigens (that have been used as targets in other forms of immunotherapy, such as monoclonal antibodies) are now accessible to T cell response. Although far from ideal targeting, because of substantial toxicities, chimeric antigen receptors (CARs) have demonstrated superior antitumor effect compared to any standard therapies, especially in B-cell malignancies. The approval of the first genetically modified cellular product is a breakthrough, but active investigation is underway for the discovery and improvement of more candidate antigens. A balance between strong antitumor effect and low toxicities needs to be found and each type of tumor will likely be a new challenge. New candidate target antigens not only increase the repertoire of different types of cancer that can be addressed using CAR-T cells, but also refine the processes targeting those antigens, with better recognition of tumor cells without destruction of normal and vital tissues, reducing off-tumor toxicity.

This thesis describes the work developed during the PhD studies and born out of the intention to learn and contribute to this exciting medical field that now seems to rapidly be reaching its maturity. This work was initially started as collaboration between Instituto Português de Oncologia de Lisboa Francisco Gentil and the Stem Cell Engineering Research Group of Instituto Superior Técnico, which in the past was essential for the development of other types of cellular therapies. The involvement and contribution of the Center for Cell and Gene Therapy at Baylor College of Medicine, which has intensive research and clinical experience in several different forms of adoptive T cell therapy provided access to the state of the art in CAR-T cell therapies.

The first chapter summarizes the theoretical background important for better understanding of the work. The following chapters describe the work developed to generate, for the first time, a CD7-specific CAR that can be used to target T-cell malignancies (chapter II) and acute myeloid leukemia (Chapter IV), and a CD3-specific CAR for T-cell lymphomas (chapter III). CD7 is expressed, although differently, in both types of tumors. The antitumor potential of CD7 CAR-T cells is demonstrated but also, due to CD7 expression in normal T and NK cells, expected off-tumor effects together with potential solutions for their mitigation are presented. CD3 is also expressed in T cells and a potential target in T-cell malignancies

but, owing to an undesired effect, CD3 CAR T cells are prematurely eliminated, preventing their future use in the clinic.

Despite a consensual design of second generation CARs currently being used in clinical trials, improvements can still be made to increase CAR-T cell potency. Even the most used CAR – CD19 CAR – reveals different activation properties and differential potency among different clinical trials. This demonstrates that its functionality it is still not fully understood. In chapter V, we demonstrate an unexpected toxicity of CD19 CAR with a 4-1BB endodomain. Changes in CAR design and T cell engineering prove that improvements can still be made to produce a CAR-T cell with better properties, including a stronger anti-tumor effect.

Complete List of Publications:

- Gomes-Silva D., Srinivasan M., Sharma S., et al.; *CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies;* **Blood;** 2017; 130, 285-296
- Gomes-Silva D., Mukherjee M., Srinivasan M., et al.; *Tonic 4-1BB Costimulation in Chimeric Antigen Receptors Impedes T Cell Survival and Is Vector- Dependent*; **Cell Reports**; 2017; 21, 17–26
- Gomes-Silva D., Ramos C. A.; *Cancer Immunotherapy Using CAR-T Cells: From the Research Bench to the Assembly Line*; **Biotechnology Journal**, 2018, *13, 2*.
- Mamonkin M., Mukherjee M., Srinivasan M., Sharma S., Gomes-Silva D., et al.; *Reversible Transgene Expression Reduces Fratricide and Permits 4-1BB Costimulation of CAR T Cells Directed to T-cell Malignancies;* **Cancer Immunol Res**; 2018; 6 47-58
- Gomes-Silva D., Tashiro H., Srinivasan M. et. al; *CD7 CAR T Cells for the Therapy of Acute Myeloid Leukemia*; **Molecular Therapy** (accepted)

List of oral communications

- American Society for Gene and Cell Therapy 2017, Washington, USA
- 25th Porto Cancer Meeting, 2018, Porto, Portugal
- XLIV SPI (Sociedade Portuguesa de Imunologia) Annual Meeting, 2018 Lisbon, Portugal

List of poster communications

- American Society for Gene and Cell Therapy 2016, Washington, USA

- American Society for Gene and Cell Therapy 2017, Washington, USA
- American Society of Hematology, 2016, San Diego, USA
- American Society of Hematology, 2017, Atlanta, USA

List of awards

- Outstanding Poster Award in American Society for Gene and Cell Therapy 2016
- Outstanding Poster Award in American Society for Gene and Cell Therapy 2017
- Best oral communications in XLIV SPI (Sociedade Portuguesa de Imunologia) Annual Meeting, 2018

Chapter I.Theoretical Background

Immunity – the exemption of disease or immunis *– is an old concept whose foundations have been unraveled during the last centuries, leading to an important medical field. Understanding the immune system and its properties not only allowed comprehending numerous diseases and improving their treatments, but also ignited the idea of Immunotherapy – a form of treatment that relies on the natural ability the immune system has to recognize foreign antigens and tries to re-educate it to specifically recognize and fight diseases such as cancer. Immunotherapy is not entirely new, neither as a theoretical concept nor in its practical use. Pioneer work in the 19th century using bacterial injections with inactivated Streptococcus led to the first observed tumor regressions, presumably because it triggered an attack by the immune system. During the following two centuries, several immunotherapeutic tools have become available for use in the clinic, such as monoclonal antibodies and pharmacological grade cytokines. Nonetheless, it is commonly thought that immunotherapy has not yet reached its full potential. An intense interest in immune cellular therapeutic approaches has been stedily growing during the last couple of decades. The immunotherapeutic approaches that sparkled two centuries ago relied only on the innate ability of the immune system to recognize some cancer cells. However, with the introduction of genetic engineering and synthetic biology, this limitation has recently changed…*

Part of this chapter is published as:

Gomes-Silva D., Ramos C. A.; *Cancer Immunotherapy Using CAR-T Cells: From the Research Bench to the Assembly Line*; **Biotechnology Journal**, 2018, *13:2*.

I.1. A brief overview of the immune system

As a response to the rapidly evolving microbial pathogens, evolution endowed multicellular organisms with a versatile and complex immune system to protect them. The immune system allows a fast and precise response against any microorganism or agent capable of inducing disease (from small viral invaders measuring 30 nm to giant parasitic worms that can reach 100 cm in length). An immune system must be capable of recognizing and killing a diverse array of pathogens while sparing the normal tissues of the host (i.e. must be self-tolerant) [1]. The first observation of leukocytes at the site of inflammation was performed in 1774 by Silvertein, [2] although it took almost a century until the first observation of the engulfment of fungal spores by some cells in invertebrates that preceded the publication of Metchnikov's cellular theory of immunity in 1884 [3]. The notion of chemotaxis, that is, the attraction of leukocytes by chemical substances to inflammation sites arose a few years later. Step by step, the several types of immune cells and their functions were discovered in the last century. Such identification led to a traditional division of the immune system into two different but cooperative branches: the innate and the adaptive immune systems [4] (Figure I-1)

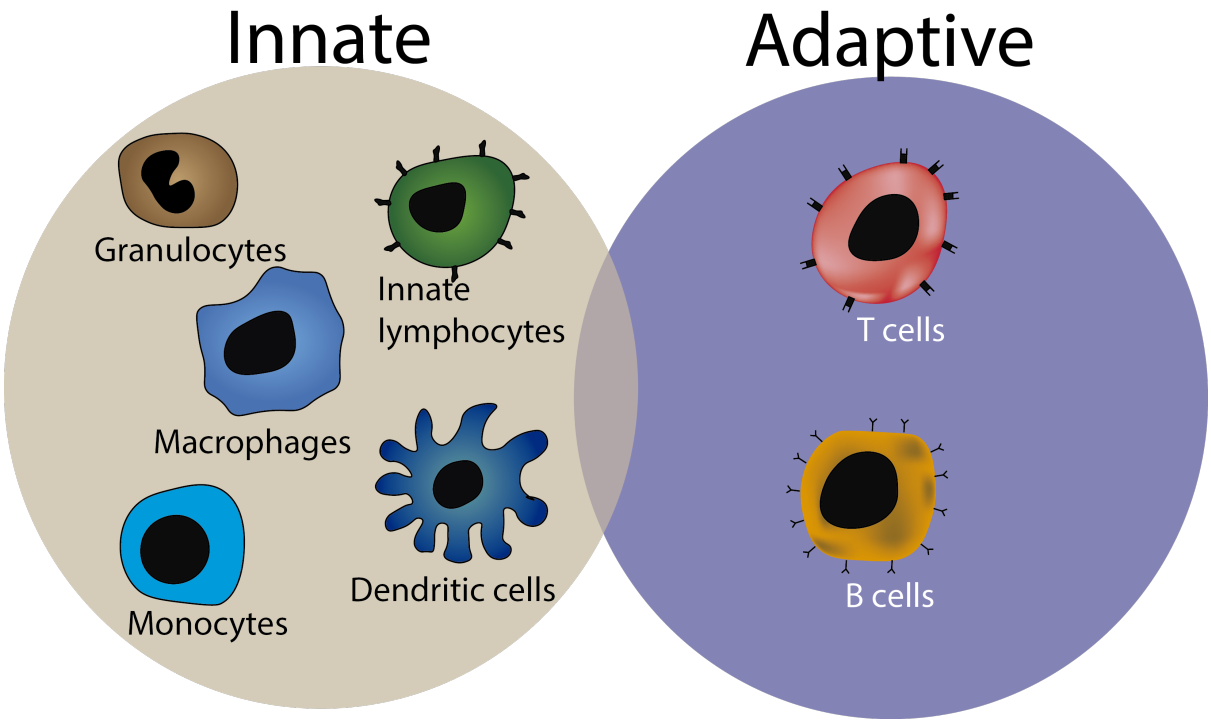

Figure I-1- Classical characterization of immune cells according with their intrinsic characteristics in innate and adaptive immunity.

I.1.1. Innate Immune System

The innate immune system includes cellular and humoral elements and is considered, from an evolutionary perspective, more primitive. Its role is to eliminate invaders as fast as possible using common and easily recognized signatures found in most pathogens. Most multicellular organisms exclusively depend on this type immune system. This response is stereotyped, conferring a broad protection against pathogens and, in a second encounter, the response is not different from the first.

The first line of defense of the innate immune system is any physical barrier (for example, the skin in mammals and the exoskeleton in insects) that is capable of prevent entry of an infecting agent into an organism. If any of these physical barriers are breached, the immune system has humoral and cellular mechanisms capable of starting a response, sometimes in a few seconds, using cells that circulate in the blood and traffic through tissues, and that in the first hours should contain any pathogen infiltration and spreading, preventing significant damage to the host Most of the innate cellular responses depend on the phagocytic capacity of myeloid cells, which can be divided into granulocytes and mononuclear myeloid cells although some populations of lymphocytes are also included in the innate immune system.

I.1.1.1.Granulocytes

Granulocytes are a diverse category of cells, all of which contain cytoplasmic granules containing antimicrobial compounds. Neutrophils are the most frequent type of leucocytes in circulation, and one of the first type of immune cells to reach the site of infection, where they phagocytose (engulf) bacteria very effectively. They have a multilobed nuclei, which explains their alternative designation of polymorphonuclear phagocytes, and have a short life span of just a few hours or days. Basophils and mast cells (nonphagocytic granulocytes) and eosinophils (essential in the defense against multicellular parasitic organisms) are rare in circulation and in tissues (usually less than 5% of total leukocytes), but play an important role in the production of molecules that can shape the inflammatory milieu [5].

I.1.1.2.Mononuclear Myeloid Cells

Contrasting with the polymorphonuclear phenotype observed in granulocytes, monocytes, macrophages and dendritic cells are myeloid cells with a single round nucleus (mononuclear). They have an intense capacity to present antigens to lymphocytes and thus are called professional antigen presenting cells (APCs). This APC function acts as a bridge between the innate and the adaptive immune system and, although their first step in controlling infections consists of phagocytosis of the pathogen, their role goes beyond the

simple destruction of the invader. By presenting microbial antigens to lymphocytes, they can shape dramatically the continuation and progression of immune response. These types of phagocytic cells are more complex and less disposable than granulocytes, having a much longer life-span.

Monocytes represent 5-10% of leukocytes in the peripheral blood. Despite having phagocytic and APC functions, their role seems to be more directed at constant patrolling of the blood stream [1]. Macrophages are professional APCs thought to differentiate from monocytes that migrated from the blood into the parenchyma of major organs, and thus being tissue-restricted. Macrophages can perform an array of functions that range from initial elimination of pathogens in tissues through phagocytosis and removal of cell debris to the induction of the initial steps of tissue repair and regeneration [6].

Dendritic cells are found mostly in lymphoid organs and organ tissues, and rarely in peripheral blood, where they can uptake and present antigens more powerfully than any other immune cell [7], [8]. The APC function of dendritic cells is not only essential for controlling pathogens but, due to the ability to present mutated self proteins found in tumor cells, also likely important for antitumor responses, a property that has been exploited for cancer therapy[9]. After activation, dendritic cells abandon the peripheral tissue or blood and migrate to lymph nodes, where they can activate lymphocytes [10].

I.1.1.3. Innate and innate-like lymphoid cells

Although lymphocytes are the main cellular effectors of the adaptive immune system, several types of lymphocytes participate in an innate type of immune response.

Natural killer (NK) cells are large granular lymphocytes known for their fast cytotoxic activity against virally infected and transformed cells, and their massive ability for cytokine (secreted molecules capable of regulating the growth, differentiation and activation of immune cells by interaction with their receptors) production, such as interferon-γ (IFN-γ) [11], [12]. NK cells rely on a fixed number of inhibitory and activating receptors to detect patterns associated with diseased cells. Nonetheless, NK cells, like other myeloid cells, efficiently recognize antibody-bound antigens [13].

Natural killer T (NKT) cells are a heterogeneous group of T cells that share properties of both T and NK cells[14]. Although they are considered a subset of T cells because express the same type of antigen receptor as conventional T cells, NKT cells are not considered part of the adaptive immune system express the same type of antigen receptor as conventional T cells because of the limited diversity their receptors. The most common subset of NKT cells recognize self and foreign lipids presented by CD1d, a non-polymorphic antigen-presenting molecule [15]. Although less frequent than any other

lymphocyte in peripheral blood, NKT cells have a protective role in microbial infections, some auto-immune and allergic diseases, and cancer [16].

Other forms of innate T cells, such mucosal-associated invariant T cells and some forms of gamma delta (γδ) T cells have been discovered. γδ T cells express a γδ T cell receptor (TCR) and, although they have somatic gene rearrangement and can give rise to memory-like conventional T cells, their TCR recognition and activation is independent of antigen presentation, at least in the most common subsets. Instead, they recognize small non-peptidic prenyl-pyrophosphate metabolites of isoprenoid biosynthesis, termed phosphoantigens[17]. Because γδ T cells share several characteristics with T cells (adaptive system) but rely on a type of response more similar to that of NK cells, although some authors classify them as part of the adaptive immune system, the term innate-like has emerged to distinguish this type of cells, which are probably positioned as a bridge between both types of response [18], [19]. γδ T cells have both innate cytotoxic functions and antigen-presenting capabilities [20], [21].

Although fast and able to recognize a myriad of pathogens, innate immune responses are not specific, being incapable of recognizing minor differences in foreign antigens, and cannot adapt to different types of invaders. In a second line of defense, lymphocytes, adaptive immune cells, arrive and, after proper activation, undergo clonal expansion to produce highly specific effectors that allow achievement of a highly effective response.

I.1.2. Adaptive Immune cells

The acquired or adaptive immune response evolved from the older innate system, by which it is controlled and assisted. Without the presence and assistance of the innate immune system, the acquired immune response offers weak protection.

B and T cells, due to intrinsic characteristics, are considered immune cells belonging to the adaptive immune system. While T cells are important for self/non-self discrimination, B cells produce antibodies that recognize antigenic profiles in soluble proteins, carbohydrates or nucleic acids [22]. Despite having distinct roles in an immune response, they both express antigen-specific receptors, whose genes are rearranged somatically in a random fashion so that one organism has innumerable clones in circulation that can virtually recognize any foreign molecule. This gives them the ability to recognize minor changes (e.g. mutations) in pathogen-associated patterns that innate cells cannot detect. Furthermore, after stimulation, adaptive cells can not only expand but also differentiate into memory cells, which in a second encounter with the same pathogen trigger a faster immune response [23].

I.1.2.1.B cells

B cells express in their cell surface the B cell receptor (BCR), a transmembrane form of an immunoglobulin (antibody) molecule. Each B cell expresses a BCR with unique specificity to a certain antigen. Each host has B cells with a variety of amino acid sequences in their BCRs that leads to subtle differences in structure allowing antibodies to bind specifically to an equally vast variety of antigens. Upon binding of the BCR to the antigen, B cells differentiate into plasma cells and start to synthetize and secrete the antibody [24]. Since antibodies are the soluble form of the BCR, the antigen that activates a given B cell becomes the target of the antibodies produced by that cell's progeny. These soluble molecules, after being released by plasma cells (activated B cells), can bind to pathogens. The part of the antibody molecule that is not involved in antigen binding is common to all antibodies. These constant portions (referred to as Fc) bind to cells that express Fc receptors, such as NK cells and APCs. Upon activation of the Fc receptors, these cells can eliminate the antigens bound to antibodies through cytotoxic or phagocytic processes, respectively. Antibodies have several other roles in the immune response, such as activation of complement, and can by themselves neutralize a pathogen by binding directing to it and inhibiting the initial pathogenic steps of infection [25]. Immune responses dependent on antibodies are called humoral responses.

Although there are several types of immunoglobulins, they all share the same structure of 4 polypeptide chains consisting of two identical light and heavy chains. Each light chain is connected by a disulfide bond to a heavy chain. An antibody molecule is commonly illustrated as a Y, with each antigen-specific domain present in each arm (Figure I-2). While the variable regions are in the N terminal of both light and heavy chain, the constant region is present in the C terminal of the light chain and spans the majority of the heavy chain. The variable regions so called because they have diverse sequences that allow them to recognize different antigens, while constant regions are important for activation of other immune effectors, such as macrophages and NK cells or the complement system, when pathogens are coated (opsonized) by antibody molecules [26].

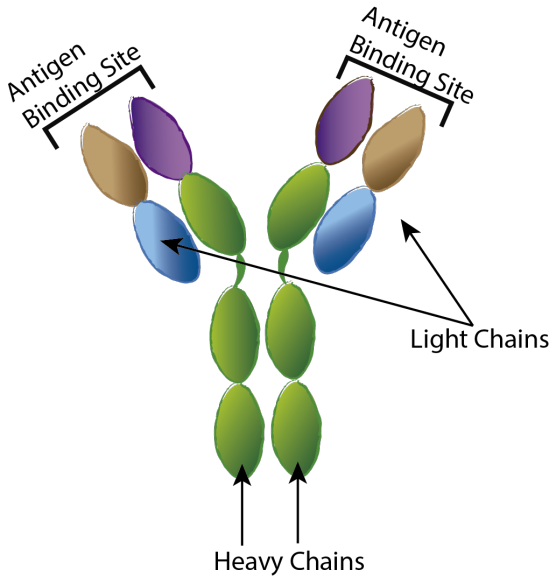

Figure I-2- Common structure of an antibody highlighting the two heavy and two light chain and the variable regions in the N terminal of both types of chains.

I.1.2.2.T cells

T cells are the other type of cells belonging to the adaptive immune system. Like B cells, they express their own antigen-specific receptor (T cell receptor – TCR), the gene for which is rearranged somatically during maturation of T cells in the thymus. While B cells are responsible for the humoral response, T cells are the key players in the cellular adaptive immune response. TCR stimulation leads to T cell activation and release from cytoplasmic granules of proteins capable of punching holes in the membrane of the target cell (such as perforin) and induce apoptosis (such as via granzyme b, which activates caspase molecules in the target cells) [27]. Relegating T cells to their cytotoxic function is, however, giving an incomplete view of their role in the overall immune system. While T cells expressing CD8 in their cell surface are indeed associated with degranulation and cytotoxicity upon activation, T cells expressing CD4 (corresponding to approximately 60% of all T cells in peripheral blood) produce cytokines whose primary role is to modulate the entire immune response.

T cells are subject to clonal selection, similar to B cells. After activation, a T cell proliferates to build up a clone of T cells with the same antigen specificity, which allows an adaptive immune response to emerge specifically against a particular antigen. While effector T cells undergo an exponential proliferation that takes several days, they also differentiate into memory cells so that upon a second encounter with the same exact pathogen the response is faster and stronger due to immediate activation of memory cells [28]. T cells are

usually phenotypically divided into 4 stages of differentiation and activation: Naïve (N), central memory (CM), effector memory (EM) and effector memory RA (EMRA), which can be characterized according to certain cell surface markers [29]. Naïve cells, the less differentiated subset, lose CD45RA while transitioning to the central memory state. Both N and CM cells express CCR7. After antigenic re-stimulation, T cells convert to an effector phenotype (EM), losing CCR7, and as a last step they reacquire CD45RA (EMRA) becoming terminally differentiated. At this point, T cells lose almost all capacity to proliferate but become effector killer cells [30]. The canonical perspective is that memory cells are responsible for the initiation of a T cell response after antigen stimulation and effector cells mediate cytotoxic responses [31]. Other markers, such CD62L, CD45RO and CD95, are being used to classify the T-cell differentiation profile, and new subsets such as stem cell memory T cells (T_{SCM}) have been described [29], [32]. Regardless of the markers used for the classification, it is well established that less differentiated cells are long-lived but that as they lose their proliferation abilities, they acquire a stronger capacity to kill target cells and produce cytokines. For example, central memory T cells have a long life span and are thought to increase engraftment in preclinical trials [28]–[30], [33]. Undoubtedly, this is an area of intense research and new findings are expected in the coming years.

Differentiation of T cells is not only based on memory-effector polarization. T cells can also be polarized according to the type of cytokine response that is required. The most classical vision splits T cell responses in T_H1 (directed towards cytotoxicity and cellular immunity capable of responding viral infection and tumor cells) and T_H2 (B-cell activation and antibody production against parasites). However other phenotypes have already been identified, such as T_H17 [34].

I.1.2.2.1.T cell receptor

Interestingly, although BCR and TCR are distinct in many ways, both possess immunoglobulin domains and are hetero-oligomers [35]. A TCR is composed of two heterodimer subunits (α and β), each having a constant and a variable region. Both α and β chains are rearranged at the DNA level similarly to BCR, efficiently creating an extremely broad somatic diversity. This rearrangement of the variable regions of the TCR chains during T cell development in the thymus confers specificity to a particular TCR [36]. An it is precisely in the thymus that a clonal selection process occurs [37]: T cells whose TCRs have good binding to major histocompatibility complex (MHC) molecules are positively selected, but if those cells recognize self-antigens, they are removed. This negative selection of self-reactive T cells is essential to avoid auto-immune diseases [38].

The TCR proteins do not have an endodomain with intrinsic signaling capacity. Each TCR is expressed on the cell surface of all T cells in association with three dimers created from three different CD3 subunits (γε, δε and ζζ) [39]. Each CD3 subunit has immunoreceptor tyrosine-based activation motifs (ITAM), with each ζ subunit having 3 ITAMs, which upon TCR activation trigger the Src kinase family member Lck to bind to the CD3, initiating a signaling cascade [40] that will activate and prime T cells for killing the target cell. Phosphorylation of CD3 subunits leads to activation of the Ras-Erk pathway, NF-kB activation and Ca^{2+} influx [41].

Contrary to the BCR, which can be activated by molecules in solution, the TCR is exclusively activated by peptides presented by APCs in association with MHC molecules. MHC class I proteins are members of the MHC superfamily and are expressed in all nucleated cells. Thus, all cells but red blood cells can in theory present antigens to $CD8^+$ T cells. MHC class II proteins are only expressed by specialized APCs (dendritic cells, B cells, macrophages, and monocytes among others) and present peptides to $CD4^+$ T cells [42], [43]. In APCs, after phagocytosis of targets, the ingested proteins are processed into small peptides and loaded into MHC class II molecules, which have a special groove that encloses those small peptides. In nucleated cells, cytoplasmic proteins are similarly loaded into MHC class I melecules. The small peptides (8-11 amino acids for MHC class I and 13-17 amino acids for class II) are generated by the proteasome and transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP) protein, where they associate with the MHC forming a complex (pepMHC). This pepMHC complex is then transported to cell surface [44]. On the cell surface, the pepMHC complex can then interact with a specific TCR [45], a process known as antigen presentation to T cells. Although the activation of TCR via MHC antigens is the main event in antigen recognition, it is not enough to ignite a systemic T cell response. T cells in lymph nodes are initially activated through a TCR-MHC interaction providing the first, antigen specific, bond between the APC and T cells. However, subsequent co-stimulation steps are needed for full T cell activation [46], [47]. Antigen presenting cells also express molecules such as B7, 4-1BBL or CD40L on their surface, which are required to activate a second signaling event in T cells that is required to full activation of T cells. Indeed, TCR stimulation alone with lack of co-stimulation leads to T cell unresponsiveness (a state called anergy) [48].

I.1.2.3. New visions of the immune system

Although from a traditional point of view, it is thought that a true adaptive immune system is only present in vertebrates, new findings may change this stance. Although more recent from an evolutionary perspective, adaptive immunity may not be an exclusive luxury

of more advanced multicellular organisms, with some unicellular organisms also having their own form of adaptive immunity. One example of a rudimental form of adaptive immunity is the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 complex, which protects bacteria from being infected and genetically modified by exogenous nucleic acids. A CRISPR system is composed of two guide RNAs (gRNA) – crRNA and trRNA – that in combination can guide the nuclease cas9 to cut foreign DNA that is introduced during phage DNA injection. This primitive form of immunity can be considered adaptive since each created RNA is target-specific and bacteria become resistant to a second encounter with the same DNA [49].

Although innate and adaptive immune responses are most of the time considered two independent forms of immune response, in reality both branches work side by side to ensure the host's homeostasis. In fact, new findings have blurred the classical sharp distinction between innate and adaptive immunity. For example, new insights into NK [50] and γδ T cell [51] biology suggest they are responsible for a response that instead of fitting in one of the classical branches seems to occupy an intermediate position, demonstrating a continuum of both innate and adaptive immunity. An immune response starts with a set of cells capable of reacting against and containing the invading antigens in the first minutes or hours, allowing a more robust and specific response to be prepared that requires not only T and B cells but also interaction among all cells.

The elimination of pathogens or diseased cells, although considered the main goal of the immune system, is a simplified vision of its the role in host homeostasis. For example macrophages exhibit critical regulatory activities at all stages of tissue repair and fibrosis [52]. The importance of the immune system in neurologic [53], aging, obesity and other metabolic diseases [54] is now being elucidated, demonstrating that the role of the immune system goes beyond its anti-pathogenic or anti-neoplastic effects. The full integration of the roles of the immune system may provide the missing link for understanding particular diseases.

I.2. Immune system and cancer: the missing link

Cancer is a leading cause of death in developed countries. Malignancy arises as a consequence of dynamically evolving genomes, which are constantly undergoing mutation in somatic cells [55]. Such mutations are usually directly or indirectly associated with loss of function or overexpression of genes. Cancers, however, contain not only malignant cells but also many healthy (non-transformed) cells like stromal cells [56], vascular cells involved in neoangiogenesis [57], and immune cells among others, which surrounding the malignant

cells and play an important role in an interconnected crosstalk that is associated with cancer progression [58]. The presence of immune cells in the cancer microenvironment has been the subject of intense debate and is not completely understood. The link between inflammatory state and cancer, although not always consensual, has been known since the 19th century [59], [60]. Chronic inflammation is considered one of the hallmarks of cancer growth [58]. For example, aberrant expression of NF-κB, an important orchestrator of inflammation, has already observed in several different types of cancer [61].

A variety of immune cells can be found in tumors, including myeloid cells, B cells and all subsets of T cells, with the presence of more pro- or anti-inflammatory cells possibly dictating different outcomes in patients with the same underlying malignancy. If the immune system is able to actively recognize cancer cells, tumor growth may be inhibited [62]. However, tumor-associated macrophages and regulatory T cells, among other inhibitory immune cells, can facilitate tumor growth through the production of cytokines that influence immune responses negatively [63]–[65]. This dual role of immune system in cancer leads to a process called immunoediting, which is though to occur in three stages: elimination (immune cells mount an immune response against tumor cells); equilibrium (some tumor clone variants are not efficiently destroyed and stay in a limited growth state due to immunological mechanisms) and escape (tumors become clinically relevant when immune cells fail to recognize tumor cells, which can growth by recruting immunosuppressive mechanisms) [66].

I.2.1. Immunosurveillance

Immune cells from both innate and adaptive immune system appear to be able to recognize aberrant antigenic patterns that occur during malignant transformation and may eventually mount an inflammatory response geared towards eliminating cancer cells, a concept known as immunological surveillance, initially proposed by Burnet. According to Burnet "*in large long-lived animals (…), inheritable genetic changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy*" and "*there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character*" [67]. Indeed, several decades after Burnet's theory, an intense effort was made not only to understand in detail such mechanisms, but also to enlist them for use as a therapy. Also noted by Burnet, the lack of a competent immune system – such as in immunodeficiency states – is associated with malignancy [68]. For example, patients with a defect in humoral immunity have an increased incidence of lymphomas and epithelial tumors of the stomach, breast, bladder, cervix [69]–[71]. Similar examples can be found in mice strains that lack some components of immune system. Mice that lack both T and B cells are

more susceptible to spontaneous and carcinogen-induced carcinomas [12]. Mice with a defective FAS gene have frequent B-cell lymphomas [72]. FAS is a member of the tumor-necrosis-factor family and functions as a death receptor that helps maintaining B-cell and T-cell homeostasis [73]. Other correlations between cancer and the absence of specific immune system effectors have been extensively documented, which collectively reveal a consistent association between cancer and defective immune surveillance.

Another clinically relevant example is the association between transplantation and an increased risk of developing cancer. The discovery of immunosuppressive drugs was a breakthrough in organ transplantation as a way to prevent organ rejection by the host's immune system. However, these drugs exert a general suppression or reduction of the immune function rather than specifically suppress those mechanisms responsible for organ rejection. Therefore, there is an association between the use of immunosuppressive drugs and increased incidence of cancer in several types of transplant patients, as demonstrated by several large cohort studies [74]–[76].

The complexity of the interaction of the immune system in cancer is further demonstrated by a protective effect seen in some tumors – an association between increased infiltration of immune cells and tumor cell regression. The role of T cells (and other antitumor immune cells) in immunosurveillance has indeed been demonstrated in different mouse models of, for example, UV-induced skin cancers [77] or chemically-induced carcinomas [78]. More importantly, a correlation between T-cell tumor infiltration and better prognosis has been suggested in several types of human cancers, including ovarian [79], colorectal [80], non-small cell lung [81], breast [82], and esophageal carcinomas [83], just to name a few.

The ability of T cells to eliminate tumor cells is dependent on the recognition of tumor antigens by the TCR. Since malignancy is associated with point mutations in somatic cells that range from just 30–60 non-synonymous mutations in protein-coding genes in less immunogenic tumors to ~150 in melanomas (a highly immunogenic type of tumor), T-cell antitumor response must be capable of recognizing specifically these transformed proteins, which are displayed by tumor cells during antigen presentation. Since antigen presentation by tumor cells reflects directly their internal mutated proteome, tumor cells can present such aberrant peptides originated from mutations to T cells through MHC. Although several different events can create a tumor antigen, not all are associated with aberrant function or are crucial for tumor survival and most of them have, probably, a more passive role [84]. The number of known tumor-specific antigens that arise from mutations is small. Usually, tumor cells are weakly immunogenic since most antigens present in tumors are self-antigens (tumor-associated antigens) and few are associated specifically to malignancy [85]. Viral antigens, mutated antigens, breakpoint peptides or any *de novo* protein are interesting

targets for an anti-tumor response because are not expressed by normal cells [86]. In any case, although *de novo* mutations that are unique to tumor cells, such mutated β-catenin gene 3 or BCR-ABL, are ideal targets, most tumor antigens are associated with cancer due to an aberrant expression of non-mutated genes, such as NY-ESO-1, [87] which are normally only expressed in testes and fetal tissue. Other antigens like MART-1 [88] or gp100 [89] are also non-mutated but lineage-specific and aberrantly expressed by cancer cells. Because these are also expressed by normal cells, they are considered self-antigens by the immune system and developing T cells commonly become tolerant due to the negative thymic selection. When recognition of those antigens is artificially augmented as, for example, in clinical trials directed against MART-1^{+} tumors, although patients had 30% objective response, many developed skin rashes followed by depigmentation and vitiligo [90]. In contrast, T cells capable of recognize tumor-specific antigens have been documented and are good example of cancer immunosurveillance that is not likely to be associated with off-tumor toxic effects [91]–[93].

I.2.2.Tumor escape from T-cell recognition

Although the recognition of cancer cells by T cells is achieved through antigen presentation by tumor cells, to be effective such event must be preceded by an antigen presentation from APCs, usually dendritic cells. The crucial role of dendritic cells in immunosurveilance is dictated by their unique property as potent antigen presenting cells with strong capacity to differentiate naïve T lymphocytes into active tumor-specific immune effectors that ultimately lead to tumor rejection [94]. After naïve T cells are activated by APCs and undergo an expansion, effector T cells can then recognize the same tumor peptides presented by tumor cells. Nevertheless, as cancer cells are constantly evolving, point mutations in key genes of the MHC class I antigen presentation pathway are common and are an efficient mechanism to avoid T cell recognition [95]. Mutations in the β2-microglobulin gene, which is required for MHC class I expression on the cell surface is also commonly observed leading to downregulation of MHC/antigen presentation [96], [97]. Many other mutations in genes involved in the MHC class I antigen presentation pathway (human leucocyte antigen (HLA-A, HLA-B, or TAP1) were also found across 21 cancer types [98]. And even the complete or partial loss of chromosome 6, which harbors the MHC class I and II genes, was also observed on various tumors [99] (Figure I-3). Notably, such mutations most likely do not confer any advantage from a strictly growth perspective but may be selected as a result of selective pressure to render these cancer cells invisible to an immune system that otherwise could recognize and kill them. Nevertheless, even effects unrelated to genetic mutations can change antigen presentation. Most tumors use anaerobic glycolysis (Warburg effect), which decreases RNA and protein expression of MHC class I genes [100].

Tumor associated genes, such overexpression of epidermal growth factor receptors [101], [102], can also lead to decreased expression of antigen presenting genes. Other mechanisms that facilitate tumor escape are the absence of highly avid TCRs recognizing proteins that self-antigens, and incomplete T cell activation by tumor cells, which do not usually express co-stimulatory ligands. (Figure I-3)

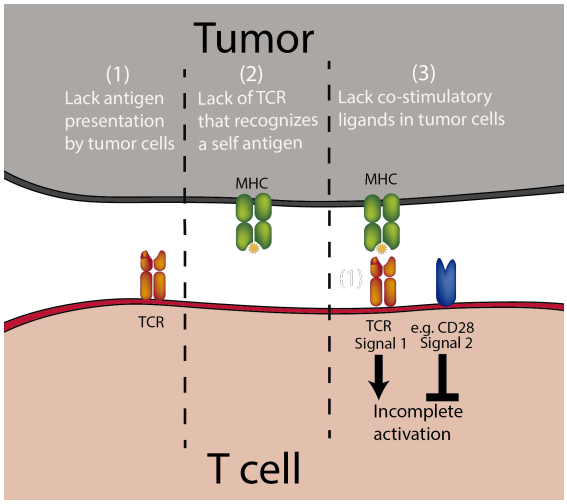

Figure I-3- Common events that can cause lack of proper activation of T cells by tumor cells

Although cancer initiation can be considered an accumulation of genetic mutations leading to an aberrant phenotype, tumor growth cannot occur, however, without its ability to escape the immune system. Even when T cell recognition of the malignancy occurs, tumor cells and its neighbor cells enlist several mechanisms to create a hostile tumor microenvironment for T cells, preventing them from effecting complete tumor elimination. Indeed, several different types of cells that, although not genetically mutated, are recruited to tumor microenvironment are of extreme importance for the tumor's biology and can dramatically change its fate. Therefore, a tumor should not be perceived as "just" a mass of malignant cells but almost as a complex "rogue" organ [103].

I.3. When the immune system goes rogue

Cancers can arise from the hematopoietic compartment (bone marrow, blood, and lymphatic system), in which case they are classified as hematological malignancies, or from any other tissue, being called solid tumors. Although the initial conceptualization of cancer understood tumors as a group of homogenous malignant clonal cells, all with a similar ability to initiate cancer, a more recent view considers that tumors are composed of a very heterogeneous population of cells, with only a small subset of cells possessing tumor-initiating abilities. These so-called cancer stem cells, because of their self-renewal properties

(similar to normal stem cells), are thought to be able to differentiate into less tumorigenic cells [104]. Tumor stem cells were initially demonstrated in the bone marrow for AML [105], but other putative cancer stem cells have been isolated from several forms of solid tumors [106], [107]. This small but important population of tumor cells is more resistant to chemotherapy and radiotherapy due to several mechanisms and is thought to be one of the main reasons for tumor recurrence after treatment [108].

It is known since the middle of 18th century that blood cells arise from the bone marrow [109]. While most blood cells have a finite life-span, a more primitive pool with continuous self-renew ability resides in the bone marrow – the hematopoietic stem cells [110]. Somatic mutations in any type of hematopoietic cell (from stem to lineage-committed cells) can lead to neoplastic transformation. Even though the proliferative capacity of hematologic malignancies does not directly depend on the level of differentiation of the originally transformed cell, it is believed that the properties of that cell define the type and characteristics of the tumor. Depending of the type of cell of origin and the clinical presentation, hematological malignancies are classified as lymphomas (Hodgkin or non-Hodgkin), leukemias, and multiple myeloma.

Leukemias present clinically with malignant cells in circulation and crowding the bone marrow. They are split into myeloid or lymphoid, according to the specific hematopoietic lineage affected. A second classification is based on their acute or chronic presentation. There is an acute and a chronic myeloid leukemia and an acute and a chronic lymphoid leukemia. Several subtypes further defined depending on the type of cell of origin and the characteristics of the malignant cells, commonly referred to as blasts.

B-cell acute lymphoblastic leukemia (ALL) is the most common type of cancer among pediatric patients (60% of all cases are in patients with less than 20 years), although it occurs also in adults [111], [112]. In these patients, tumors typically arise from a cell in the pro-B stage whose development is halted, with tumor cells retaining primitive characteristics without further differentiation [113]. Chemotherapy is the first line of therapy and, although intensive, when used in combination with radiation therapy and hematopoietic stem cell transplantation has dramatically improved overall survival. Nonetheless, long-term health problems frequently arise, particularly among children [114], [115]. For example, development of myeloid leukemia in pediatric patients in remission from B-ALL is a known long-term complication [116]. Incomplete eradication of the primary tumors can result in residual disease, leading to emergence of malignancies resistant to conventional therapies [114], [117]. Drug-resistant relapses occur in 65% and 20% of adult and pediatric patients, respectively, due to residual disease [118]. Relapses are very difficult to treat, even in children, with a cure rate of only 25-40% [119]. Apart from secondary cancers, organ impairment is also a long-term side-effect that can be observed in patients due to aggressive

induction treatments. If a similar malignant transformation occurs in T-cells, a T cell acute lymphoblastic leukemia (T-ALL) occurs instead, which accounts for less than 25% of all ALL, and is usually associated with worse prognosis relative to B-ALL [120]. Like in B-ALL, residual disease is a main reason for relapses. In contrast to the experience with B-ALL, so far there is no adoptive cell therapy for T-ALL.

Chronic lymphocytic leukemia (CLL) occurs at later time in life and is the most common type of leukemia in western countries. It is thought to originate from activated or memory B cells. CLL progresses slowly and is less aggressive than ALL, although also potentially deadly [121]. Few therapies are truly curative, but combination of the chemotherapy, monoclonal antibody therapy, and stem cell transplant can extend survival several years [122].

Acute myeloid leukemia (AML) results in accumulation of abnormal cells that fail to differentiate into mature granulocytes or monocytes. Although circulating leukemic blasts in blood have limited proliferation ability, a small population of self-renewing, tumor initiating cells is responsible for maintaining the continuous growth of the neoplasm [123]. Although chemotherapy, radiation and hematopoietic stem cell transplantation have been the standard therapies, 5-year overall survival from AML still remains poor over the last three decades, particularly in adults and in the elderly, who account for 80% of cases [124]. Although the majority of the patients under 60 years reach complete remission after intense chemotherapy [125], relapses are common and only 20-40% of adult patients have disease-free survival greater than 5 years after chemotherapy. Advances in AML treatment have improved the outcome in young patients but have not substantially improved the prognosis in the elderly [126]. With the approved treatments used today, 70% of patients with more than 65 years die within 1 year of diagnosis [127].

Chronic myeloid leukemia (CML) is associated with a unique chromosomal rearrangement that leads to the emergence of an aberrant chromosome, the Philadelphia chromosome [128]. Since an abnormal kinase encoded by a BCR-ABL fusion gene is a signature feature in these patients, specific tyrosine kinase inhibitors were a breakthrough therapy not only in CML treatment, but also introduced a new era of genomic-driven therapies for cancer. Nonetheless, CML is an incurable form of cancer since treatment must be given continuously for several years, and its discontinuation almost always results in a rapid reappearance of large numbers of leukemic cells. Moreover, even with treatment, 10–20% of patients in early chronic phase and up to 40% with advanced phase disease will fail to achieve an initial response [129].

Lymphomas are more common that leukemias and comprise a heterogeneous group of hematologic tumors, with more than 35 subtypes, that arise from lymphocytes residing in lymph nodes or the thymus, although they can also develop in extranodal tissues. The cells

involved in the malignant transformation can be either B cells or T cells in varying stages of differentiation, but 90% of the lymphomas are of B-cell origin [130] Lymphomas differ from leukemias due to lack or minimal evidence of peripheral blood or bone marrow involvement (<25% blasts). While several subtypes are curable, relapsed forms have poor prognosis [131].

Multiple myeloma results from the expansion of dysfunctional plasma cells (activated B cell) in the bone marrow. Due to increased number of plasma cells, patients with myeloma have high levels of monoclonal immunoglobulins. Multiple myeloma is associated with hypercalcemia, renal failure, anemia and bone lesions [132]. Immunosuppressive and immunomodulatory drugs, proteasome inhibitors, and antibodies such as daratumumab (anti-CD38) are among the most active therapeutic options [133], [134]. However, despite progress in treatment, this form of cancer is still incurable owing to the development of drug resistance clones.

The cornerstone of treatment for hematological malignancies consists of intensive chemotherapy, which in some patients may be followed by stem cell transplantation. Better diagnostic tools have demonstrated that a small number of cancer cells can still frequently be detected in the bone marrow of patients that are considered to be in remission. This minimal residual disease (MRD) state often precedes leukemia relapses, and there are no fully effective therapeutic modalities to eliminate it. Relapses can lead to the use of hematopoietic stem cell transplantation (HSCT) [135], which can be curative for some patients but is often quite toxic. HSCT can also be used as a first-line therapy usually alongside with chemotherapy for patients with high-risk disease [136]. However no effective conventional treatment options exist for recipients who relapse after HSCT. As an example, the 5-year overall survival for adults with ALL who relapse after stem cell transplantation is less than 20% [137].

For several types of hematological malignancies, standard treatments, apart from not being very effective, are also not cell specific and can cause many side effects, demonstrating the need for new therapeutic modalities. Recently, the focus of cancer treatment has shifted toward targeted therapies, including immunotherapy, which allow better individualization of care and are hoped to increase the probability of success for patients.

I.4. Immunotherapy

In the last decades, accumulating preclinical and clinical data using many different forms of immunotherapy, ranging from antibodies and cancer vaccines to adoptive cell

therapy, suggest that these approaches have the potential to treat cancer. William Coley was the first to propose and test the concept of using the immune system to treat cancer patients, in the 19th century [138]. While observing that tumors could shrink in patients who developed bacterial infections, he hypothesized that the type of immune response against these infections could also be directed against tumors. To test this, Coley formulated a streptococcus derived vaccine (a preparation known today as Coley's toxins) that he administered to cancer patients, some of whom experienced remission of their sarcomas. Although such procedure is no longer used, its principle is still being exploited. For example, intravesical instillation of Bacillus Calmette-Guérin (BCG) is used to treat bladder cancer [139]. However, the focus of immunotherapy field is to induce a specific immune response against tumor antigens. Although there have been developed several diiferent types of immunotherapy immune checkpoint inhibitors, monoclonal antibodies and more recently adoptive cell therapy demonstrated more impressive results in clinical trials.

Monoclonal antibodies are probably the most common and widespread form of immunotherapy. Antibodies with specificity to tumor-associated antigens can be injected in patients to redirect the immune system against tumor cells. Nonetheless, the use of monoclonal antibodies has some drawbacks. Low, heterogenous expression of the targeted antigen or binding of the antibody in a suboptimal fashion can lead to failed recruitment of effector cells to kill opsonized cells. A second limitation is their limited half-life and, more importantly, physical barriers such endothelial cells and extracellular matrix can prevent adequate biodistribution to different tumor sites [140], [141]. This limitation seems to be more relevant in solid tumors, where a very small fraction of the intravenously infused antibody (<0.1% per gram of tissue) can be detected within the tumor [142]. In contrast, T cells and other leukocytes can, when activated, migrate into tumors due to their ability to extravasate through epithelial barriers [143] following a chemokine gradient [144] and reach the tumor sites, where they can mount an anti-tumor response. The detection of virus-specific T cells infused in patients with Epstein-Barr-virus-related post-transplant lymphomas for 9 years after adoptive transfer [145] demonstrates that, after injection, tumor-specific T cells can be useful for several months or years, as opposed to the half-lives of antibodies of just a few days.

I.4.1. Adoptive Cell Therapy

The first form of adoptive cell therapy still being used today with extreme success is hematopoietic stem transplantation. Although initially unappreciated, T cells co-transplanted with stem cells are, in some cases, able to recognize antigens present in leukemic cells and thus elicit a graft versus tumor effect , which together with the high dose chemotherapy used as conditioning regimen is one of the main reasons for its success as a cancer treatment.

Since 1965, when the first patient with ALL was successfully treated with this form of cell therapy [146] that allogeneic stem cell transplantation has been perfected and is, in some instances, the last possible curative option for several hematologic malignancies. However, the toxicity that this type of transplantation carries, including the risk for severe graft versus host disease (GVHD) and opportunistic infections, has provided the impetus to push the immunotherapeutic field towards even more specific approaches.

The recognition of the anti-tumor role of T cells co-infused with stem cells in hematopoietic stem cell transplants [147] together with data obtained in experimental therapeutic approaches, has put these cells in the spotlight of cellular immunotherapy. Adoptive T-cell therapy is a form of immunotherapy that involves the isolation of lymphocytes with the intent to stimulate and expand ex vivo potent antigen-specific T cells that are subsequently infused into a patient to treat a disease. This form of immunotherapy has become very attractive due to its high specificity and the possibility of long-term protection. Adoptively transferred T cells are a “living” drug that has potential advantages over conventional therapies: T cells recognize tumor antigens through the TCR allowing them to mount a strong specific immune response potentially capable of eliminating tumors in a short period of time and, in addition, are able to proliferate and survive in vivo for several month or even years [148].

Since the presence of tumor infiltrating lymphocytes (TILs) has been associated with a better prognosis [149], [150], one of the first attempts to induce a specific and controlled antitumor response using T cells was the isolation and in vitro expansion of these lymphocytes naturally present in tumors [151]. The rationale for this approach was that T cells isolated from tumor biopsies should have been naturally “selected” to recognize tumor antigens through their TCRs. Since the TCR mediates antigen-specific recognition and activation of T cells, expansion of TILs allowed the reinjection into the patient of a high number of potentially tumor-reactive lymphocytes. TILs were initially isolated from transplantable mouse sarcomas and cultured in high levels of IL-2. These lymphocytes demonstrated specific lytic activity against tumor cells in vitro and also mediated tumor regression when transferred into tumor-bearing mice [152]. Human TILs were first tested in melanoma [153], with tumor regressions being demonstrated in some patients. Although clinical trials using TILs have been tested mostly in melanoma patients [153]–[155], some trials also explored this therapeutic approach in renal cell carcinoma [156] and glioma [157].

The isolation of TILs can be problematic, as seen since the first clinical trials, not only in patients affected by melanoma but especially other tumor types. Indeed, not all patients have tumors that can be resected. But even in those with resectable tumors, not all can be used to expand lymphocytes. Melanoma lesions, due to increased immunogenicity and relative ease of for T cell collection, seem particularly suited for this approach. Nonetheless,

even after lymphocyte collection and expansion in vitro not all TILs have anti-tumor effect after re-injection into the patient; moreover, patients may not survive the 2-3 months that are usually required to expand enough cells to induce an anti-tumor response.

Although the process is laborious and time consuming, recent refinements in methods for isolation and growing of TILs have resulted in the generation of autologous tumor-reactive lymphocytes in nearly 80% of patients (36 patients) in a recent trial [158]. The success of this form of immunotherapy has been limited. Despite near 30-50% objective clinical responses in some human trials [159], [160] some are transient and there is limited persistence of the transferred cells. More recent clinical trials employing this form of adoptive cell therapy have demonstrated an objective clinical response of up to 70% [161], [162]. Use of preconditioning regimens with lymphocyte-depleting chemotherapy or whole body irradiation seems to be one of the reason for this improved efficacy [163]. One of the most exciting results using TILs was recently published in a report from an ongoing phase 2 clinical trial demonstrating a durable (2 years) complete remission of a patient with chemorefractory late-stage breast cancer after several multiple failed treatments using conventional therapies [164]. Although slow, continuous research and development of TILs as a therapy has reached an international phase 3 randomized human trial treating patients with metastatic melanoma (NCT02278887), and biotech and pharmaceutical companies like Iovance Biotherapeutics are now interested in this technology for commercial use.

The limitations observed in the first clinical trials using TILs led to the search for new forms of adoptive T-cell therapy using lymphocytes that, although collected from easier but tumor-unrelated sources such as peripheral blood, could be redirected in vitro to recognize tumor antigens. The observation that TILs from different patients show high responses against MAGE, MART-1 or gp100 [165], [166], together with advances in genetic engineering tools, led to the development of TCR-transduced T cells. When the individual TCRs responsible for tumor recognition are known, T cells can be engineered to express those TCRs, making T-cell therapy more widely applicable. T cells expressing transgenic TCRs are usually autologous T cells collected from peripheral blood and modified with a gene encoding a TCR specific for an antigen present in certain cancers (mostly tumor associated antigens) such as ErbB2/HER2 [167], MAGE [168], NY-ESO-1 [169], gp100 and MART-1 [90]. Using this approach, T cells can in theory be redirected to any type of tumor with the appropriate TCR, provided an adequate tumor antigen exists, and expanded in vitro. For instance, T cells expressing a TCR that recognizes MART-1, a melanoma associated antigen, demonstrated tumor regression in some patients and T-cell persistence for at least 1 year [168]. Other clinical trials for other tumors besides melanoma were conducted using for example NY-ESO-1 TCR-engineered T cells in patients with synovial cell sarcoma with 61% objective responses in 18 patients [170]. Although much easier to produce than TILs,

manufacture of transgenic-TCR engineered T cells is still complex and their clinical use has several limitations.

First, because the endogenous (native) TCR continues to be expressed by the T cells and because TCRs are composed of 2 independent polypeptide chains, mispairing between endogenous and transgenic TCR chains can occur and lead to failure of antigen recognition or, worse, unwanted reactivity against non-tumor antigens. Indeed, lethal GvHD has been already documented in mice due to the formation of self-antigen-reactive mixed TCR dimers between the endogenous and introduced TCRs [171]. The GvHD was manifested as cachexia, anemia, loss of hematopoietic reconstitution, pancreatitis, and colitis, leading to death. However, it is worth to mention that this toxicity was never observed in human trials. A potential solution for this problem is the creation of hybrid TCR genes where the human constant region is replaced by its murine counterpart to prevent undesired pairing of endogenous and introduced TCRs. This has already been tested [172], although the immunogenicity of transferred cells may increase due to the presence of mouse sequences. Another solution is the knockout of endogenous TCR α or β genes using any gene editing tool available.

Second, antigen recognition by the TCR requires that a portion of that antigen be presented by a specific major histocompatibility complex (MHC) molecule that is only present in individuals that share the same human leucocyte antigen (HLA) type; thus, a particular T cell product can only be used in a select group of patients.

Third, as stated before, downregulation of MHC in tumor cells is a commonly observed mechanism of immune evasion, so malignant cells can rapidly become invisible to the TCR-engineered T cells. Therefore, the efficacy of adoptively transferred tumor-reactive T cells can decrease upon the loss of antigen expression by malignant cells, which occurs frequently in response to the selective pressure of therapy itself.

Finally, sole stimulation through the TCR is not enough for full activation of T cells, which requires not only the engagement of the TCR complex (signal 1) but also a second stimulatory signal (signal 2) arising from a co-stimulatory receptor (e.g. CD28, CD80, CD86 or 4-1BB) engaging with its ligand [173]. While professional antigen presenting cells express a plethora of these co-stimulatory ligands, tumor cells do not. Therefore, transgenic-TCR engineered T cells may fail to provide antitumor effect due to the lack of required molecules (MHC and/or co-stimulatory ligands) on the surface of tumor cells [174], [175].

I.4.2. Chimeric antigen receptors

Adoptive cell therapy has recently entered a new phase in which genetic transfer of tumor-specific receptors into T cells can convert them into effective cancer cell killers. Some of the limitations seen with earlier types of adoptive T cell therapy can be overcome by

redirecting T-cell activity towards any antigen using a recognition system that relies on the antigen binding abilities of an antibody molecule. A chimeric antigen receptor (CAR) is a synthetic molecule that fuses an extracellular, antibody-derived antigen recognition domain with an intracellular TCR/CD3-derived, activating domain [151] (this chimeric molecule was initially called a T-body – part T-cell receptor, part antibody) (Figure I-4). The extracellular binding moiety provides the antigen specificity and is commonly a single-chain fragment variable (scFv) originated from a monoclonal antibody. The use of scFvs takes advantage of the high specificity of antibodies. Another benefit of using of scFvs is that several therapeutic monoclonal antibodies for tumor-associated molecules already exist and have been tested in the clinic. Separating the scFv and the endodomain, a CAR has usually a spacer domain, which provides flexibility and optimizes T cell and target cell engagement, and a transmembrane domain.

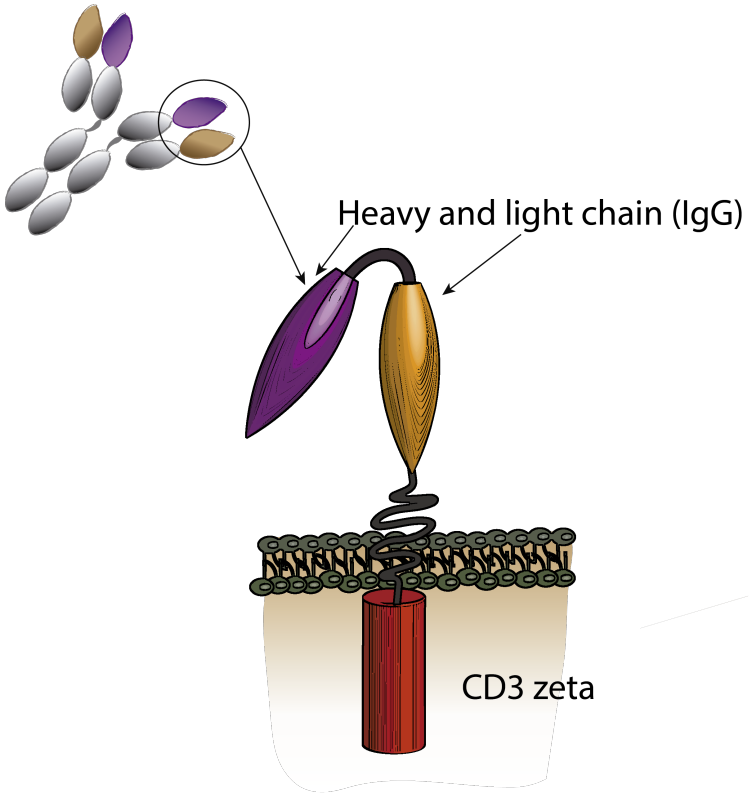

Figure I-4- Schematic representation of a Chimeric Antigen Receptor (CAR). Most CARs use an extracellular antigen recognition domain (here represented by an antibody-derived single-chain variable fragment, scFv). To activate T cells a CAR must have the CD3 subunit zeta from TCR/CD3 complex.

This concept was developed in the 1980's, when several groups designed a synthetic receptor fusing an scFv and the endodomain of TCR/CD3, more specifically the CD3 zeta endodomain [151], [176]. After demonstrating in vitro that T cells can be engineered with a primitive form of a CAR, Gross, Waks, and Eshhar wrote: "*chimeric T cell receptors with anti-tumor specificity will enable testing of the feasibility of this approach in combating*

human tumors." A few decades later, clinical trials proved the feasibility of this approach for the first time.

When T cells are engineered to express a CAR, after binding the target antigen via the scFv, they get activated through the signaling components included in the CAR. Since CARs provide MHC-independent antigen recognition, some of the mechanisms used by tumor cells for immune escape, such as downregulation of MHC molecules, can be mitigated; in addition, a CAR specific for a certain antigen could be used in patients regardless of their specific HLA type (a common limitation of other engineered T cells). Another advantage of CAR-mediated antigen recognition is that antigens other than proteins, such as carbohydrates and lipids, can be recognized [177], [178].

Nonetheless, CAR-T cells have some drawbacks. Whereas traditional T cell responses through TCR and MHC interaction can recognize intracellular proteins (which account for more than 90% of potential tumor-associated antigens) that are processed and presented by the MHC molecule, binding of the CAR is limited to molecules that are present on the surface of tumor cells. This is actually not completely accurate since scFvs have been developed that can recognize intracellular peptides in the context of HLA molecule, making them targetable by antibodies and antibody derived immunotherapies like CARs. However, such scFvs are so far uncommon. For example, a CAR against Wilms Tumor 1 (WT1) using TCR-mimic (TCRm) antibody has been created and shown to be able to lyse tumor cells [179]. This recognition is in the context of HLA-A*02:01 and thus not MHC-independent, in contrast to most other CARs. Nonetheless, in spite of being unable to target intracellular proteins, since in principle any cell surface molecule can be targeted, conventional CARs can expand the repertoire of T-cells reactivity and override tolerance to self-antigens.

Another potential disadvantage of current CARs is that, because scFv sequences are usually derived from murine proteins, humoral and cellular immune responses can occur against this portion of the molecule and lead to elimination of CAR-T cells from circulation [180]. This problem, which has been previously documented in some patients, may be minimized by using scFv whose amino acid sequences have been fully humanized [181].

The first clinical trials with T cells expressing CARs targeted folate receptor for ovarian cancer patients [182], carbonic anhydrase IX (CAIX) in patients with renal cancer [183] and CD171/L1-CAM in pediatric patients with neuroblastoma [184] and were associated with lack of T-cell persistence and minimal to no antitumor effect. These early CARs contained only one activating domain (usually a portion of the ζ chain in the TCR/CD3 complex) and thus emulated only TCR activation (signal 1). A similar lack of antitumor effect was observed in clinical trials using CARs for hematologic tumors targeting CD20 [185]. Despite the use of lymphodepletion to deplete endogenous anti-inflammatory regulatory T cells and in some cases concomitant IL-2 administration, T cell persistence was no longer than a few weeks in

circulation in some patients. This first generation of CARs, even though capable of activating T cells to eliminate tumor cells in vitro, failed to induce a strong anti-tumor response in vivo, probably due to lack of long-term T-cell activation and expansion. Although able to initiate phospatidyl-inositol and tyrosine kinase pathways and calcium influx similar to TCR/CD3 activation [186]–[188], first generation CARs are prone to undergo anergy, as demonstrated in mouse models [189]. This seems to be due to defective cytokine production, such as IL-2, and lack of proper T cell expansion after continuous exposure to the same antigen. Since tumors do not generally express ligands to activate costimulatory receptors, addition of costimulatory domain to the CAR molecule increases the strength of the signal and leads to higher T cell persistence when compared with first generation CARs in human trials [190]. The costimulatory domains most commonly included are CD28, 4-1BB (CD137) or OX-40 (Figure I-5). These second (with one additional activating domain) and third (with two or more additional domains) generation CARs are associated with increased CAR-T cell proliferation and cytokine production both in preclinical models and in clinical trials [190]–[193]. After infusion, CAR-T cells with co-stimulatory domains can expand by 100- to 1000-fold [194].

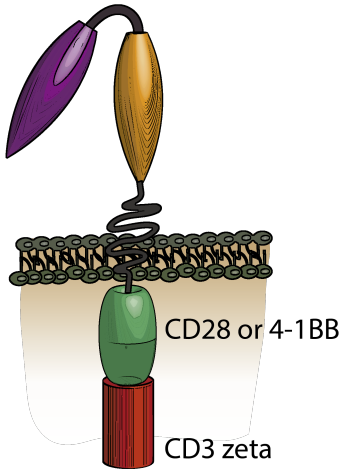

Figure I-5- Improved CAR design with two endodomains to provide signal 1 and signal 2 (second generation) and with one extracellular domain used in most successful clinical trials.

So far, clinical trials using second generation CAR-T cells to treat patients with B-cell malignancies have demonstrated the most exciting results. While CAR-T cells can in theory be redirected against any surface-expressed target of choice, most clinical trials so far have been directed at CD19, an antigen that is expressed in B-cell malignancies [195], [196]. CD19 is a transmembrane glycoprotein which is expressed on cells of the B-cell lineage, from early pro-B-cells in the bone marrow to mature B-cells in blood and tissues, but not on hematopoietic stem cells. CD19 increases pre-B cell receptor signaling thereby promoting

the proliferation and differentiation of late pro-B cells [197], [198]. CD19 is a good target since, although is not tumor specific it is shared between tumor, and non-crucial cells (B cells only). Individuals with congenital absence of B cells have been described and can live normal lives if prophylactic infusions of gamma-globulin are accessible [199].

In 2010, the first reports of clinical trials using CD19 as a target were released. Steve Rosenberg's group published initial results using a second generation (CD28) CAR, updated in 2012, describing 6/8 patients experiencing clinical remission and 4/8 developing long term B-cell aplasia [200], [201]. In 2011, Carl June and colleagues from the University of Pennsylvania published the outcome of three patients with chronic lymphocytic leukemia (CLL) treated with CD19 CAR-T cells containing 4-1BB as second endodomain, and demonstrated for the first time a complete remission in this disease [202]. It was the beginning of several phase 1 and 2 clinical trials in which an increasing number of patients was infused with CD19 CAR-T cells. These have consistently shown high antitumor efficacy, with complete response rates of 70–90% in pediatric and adult patients with relapsed or refractory, chemotherapy-resistant B-cell malignancies [181], [203]–[206].

It is difficult to accurately calculate the number of recipients of these cells, but is estimated that a few hundred patients have received some form of CAR-T cells. These numbers will dramatically increase now that these encouraging results attracted the interest of pharmaceutical and biotech companies and resulted in the approval of CD19 CAR-T cell products by the FDA for commercial use outside clinical trials. More companies are likely to see their products approved in the next years. Different institutes have different approaches, which makes the comparison and attempts at findinding the best protocol impracticable. However, all clinical data clearly demonstrates that patients should receive lymphodepletion, at least two activating endodomains are required in the CAR construct, patients often develop significant side effects such as the cytokine release syndrome, and there are no clear correlations between the number of infused T cells and the final outcome as well as between initial disease burden and the likelihood of the response [207].

Meanwhile, the success of CD19 CAR-T cells started paving the way for clinical trials targeting other diseases, both hematological malignancies (such as using BCMA [208] for Multiple Myeloma; CD20 [209] for B-cell malignancies; CD33 [210] or CD123 [211] both for acute myeloid leukemia) and solid tumors (such EGFRvIII [212] for gliobastoma/glioma; GD2 [177] for neuroblastoma; PSMA [213] for prostate cancer; and HER2 [214] for several types of cancer such as breast, ovarian, lung cancers and gliobastoma). Targeting solid tumors with CAR-T cells has shown less encouraging data so far. For example in a clinical trial conducted at Baylor College of Medicine using a GD2 CAR (first generation), 3 out of 13 pediatric patients with neuroblastoma achieved complete remission [177], demonstrating the

feasibility of using this technology against such tumors. Clinical trials using second and third generation CAR-T cells against solid tumors are under way.

I.4.2.1. Clinical data beyond CD19 CAR

Although CD19 is not expressed by plasma cells, CD19 CAR-T cells are also being used to treat multiple myeloma and an initial report demonstrated a complete response in one patient [215]. However, for this type of tumor, CD138 and BCMA seem to be the most promising targets. Initial reports of the use of CD138 CAR-T cells demonstrated 4 out of 5 patients achieving stable disease [216]. Because BCMA is only expressed by plasma cells, both malignant and normal, it is considered a good target for multiple myeloma. Clinical trials using CAR-T cells to target BCMA have shown very promising results, with 100% overall response rate at higher T cell doses and 89% in all eligible patients treated [217].

Although CD20 CAR-T cells could in theory also be used to treat some cases of Hodgkin lymphoma, because of the almost universal expression of CD30 in Hodgkin lymphoma cells, this target has also been explored in CAR-T cell therapy. Results from a phase 1 dose escalation study of CD30 CAR-T cell trial for refractory/relapsed EBV-negative CD30+ lymphoma were recently released and a few more clinical trials have now started. No toxicities were observed and in 7 treated relapsed HL and 2 T-cell lymphoma patients, two entered complete remission (one with HL and one with T-cell lymphoma), and 3 achieved stable disease [218]. The authors mention that, although CD30 is expressed by endogenous T cells, no impaired virus-specific immunity was detected. Expression of the target molecule in T cells could lead not only to elimination of endogenous T cells but also to self-elimination of CAR-T cells during their ex vivo coculture, since they do not discriminate between normal and malignant cells, a phenomenon called fratricide. Notably, this did not occur. Interestingly, similar results were seen in a preclinical study using CD5-specific CAR-T cells, being developed for T-cell malignancies [219]. In these case, the authors reported transient toxicity due to fratricide immediately after the CD5 CAR was introduced but, later, due to complete downregulation of CD5 in the transduced cells, they grew well, and anti-tumor effect was observed in a xenograft mouse model. Results from a CD5 CAR-T cells clinical trial that was recently launched (NCT03081910) will inform if these cells have a low toxic profile similar to that observed in the CD30 CAR trials, or if endogenous T cells will be indiscriminately targeted leading to T-cell aplasia. Regardless, extending the success of CAR-T cells to T-cell malignancies can be a challenge due to shared target antigens expressed by malignant and normal T cells, which may preclude their normal expansion and limit their anti-tumor effect. This problem will be discussed in Chapters II and III.

Other types of hematologic cancers can be more difficult to target, especially myeloid malignances due to their complexity and variability of phenotype and cell of origin of

malignancy. Clinical trials of CAR-T cells targeting CD33, CD123 and Lewis Y antigen are in progress. CD33 is a target that, although expressed by malignant cells, is also found in hematopoietic stem cells, leading to potential prolonged cytopenias and delayed hematologic recovery [220]. Targeting of CD33 can also cause liver toxicity since is also expressed in Kupffer cells in this organ [221]. Because of the toxicity observed in the first report using CD33 CAR-T cells, their use is now being proposed as a tumor debulking strategy before hematopoietic stem cell transplantation [220]. CD123 is also expressed in malignant myeloid cells as well in normal myeloid cells and endothelial cells. Toxicity using CD123 is not yet fully established although some initial reports show safe profile and anti-tumor effect [211], [222]. Interestingly, both therapeutic monoclonal antibodies targeting CD33 and CD123 demonstrate a favorable safety profile [223], [224]. Lewis Y antigen is expressed on a portion of AML cells but also on gastrointestinal and pancreatic cells. Initial studies demonstrated migration of Lewis Y CAR-T cells to the bone marrow and some observed antitumor effects, but all four patients ultimately relapsed [178]. Although safe, the authors concluded that single therapeutic targeting of Lewis Y is not ideal since it is only expressed by a proportion of AML blasts. Development of a novel CAR for AML will be the subject of chapter IV.

I.4.2.2. CAR design

Despite encouraging clinical results with CD19 CAR-T cells, there is considerable heterogeneity in the structure of CARs [225] used across trials in different institutions. Yet, it is unclear whether one type of CAR has advantages over another, even when targeting the same antigen. CARs have been designed empirically; distinct backbones, types and number of signaling endodomains, and methods of CAR delivery into T cells likely affect antitumor activity. For instance, the spatial interaction between a cancer cell and a T cell depends on the exact location of the epitope being recognized on the target molecule and the structure of the extracellular domain of the CAR. Differences in T cell physiology and activation owing to the use of distinct scFvs recognizing the same antigen, different co-stimulatory endodomains [226] or different lengths and sequences of the CAR extracellular domain [227] have been documented.

Little is known about the best properties or best way to choose an scFv. The position of the epitope or the distance to the cell surface are important aspects that will influence CAR binding and formation of an immune synapse [228]. It has been demonstrated that a CD22 CAR built with an scFv that recognizes an epitope proximal to cell surface has higher anti-tumor effect than with an scFv that binds to a membrane-distal epitope [229]. Although data about the best characteristics are scarce, it has also been demonstrated that a higher affinity scFv in a CAR against ROR1 conferred superior antitumor effect than an scFv

against the same molecule but with lower affinity [230]. However, such observations cannot be construed as a rule applying to all CARs.

Although most of the CARs created so far use an scFv as exodomain, other molecules can be used as ligands/receptors that can interact with compatible molecules in tumor cells, such as IL13Rα2 [231] interleukin-13 receptor subunit alpha 2 that can be bound by a modified form of IL13 expressed on T cells, or NKG2D, a NK receptor that when fused with CD3ζ allows activation of T cells by ligands overexpressed by tumor cells [232]. The search for new and better targets is under intense research, which may lead to new CAR designs that may require new exodomains beyond scFvs and antibody-related structures. Because these alternative exodomains are usually derived from human sequences, they avoid production of anti-idiotype antibodies against the CAR. However, their reduced ability to modulate affinity, avidity and synapse formation such as that observed scFv-containing CARs may hinder further optimization. Nonetheless, it is indisputable than new targets and CARs will emerge in the next years.

Spacer sequences (the part of CAR molecules that links the scFv and the transmembrane domain) vary in length and composition and can be derived from IgG, CD28 or CD8α genes, among others. This part of the CAR was until recently considered inert or less important, but increasing preclinical and clinical data suggest it is an important aspect of CAR design. The importance of the spacer characteristics in CAR design was recently demonstrated by several groups who studied the effector function of CAR-T cells [233]–[235]. Comparison of different CARs (CEA, NCAM, 5T4 and CD19) using different spacer sizes (derived from the IgG1 sequence) demonstrated interesting, albeit conflicting, results. While 5T4 and CD19 CAR showed stronger anti-tumor effect with a longer spacer, CEA and NCAM demonstrated to be superior when no spacer was used [236]. A similar result comparing three different lengths of spacers derived from the IgG1 sequence demonstrated that ROR1 CAR-T cells have superior antitumor effect when the shortest sequence is used in the CAR [230]. The reason for this discrepancy seems to be related to the specific properties of the scFv, targeted epitope and spacer, and of their interaction during formation of an immune synapse. While it is conceivable that there is an optimal distance and angle of interaction between CAR-T cells and target cells for proper T cell activation, these properties are often impossible to predict. So far, due to lack of enough data, an algorithm or even some general predictable rules have not emerged and construction of an optimized CAR has been done empirically.

Which co-stimulatory endodomain is the best is also still unclear and it is possible that different domains need to be used in different clinical contexts. In vitro and preclinical data have failed so far to predict the best endodomain. The most successful CARs used in the clinic rely on CD28 or 4-1BB as co-stimulatory domains. CD28 is expressed in 95% of

human $CD4^+$ T cells and 50% of $CD8^+$ T cells and is a member of the immunoglobulin super family of co-stimulatory and inhibitory receptors, which also includes among others ICOS, which is used in some CAR constructs as well [237]. CD28 is recruited to the immune synapse that is formed between T cells and APCs after pepMHC recognition by the TCR. The main function of CD28 is to augment TCR signaling [238], [239], resulting in increased cytokine production [238], [240], clonal proliferation [241], differentiation and survival [242]. Activation of CD28 leads to activation of (PI3K)–AKT pathway, which is important for cell proliferation, as well as the activation of the kinases PKCθ, LCK and RAS [243]. LCK and PKCθ recruitment induces Ca^{2+} influx and activation of the transcription factor NFAT, resulting in upregulation of T-bet, a transcription factor that induces cytokine production [240].

4-1BB belongs to a different family of receptors that are part of the TNF receptor superfamily, together with OX40, CD27, and CD30 among others [244]. 4-1BB is expressed only in activated T cells and its expression is transient [245]. When activated, 4-1BB enhances T cell proliferation, cell cycle progression, cytokine secretion and cytolytic potential as well as prevention of clonal deletion and activation-induced cell death [246], [247]. Like CD28, it enhances TCR signaling, in this case through tyrosine phosphorylation of the adaptor protein SLP76, the TCR signaling subunits CD3ε, CD3ζ and the tyrosine kinase LCK, as well as through the recruitment of PKCθ and an increase in intracellular Ca^{2+} levels [246]. Additionally, it enhances the NF-κB pathway [248]. TRAF2 binds to 4-1BB endodomain and is essential for the activation of p38 mitogen-activated protein kinase (p38 MAPK) and induction of NF-κB-dependent activation of the anti-apoptotic mediators BCL-X_L and BFL-1. Interestingly, although 4-1BB stimulation can rescue T cells from anergy and exhaustion, early activation after viral infection can have a detrimental effect by induction of activation-induced cell death through prolonged upregulation of TNF and FAS [249]. A similar deleterious effect was observed in a CD19 CAR containing 4-1BB, and its optimization will be described in chapter V.

Despite clear evidence of the activating effect of both endodomains in T-cell biology, it would be a mistake to directly extrapolate the physiological functions of these natural receptors to CARs. Indeed, when CARs employing CD28, 4-1BB or combinations thereof were compared, the outcome depended of the type of tumor and target, and the mouse strains that were used [192], [193]. Nonetheless, there are clinically relevant differences between CARs containing either co-stimulatory domain. While both endodomains augment cytokine secretion, mostly of T_H1 type (such as IL-2, IFNγ, TNF, and GM-CSF), CD28 elicits higher IL-2 production [250], [251]. Preclinical and clinical data with CD19 CAR suggest that, while anti-tumor effects of CD19 CARs bearing CD28 or 4-1BB are similar, CD19 CAR-T

cells with 4-1BB accumulate over time, possibly even by antigen-independent activation [192]. Although inclusion of a secondary domain increases T cell survival in general, CD28 induces stronger effector functions whereas 4-1BB directs greater longevity. A lower level of telomerase activity after activation of CD28 CARs may be responsible for lower survival when compared with CARs with 4-1BB [192], [252]. Thus, CARs with CD28 can be used when long persistence of T cells is not be desired, and 4-1BB can be a preferable choice if side effects are manageable. However, which endodomain should be used in a given CAR may need to be decided by performing human trials. Clinical trials comparing the anti-tumor effect and fate of two CAR-T cell populations bearing different endodomains that are injected simultaneously in the same patient are now being reported but more data are required [190].

Although CARs with 3 endodomains (CD3ζ plus two co-stimulatory endodomains) are also possible to generate and already tested, an increased anti-tumor effect compared with second generation CARs was not clearly demonstrated, despite a suggestion of potential synergistic effect [253]–[255]. Preliminary data from a clinical trial comparing 2nd and 3rd generation CD19 CAR-T cells demonstrated increased persistence of the 3rd generation, although this may be due to the inclusion of 4-1BB specifically and not just to the presence of an extra endodomain [256]. Superiority of 3rd versus 2nd generation CAR-T cells was, however, never clearly demonstrated. Nevertheless, it is clear that the success of CAR-T cells in the clinic is intimately correlated with the ability of one molecule to induce at least two independent but related signal pathways for full T cell activation [257]

In summary, although the backbone of the CAR and the number and type of its endodomains are an absolute determinant of the overall antitumor effect observed in patients, their optimization is currently a laborious and trial-and-error procedure, with multiple iterations of “bench to bedside and back again” approaches. This process makes translation to the clinic difficult and time consuming. It is clear, however, that the CAR design process does not follow a “one size fits all” model and that each CAR must be designed, optimized, and tested for the specific target molecule in order to generate the ideal CAR for a particular tumor

I.4.2.3. CAR-T cells generation

After a CAR is designed, its gene needs to be permanently introduced into T cells. The most commonly used vectors for this purpose are replication-defective retroviruses of two types: γ-retrovirus and lentivirus. Both have an RNA genome that is reverse-transcribed into DNA after T cell transduction, which then gets integrated into the host genome. Despite the lack of any documented tumorigenic event caused by viral transduction in T cells [258], [259], because this has been observed in engineered hematopoietic stem cells used in clinical gene therapy trials for X-linked severe combined immune deficiency [260], chronic

granulomatous disease [261], and Wiskott–Aldrich syndrome [262], the concern for this has not completely vanished. Partial deletion of the U3 region of the 3 long terminal repeat (3' LTR) combined with the use of any other promoter to replace the truncated viral promoter (5' LTR) has been introduced in lentiviral vectors to increase safety. Although this approach can also be explored in γ-retrovirus [263], so far the original retroviral 3' LTR it is still used as a promoter in clinical trials, even despite the apparent worse safety profile of γ-retrovirus when compared to lentivirus [264]. Factors such as the transgene and its promoter, and the cell type being transduced seem to influence the risk of mutagenesis. Another disadvantage of using viral vectors is their high price compared with other methods.

Instead of viral vectors, which require extensive and expensive biosafety testing, transposon/transposase systems, which are usually delivered into T cells by electroporation with plasmids, can be used [265]. These systems have lower costs, simpler manufacturing procedures, and are potentially safer since insertions have a nearly random distribution [266] While viral vectors prefer highly expressing regions of the genome, non-viral vectors like Sleeping Beauty can integrate outside the intragenic regions of the genome avoiding interference with endogenous gene expression of modified cells [267]. However, these systems have disadvantages, including lower transduction efficiency, which may require prolonged periods of CAR-T cell expansion (sometimes twice the expansion time of viral vectors) that may potentially reduce their potential to expand and persist, and their antitumor effect in vivo. The efficacy of CAR-T cells generated using non-viral systems remains to be demonstrated, as there has been only a small number of clinical trials using them, but modest antitumor activity was observed in a clinical trial using CD19 CAR-T cells transfected with a Sleeping Beauty (the most common form of transposon/transposase) system [267].

Another option for T cell transduction is the electroporation of mRNA encoding the CAR into their cytoplasm [268], [269]. With this strategy, there is no integration of an exogenous gene into the genome, mitigating concerns regarding genotoxicity. This system has already been tested in preclinical models [270] and CAR expression was detected on the T cell surface only for up to 1 week in a xenograft mouse model of ovarian cancer. Since expression of the CAR is transient, this method may be optimal in instances where persistent targeting of an antigen may be associated with life-threatening toxicities. Clinical trials of mesothelin-specific CAR-T cells transduced using this method demonstrated transient persistence and clinical evidence of anti-tumor response [271]. A more recent report demonstrates that 2 out 6 patients with pancreatic ductal adenocarcinoma had stable disease [272]. However, a clinical trial, also from university of Pennsylvania, using mRNA as delivery system of a CD123 CAR for AML (NCT02623582) was terminated due to lack of persistence of T cells and no observable anti-tumor effect despite cytokine production and fever [273]. Therefore, more clinical trials will be needed to investigate if CAR expression

using mRNA will persist long enough to elicit antitumor effects but short enough to prevent side effects. A potential drawback of this approach is that repeated infusions of CAR-T cells may be necessary to achieve optimal antitumor effects and this can result in increased IgE antibody production against the murine-derived scFv and lead to anaphylaxis [180].

The most recent approach to genetically modify T cells uses gene editing tools such as CRISPR or TALEN to create a double break in a particular DNA site, with the CAR gene being provided as a DNA template that is introduced into that selected genomic site. This system minimizes the risk of unrestrained genomic integration and has demonstrated good antitumor effect in a preclinical model [274]. It is, however, more complex and expensive than other approaches. Nonetheless, the use of this engineering system is under intense study.

Regardless of the actual method used for T cell transduction, from a biotechnology point of view, all these vectors can be made in large quantities and frozen for several years. When T cells are available for transduction, an aliquot can be thawed and used as needed.

The first step in the generation of autologous CAR-T cells is the collection of peripheral blood mononuclear cells (PBMC) from a patient, usually through leukapheresis, whereby blood is removed from the donor and separated into different components, with the immune cells being collected while all other components returned to the donor's circulation. T cells are then cultured in the presence of a nonspecific T cell stimulus, which is required not only for ex vivo expansion but also for efficient genetic modification, since most of the transduction/transfection systems require actively dividing cells. T cells are then transduced or electroporated to introduce the CAR gene and expanded in culture for days to weeks. The primary goal of this step is to generate sufficient cells (up to 10^{11}) to inject back into the patient to produce adequate responses (Figure I-6).

During expansion, T cells are exposed to a combination of proinflammatory cytokines that promote their growth but can also prevent their full differentiation, improving their persistence, and reactivity when injected back into patients [275], [276]. Different institutions use a combination of different cytokines such as IL-2, IL-7 and IL-15, or IL-15 and IL-21, but whether any particular cocktail has advantages over the others is an area of ongoing research. Some studies, for example, point to the idea that a combination of IL-7 and IL-15 leads to higher percentage of T_{CM} [270].

Although T cells expand well in vitro, a balance is also required between maximizing T cell numbers during expansion and preventing their differentiation and exhaustion, because the latter leads to T cell senescence and limited persistence in vivo [277]. Effector T cells had been considered the ideal subset to use in adoptive cell therapy since they have potent anti-tumor effect [278]. However, more recent data from clinical trials using adoptive T cells therapy have reshaped this vision. Effector T cells, although potent killers, have limited

proliferation capability and ultimately in vivo persistence. Thus, interest has shifted to memory T cells, which can upon injection expand substantially, are long-lived and can effectively and rapidly control pathogens over prolonged period of time, even after repeated exposures [279].

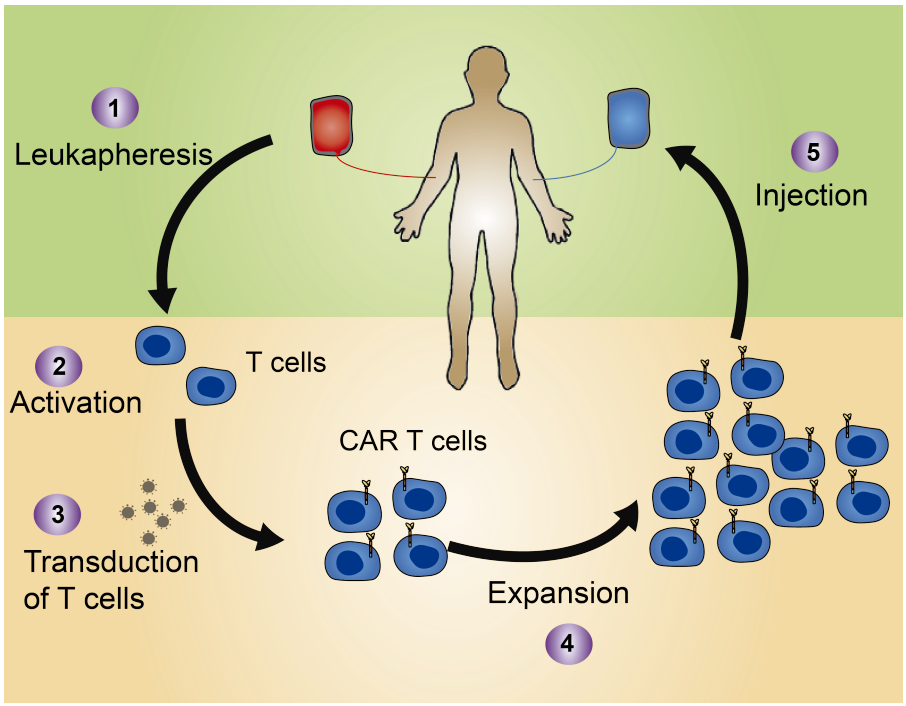

Figure I-6- Schematic representation of the manufacture process of CAR-T cells used in clinical trials. T cells are collected from the patient usually through leukapheresis (1) and then activated (2) and transduced with a retroviral vector (3). CAR-T cells are then expanded (4) to obtain sufficient numbers to infuse back into the patient a few days or weeks later (5).

I.4.2.4. Observed side effects in CAR-T cell trials

In many clinical trials, killing of tumor cells by CAR-T cells is accompanied by a cytokine release syndrome (CRS) in 30% of the patients, usually in the first days or weeks after infusion [280], [281]. Increase in peripheral blood of several inflammatory cytokines, such as TNF-α, IFN-γ and IL-2, is detected [280], [282]. IL-6 seems to be the most prominent cytokine and is associated with a macrophage activation syndrome and hemophagocytosis, the manifestations of which can be resolved with the administration of an IL-6 antibody (siltuximab) or IL-6 receptor antibody (tocilizumab) and supportive care [283]. CRS can in some patients be life-threatening with high fever, hypotension requiring intravenous fluid and pressors, transient neurologic dysfunction, severe shock and compromised respiratory function, eventually leading to multi-system organ failure; but it can also be mild, with only flu-like symptoms including fever, nausea, and chills [284].

Interestingly, the etiology of the neurological dysfunction is not clear since the targeted antigen is not known to be present in the CNS and CAR-T cells are not always found in the CNS at the time of CRS [285]. Although some authors did detect the presence of CD19 CAR-T cells in the cerebral spinal fluid of those patients with neurologic side-effects, blocking IL-6 with tocilizumab did not resolve the symptoms [286]. Nevertheless, all these toxicities are in most of the cases transient.

The inability of CAR-T cells to distinguish normal and tumor cells due to shared similar expression of the target molecule leads to potential side effects, a finding called on-target/off-tumor effect, which depends on the type of tissue destroyed by CAR-T cells and seems to be potentially worse in solid tumor than leukemias, since most of hematopoietic and immune cells can rapidly be regenerated. One patient has already died in a HER2 CAR-T cell trial probably to the expression of HER2 on lung epithelial cells [287]. This patient experienced respiratory distress within 15 minutes of CAR-T cell infusion and died a few days later. This tragedy was a harsh lesson but demonstrated how potent CAR-T cells can be and the importance of defining better and safer targets, so that clinical responses may be observed without endangering a patient. A similar toxicity was observed in CARs targeting carbonic anhydrase IX, which is overexpressed in renal cell cancer, but also present in low levels in the biliary tract, against which toxicity was detected [183]. Finding good targets for solid tumors is a bigger challenge than for hematological tumors since blood cancers are routinely classified based on expression of cell surface markers, which can then be used as target molecules to CAR-T cells. Characterization of solid tumors depends on the combination of histological and immunohistochemical tools, many of them highlighting intracellular proteins or signaling molecules associated with cancer metabolism that, although of extreme value for diagnostic proposes, are not useful as targets for CAR-T cells.

Considering the good tolerability of trastuzumab (Herceptin) while targeting HER-2 or lintuzumab while targeting CD33, it seems that CAR-T cells have a stronger antitumor effect but may also have stronger off-tumor effects than the antibodies from which the CAR is derived. These off-tumor effects, especially in trials using CAR-T cells for solid tumors, are difficult to predict using the most common preclinical models, especially considering that there had been no dose-limiting toxicity demonstrated in 17 patients treated with a HER-2 CAR-T cells in a different clinical trial [214]. This particular HER2 CAR was designed based on a different scFv, which can explain the differences in toxicity and illustrates the point that toxicities depend not only on the target molecule but also on the properties of the antibody used to create the CAR. However, other discrepancies between these two clinical trials cannot be discarded, such as differences in lymphodepletion, cell dose and use of IL-2.

While increased affinity of CARs can promote a stronger antitumor effect, specially in target molecules with lower expression, it can also increase the rate and severity of side

effects. Since some tumor-associated antigens can have higher expression in tumor cells, a perfect balance in affinity can be reached so that cells with lower expression (healthy) are spared and those with higher expression (tumor) are eliminated. Modifications in EGFR and HER2-specific scFvs to reach the affinity value that allow CAR-T cells to eliminate tumor but spare normal cells due to differential expression of the target molecule on cell surface has already been demonstrated in preclinical models [288], [289].

Other clinical trials, mostly in hematologic tumors, have been haunted by fatalities. Furthermore, despite the success of all CD19 CAR trials, patients tend to have prolonged B cell aplasia and hypogammaglobulinemia owing to the persistence of CAR T cells [203], [204], [285]. For B cell malignancies, the possibility of targeting one of the light chains of the BCR can be useful in mature B cell malignances as a strategy to spare a fraction of healthy B cells. Results from a clinical trial using a CAR targeting the kappa light chain, which is expressed on average in two thirds of B cells (the other third expressing lambda light chains) have been reported, [290] with 2 complete remissions and 4 stable diseases in 9 patients with minimal toxicities. Although difficult to assess in these patients, the majority of whom had been exposed to rituximab, lambda B cells were spared as expected. While B-cell aplasia is manageable with intravenous immunoglobulin therapy to prevent infections, this issue would be much more problematic in cells whose regeneration is not possible or whose immediate tissue destruction is life threatening.

The inclusion of a suicide or safety switch to interrupt CAR-T cell activity if the side effects threaten a patient's life is now under research. This approach requires the modification of T cells with a second gene (besides the CAR) that can specifically induce CAR-T cell elimination if desired. One of the most promising systems is the inducible caspase 9 suicide gene. In this system, a modified form of caspase 9 is only activated after injection of a small molecule (rimiducid) that induces its dimerization, activating caspase-mediated apoptosis in modified T cells only [291]. Another, less convenient, way to deal with toxicities relies on the use of immunosuppressant therapies, such corticosteroid drugs, which abrogate not only CAR-T cell functionality but also the overall immune system responses [292]. This approach is far from ideal, not only because it fails to discriminate between transferred and endogenous cells, but more importantly is accompanied by several important side effects, such as susceptibility to infections, and cardiac, kidney, and neurological damage [293].

I.4.2.5. How tumor cells avoid CAR-T cells

Despite the success of CD19 CAR-T cells in human trials, with up to 90% complete remission, relapses can occur at later time points, with only a little more than 50% of the patients being disease free after 1 year. Two reasons have been put forward for these

relapses: loss of CD19 expression by leukemic cells or poor T cell persistence/performance. Loss of CD19 was not a common finding until the use of CD19-directed immunotherapies. CD19 loss by B-cell blasts has been observed in several clinical trials of CD19 CAR-T cells in up to 31% of the patients [203], [294] and a similar effect was observed in clinical trials of blinatumomab [295]. Due to clear differences in all these clinical trials, it can be concluded that this event seems to be independent of the type of CAR (scFv, backbone or costimulatory domain) or clinical protocol used. Instead, this demonstrates that targeting CD19 imposes a pressure on tumor cells to lose the target molecule, or that there is selection of a CD19 negative clone. Several reasons have been proposed for the disappearance of CD19. One, is alternative splicing excluding not the entire molecule but only the segment with the epitope that is recognized by the scFv. Lack of exon 2 has already been seen some of these relapsed patients [296]. Another mechanism is leukemia lineage switch from lymphoid to myeloid, which has already been observed in 2 patients in a clinical trial and seems to be correlated with pre-treatment disease characteristics [297]. A similar mechanism was described in mice treated with murine CD19 CAR-T cells [298] and in one patient treated with blinatumomab [299]. Lineage switch occurs in 30% of patients and is associated with other targeted therapies, and thus not an exclusive phenomenon of CAR-T cell therapies. These events are, however, rare and need to be analyzed with caution, as they may reflect the existence of a $CD19^{-}$ clone that is selected during treatment. Nonetheless, other targets will probably experience the same difficulties, with antigen escape phenomenon already having been observed in patients who received rituximab (anti-CD20) [300].

Although antigen loss can occur in solid tumors, the less impressive results seen in initial clinical trials for this type of tumors are more likely due to the difficulty of T cells to penetrate and perform an anti-tumor response in an aggressive and immunosuppressive environment. As in any other types of T-cell therapies, CAR-T cells behave similarly to non-modified T cells regarding the limitations that the tumor microenvironment imposes. These include the inhibitory effects of tumor derived cytokines; impaired metabolism due to lack arginine or tryptophan, hypoxia or low pH; and the presence of immune suppressor cells in the tumor microenvironment [301]–[303]. These factors affect equally nonmodified and CAR-T cells in vivo [304], [305]. Premature loss of CAR-T cells in vivo is also a reason for relapse and can be potentially be resolved by further injections, which in some patients were enough to achieve complete remission [296].

Since many of these limitations are associated with any form of target therapy, future clinical trials will need to take into account the possibility of target-negative relapses. The inclusion of more than one CAR, targeting different epitopes, may prevent or mitigate this problem.

I.4.2.6. Multiple targeting by CAR-T cells

Due to the genetic instability of most tumor cells, therapies that rely on the recognition of one single antigen can be associated with relapses due to antigen loss, which is one of the major limitations of CAR-T cell therapy. These “escape mechanisms” that allow tumor cells to evade immunotherapies are, however, also the limitation of any form of monovalent target therapies.

Co-targeting of multiple markers is one of the possibilities to bypass this limitation. As an example, the combination of CD19 with CD20 or CD22 is currently being tested. Clinical trials targeting CD20 or CD22 are already ongoing (NCT03277729, NCT03244306) with 81.4% objective response and 54% complete responses for the CD20 CAR [306]. Combining different targets is an even more attractive approach for solid tumor, such as co-targeting HER2 and IL13Rα2 [307]. This may be especially important in this setting since effectively targeting one single lineage specific antigen may not be a feasible approach in non-hematopoietic tissues due to extensive toxicity. These CARs can be generated by infecting a T cell with two different viruses, each one bearing a different CAR, or creating a TanCAR (two scFvs in the same molecule linked by a small sequence) [308]. Combined targeting of MUC1 and PSCA for prostate cancer has been shown to avoid tumor escape by antigen downregulation [309]. Clinical trials with these bi-specifc CAR-T cells against CD19 and CD20 or CD22 (NCT03271515, NCT03463928) have been recently launched. Targeting three molecules with one CAR is also possible an triple targeting of CD19, CD20 and CD22 is being developed [296]. This approach has however some limitations. On one hand, increase the number of targeted molecules will most likely lead to increased cumulative side effects. If this may not be a problem with the CD19/CD20/CD22 combination since they are all exclusively expressed in B cells and may only lead to B-cell aplasia, targeting two or more tumor-associated targets may cause more normal tissues to be targeted, since all cells that express one of the targets will also be a potential target for the CAR-T cells. On the other hand, although less probable, the possibility of antigen-escape of all antigens or the presence of a double- or triple-negative tumor clone still exists. Indeed, mathematical analysis demonstrated comparable odds of capturing the bulk of tumor cells targeting just two antigens simultaneously versus targeting three antigens [310]. Thus, targeting more targets may not improve the anti-tumor effect of CAR-T cells. Targeting of more stably expressed antigens, such as fibroblast activation protein (FAP) in tumor-associated fibroblasts [311] and vascular endothelial growth factor receptor (VEGFR) [312] in the endothelial cells of the tumor vasculature, at the same time as tumor associated antigens

has already been explored in preclinical models for solid tumors and may offset some of these problems.

More complex “commands” or “instructions” can be given to T cells. While two conventional CARs allow killing by T cells if a target cell expresses one target OR another, (e.g. CD19 or CD20), changes in CAR design create the possibility of engineering T cells that follow more complicated rules. For instance, they may kill if target A AND target B are both expressed, or if target A but NOT target B is expressed, following a Boolean logic model (Figure I-7).

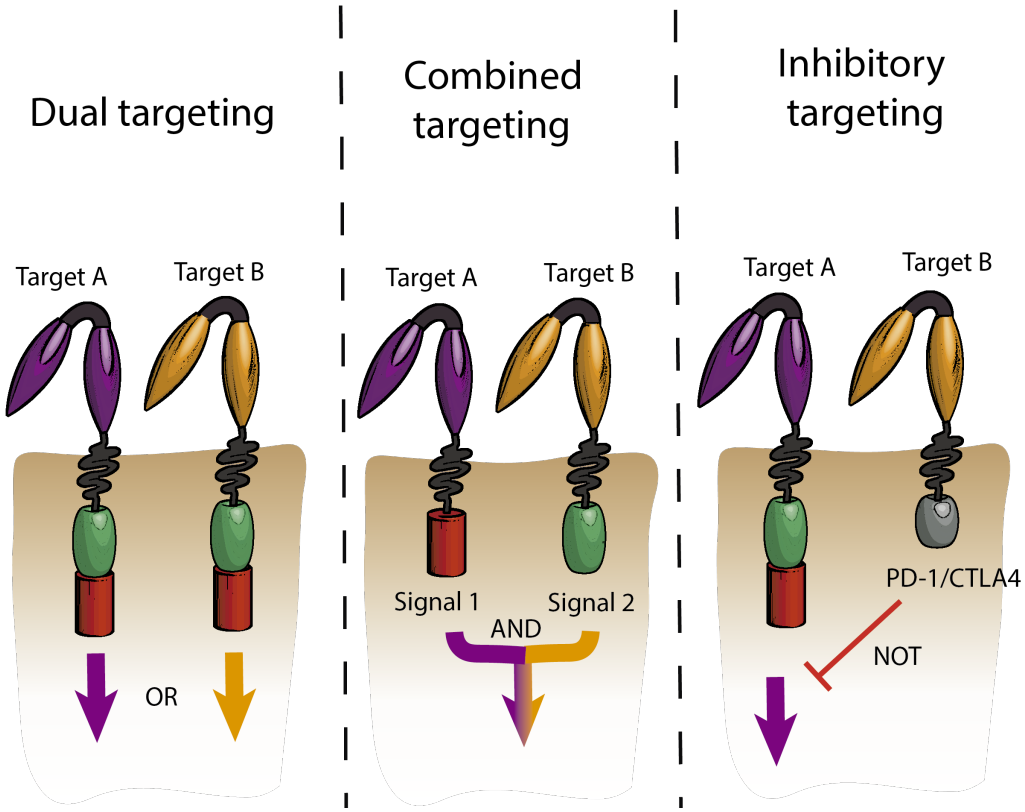

Figure I-7- Multiple strategies for multiple targeting using different endodomains in CARs.

If two CARs are introduced in T cells but only one has the CD3ζ endodomain and the other the secondary domain, full activation will only occur when both CARs bind to cells that express both target molecules. Thus, this approach can be used when both target molecules are expressed by tumor cells but normal cells only express one of them, in which case the latter will be spared [313]. Although one of the CARs is a first generation CAR, even if in vivo expansion is not large, toxicity can still occur, as it has already been demonstrated. To make a NOT command, the activating domain of one CAR is replaced by an inhibitory domain that blocks the TCR/CD3 signaling cascade and consequent T cell activation, such as portions of the PD-1 or CTLA4 molecules. In this setting, CAR-T cells can only kill a certain cell if that cell does not express a molecule that is expressed by vital cells. As an example, if a target is

expressed by leukemic blasts but also in hematopoietic stem cells, a CAR-T cell can be engineered to spare $CD34^+$ cells stem cells even if they express the target molecule. So far, this approach was only validated in PSMA CAR-T cells in a preclinical study [314] and its use has not been reported elsewhere.

I.4.2.7. Towards an allogeneic approach

Ideally, any commercial cellular therapy product should allow prefabrication, be collected from a healthy third party, be easy to store, and be quickly delivered as an off-the-shelf product. Right now, CAR-T cell manufacturing fails to meet some of these criteria since due to the presence of TCR, T cells must be used in the autologous setting and produced for a given patient on a case-by-case basis. Several solutions are already being tested to overcome the limitations of using autologous T cells. One approach is to insert the CAR into other types of cells that can also be adoptively transferred. NK, NK-T, and γδ T cells collected from a healthy donor may be able to replace autologous conventional T cells for CAR-cell generation and be used in cancer patients without causing graft-versus-host disease (GvHD). The generation of allogeneic "universal," off-the-shelf T cells are also under intense investigation. Off-the-shelf T cells can be generated in advance, in a centralized manufacturing facility, regardless of the patient's HLA and infused as needed, rather than after waiting for the T-cell product to be ready. These platforms use genome editing tools to inactivate the endogenous TCR, preventing modified T cells from producing GvHD [315], [316]. Disrupting the TCR at the same time that the CAR is introduced allows the T cell to be activated only through the CAR. Off-the-shelf CAR-T cells have shown efficacy in lymphoma xenograft models and in two infant patients who were treated for B-cell leukemia with CD19 CAR-T cells that had their endogenous TCRs ablated by TALEN technology and were magnetically depleted of TCR^+ cells (<1% TCR^+ T cells remaining) [317]. Of note, GvHD was still observed in these patients, although mild and limited only to skin, in contrast to the more widespread manifestations observed in typical GvHD; and there was evidence of alloreactivity in the marrow. In any case, this experience demonstrates that sorting of any particular subset may not be enough to totally prevent potential complications from allogeneic T cell injection. A clinical trial using these universal T cells targeting CD123 (NCT03190278) is in progress.

Chapter II. CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies

The impressive results obtained by CD19 CAR-T cells in different clinical trials have changed the paradigm of cell therapy. Never before has a genetically modified cell product been approved by a governmental regulatory agency. The road it still ahead and although it is undeniable that improvements can still be made, one of the next goals is to extend the success of this technology to different tumors. While for some it seems that the difficulty resides in find good markers, others will demonstrate to be a bigger challenge. Although our knowledge of pan-T cell markers is comparable to that of pan-B cell markers, generating CAR T cells to eliminate T cell malignancies requires innovative approaches to avoid CAR-T cell elimination even before they are infused back into patients…

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II.1.Introduction

Many patients with refractory or relapsed B-cell malignancies have achieved complete remission after receiving T cells that are redirected with chimeric antigen receptors (CARs) targeting the pan-B-cell antigen CD19 [204], [294], [318]. A number of factors have contributed to the clinical success of CAR T cells in this patient cohort, including the relative accessibility of malignant B cells, the presence of costimulatory molecules on normal and malignant $CD19^+$ B cells, and the ability to effectively manage the loss of normal B cells—a common “on-target/ off-tumor” side effect.

Broadening the success of CAR T cells to treat T-cell malignancies has proven challenging because of the shared expression of many targetable antigens between normal and malignant T cells. This shared antigenicity can cause fratricide in CAR-transduced T cells, inhibiting their proliferation and viability, and in the clinic may result in eradication of normal peripheral T cells. Such an on-target/off-tumor effect would be more profound and less treatable than is found after depletion of normal B cells with CD19 CAR T cells.

It was previously reported that the fratricide in T cells expressing a CD5-specific CAR is limited and does not impair their expansion [219]. This phenomenon was attributed to the rapid downregulation of CD5 from the cell surface of CAR T cells, reflecting the property of CD5 to internalize upon binding to a specific antibody [319]. CD5, however, is not expressed by many T-cell tumors, and even when present, expression is often dim, emphasizing the need to broaden the range of target antigens. Moreover, it is now clear that tumor immune editing leads to frequent antigen-escape relapses following CAR T-cell and other immunotherapies [204], [296], [320]. Hence, the ability to target more than one T-cell-associated antigen may be critical for the effective long-term treatment of malignancies arising from these cells. CD7 is a transmembrane glycoprotein expressed by T cells and natural killer (NK) cells and their precursors [321], [322]; it is also expressed in >95% of lymphoblastic T-cell leukemias and lymphomas and in a subset of peripheral T-cell lymphomas [323], [324] CD7 plays a costimulatory role in T-cell activation upon binding to its ligand K12/SECTM1 [325]–[327]. However, it appears not to make a pivotal contribution to T-cell development or function because genomic disruption of CD7 in murine T-cell progenitors permits normal T-cell development and homeostasis and only minor alterations in T-cell effector function [328], [329]. Notably, CD7 is internalized on ligation [330] and was previously evaluated as a target for immunotoxin-loaded antibodies in patients with T-cell malignancies [331]. Although there were no severe CD7 antibody-related permanent adverse reactions, tumor responses were limited [331]. We hypothesized that enhancing the

potency of CD7-directed cytotoxicity by substituting autologous CAR T cells for a monoclonal antibody would augment the efficacy of CD7-targeted therapy in patients with T-cell malignancies.

Here, we investigated the feasibility of targeting T-lineage malignancies by using CD7 CAR T cells. We found that unlike CD5, the internalization of CD7 from the T-cell surface following CAR expression is incomplete and leads to extensive fratricide of CD7 CAR T cells. We therefore developed a means to permit the generation of functional CAR T cells using genome editing to eliminate persisting self-target antigens in T cells. In this study, we demonstrate that targeted disruption of the CD7 gene using CRISPR/Cas9 prior to CAR expression minimizes fratricide in T cells and allows the expansion of the CD7-knockout ($CD7^{KO}$) CD7 CAR T cells with robust antitumor activity for preclinical and potential clinical application.

II.2.Material and Methods

II.2.1. CAR design and transduction

Three CD7-specific single-chain variable fragments derived from 3A1e, 3A1f, [332] and TH-69 [333] clones of CD7-specific antibodies were created using commercial gene synthesis (Bio Basic, Inc) and cloned into a second-generation backbone CAR containing CD28 and CD3z endodomains and the CH_3 domain from IgG1 Fc as a spacer region. A truncated CD7 CAR lacking signaling endodomains was used as a control. After initial efficacy studies, the best-performing clone (3A1e) was used for the remaining experiments presented here. T cells were activated by plate-bound OKT3 and anti-CD28 antibodies, transduced with gammaretroviral vectors, and expanded in the presence of interleukin-7 (IL-7) and IL-15 in cytotoxic T-lymphocyte (CTL) media, as has been described before [219].

II.2.2. Genomic disruption of CD7 in T cells

Two guide RNAs (gRNAs) for the CD7 gene (gRNA-85: GGAGCAGGT-GATGTTGACGG and gRNA-72: GGGGTCAATGTCTACGGCTC) were designed using CRISPRscan and COSMID algorithms [334], [335]. The CD7 gene was genomically disrupted in T cells according to the published protocol [336]. Briefly, the 20-nt sequence complementary to the specific gene locus was incorporated into an oligonucleotide primer and used to amplify the gRNA scaffold from PX458 plasmid (a gift from Feng Zhang; Addgene 48138). gRNAs were generated through in vitro transcription with High-Yield RNA Synthesis Kit (NEB Bio Labs) from the DNA template, following the manufacturer's

instructions, and purified using the RNA Clean & Concentrator-25 kit (Zymo Research). We electroporated 0.4 µg of gRNA and 1 µg of cas9 protein (PNA Bio) with $0.25x10^6$ of activated T cells by using the Neon Transfection System (Thermo Fisher Scientific) in 10 µL of buffer T and using 3x 1600-V 10-ms pulses. Following electroporation, T cells were incubated in CTL media supplemented with 20% fetal bovine serum in the presence of IL-7 and IL-15 overnight after electroporation. T cells were then expanded in normal media.

II.2.3. DNA analysis

Genomic DNA was harvested 72 hours posttransfection using a Qiagen DNeasy Blood and Tissue kit, according to the manufacturer's instructions. For T7EI assays, polymerase chain reaction (PCR) was used to amplify the gRNA target sites in CD7 using Accuprime High Fidelity Taq polymerase (Life Technologies), and the resulting products were purified using magnetic beads, as has been previously described [337]. T7EI digestion was performed according to the manufacturer's instructions, and the resulting cleavage products were analyzed by agarose gel electrophoresis.

II.2.4. GUIDE-Seq

For GUIDE-Seq experiments, gRNAs were generated by cloning annealed oligos containing the target sequence into pX330 (gift from Feng Zhang, Addgene 42230) [338]. U2OS cells (American Type Culture Collection [ATCC] HTB-96) were maintained in McCoy's 5a Medium Modified; ATCC), supplemented with 10% fetal bovine serum and 2 mM of L-glutamine. Cells were cultured at 37°C in a 5% CO_2 incubator. We nucleofected 200.000 U2OS cells with 1 µg of pX330 Cas9 and gRNA plasmid and with 100 pmol of dsODN by using SE cell line nucleofection solution and the CA-138 program on a Lonza 4D-nucleofector. The nucleofected cells were seeded in 500 µL of media in a 24- well plate, and gDNA was extracted 72 hours postnucleofection using a Qiagen DNeasy Blood and Tissue kit. dsODN integration at the target site was confirmed by restriction fragment length polymorphism assay with NdeI. gDNA was quantified using a qubit high-sensitivity dsDNA assay kit. We sheared 400 ng of gDNA using a Covaris LE220 Ultrasonicator to an average length of 500bp. The sheared DNA was processed as previously described [339] and sequenced on the Illumina Miseq. We analyzed GUIDE-Seq data using the standard pipeline [339] with a reduced-gap penalty for better detection of off-target sites containing DNA or RNA bulges.

II.2.5. Bioinformatic off-target identification

Potential off-target sites for the CD7 gRNAs in the human genome (hg38) were identified using the Webtool COSMID [335] with up to 3 mismatches allowed in the 19 PAM proximal bases. After off-target site ranking, 17 sites for gRNA-72 and 20 sites for gRNA-85 were selected for off-target screening.

II.2.6. Off-target validation

T cells were electroporated with recombinant Cas9 and CD7-specific gRNA, as indicated above. gDNA was extracted 7 days after ribonucleoprotein delivery, and locus-specific PCRs were performed to amplify off-target sites identified by COSMID and GUIDE-Seq. PCR primers contained adapter sequences to facilitate amplicon barcoding via a second round of PCR, as has been previously described [340]. All amplicons were pooled at an equimolar ratio and sequenced on the Illumina Miseq according to the manufacturer's instructions, except that custom sequencing primers were used for Read 2 and Read Index. Sequencing data were analyzed using a custom indel quantification pipeline [341].

II.2.7. Flow cytometry

Anti-human CD7 and CCR7 (Biolegend); CD4, CD8, CD45RA, and CD56 (BD Biosciences); and CD3, CD19, and CD45 (Beckman Coulter) were used to stain cells in phosphate-buffered saline or CTL medium. Anti-IgG Fc (Jackson ImmunoResearch) was used for CAR detection in all assays. All flow cytometry data were obtained in BD fluorescence-activated cell sorter (FACS) Canto II (BD Biosciences) and Gallios (Beckman Coulter) and analyzed with FlowJo software (FlowJo, LLC).

II.2.8. Intracellular staining assay

Tumor cells were stained with eFluor670 (Thermo Fisher Scientific), according to the manufacturer's instructions. $CD7^{KO}$ CD7 CAR T cells were cultured with stained tumor cells for 5 hours at a 1:4 effector:target ratio. Brefeldin A (BD GolgiPlug) and monensin (BD GolgiStop) were added 1 hour after plating. At the end of coculture, cells were incubated with antibodies for surface antigens and permeabilized for 10 minutes using BD FACS Permeabilizing Solution 2, followed by incubation with anti-tumor necrosis factor-α (anti-TNF α) and anti-interferon-γ (IFNγ) antibodies.

II.2.9. Cytotoxicity assay

Cell lines Jurkat (ATCC TIB-152), CCRF-CEM (ATCC CCL-119), MOLT-4 (ATCC CRL-1582), Hut 78 (ATCC TIB-161), SupT1 (ATCC CRL-1942), and Raji (ATCC CCL-86) were purchased from ATCC. NALM6 cells were a gift from Stephen Gottschalk. The cells were expanded according to ATCC recommendations. Tumor cells were stained with eFluor670 and incubated with $CD7^{KO}$ CD7 CAR T cells for 3 days without exogenous cytokines, unless stated otherwise. Cells from individual wells were collected at indicated time points. We added 7-AAD to discriminate dead cells, and we obtained absolute cell counts with CountBright Absolute Counting Beads (Thermo Fisher Scientific). In some experiments, autologous peripheral blood mononuclear cells (PBMCs) were labeled with eFluor670 and incubated with $CD7^{KO}$ CD7 CAR T cells in the presence of IL-2 (100 U/mL), IL-7, and IL-15. Cells were collected 24 hours later and stained with specific antibodies for subsequent flow cytometric analysis of $eFluor670^{+}$ cells.

II.2.10.Primary T-ALL blasts

Deidentified fresh or frozen peripheral blood samples from acute T-cell leukemia (T-ALL) patients were thawed and used immediately for analysis. Fresh cells only were used in cytotoxicity assays because of the lower viability of frozen cells on thawing. The protocol for collection of peripheral blood from T-ALL patients was approved by the institutional review board at Baylor College of Medicine.

II.2.11.ELISPOT

T cells ($2x10^5$ cells in triplicates) were plated in 96-well MultiScreen HTS IP plates (EMD Millipore, MA) previously coated with anti-human IFN-γ mAb 1-D1K (Mabtech, Cincinnati, OH), and kept overnight at 4°C. The cells were stimulated with pepmixes (15mers overlapping by 11 aa peptide libraries; JPT Technology) spanning EBV-EBNA-1, LMP-1, LMP-2, BARF-1, EBNA- 3a,3b,3c., BZLF1; Adv-Hexon, Penton; and CMV-pp65, IE-1, and with staphylococcal enterotoxin B (Sigma-Aldrich Corporation) as a positive control. After 20 hours at 37°C, the cells were incubated with anti-human IFN-γ mAb 7-B6-1-biotin (Mabtech) for 2 hours at 37°C, and avidin-peroxidase-complex (Vector Laboratories, Burlingame, CA) was added for 1 hour at room temperature. The plates were then developed with 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) substrate, dried, and sent to Zellnet Consulting (Fort Lee, NJ) for quantification. The spots formed were counted as

spot-forming cells per 10^5 cells as a measure of the number of cells releasing IFN-γ in response to viral antigen pepmixes.

II.2.12.T-ALL mouse xenograft model

Five- to 7-week-old nonobese diabetic (NOD)–Cg-Prkdc^{scid} Il2rg /SzJ^{tm1Wjl} (NSG) male and female mice (Jackson Laboratory) were engrafted with CCRF-CEM GFP-FFLuc$^+$ cells by IV injection, and 2x10^6 of CD7 CAR T cells were injected IV 3 or 6 days later. To measure luminescence, we injected mice with 150 mg/kg of D-luciferin intraperitoneally, and tumor burden was monitored by recording luminesce in an IVIS Imaging system (Caliper Life Sciences) at indicated time points. Living Image software (PerkinElmer) was used to visualize and calculate total luminescence. For analysis of tumor and CAR T cells in peripheral blood of the mice, 100 µL of blood was collected by tail-vein bleeding. After red blood cell lysis, cells were incubated with anti-human CD45, CD4, CD7, and CD8 antibodies for subsequent flow cytometric analysis. All procedures were done in compliance with the Institutional Animal Care and Usage Committee of Baylor College of Medicine.

II.2.13.Statistical analysis

Data points from individual donors are shown in all figures. Statistical significance in pairwise comparisons was determined by an unpaired two- tailed Student t test and in multiple comparisons by a one-way analysis of variance with posttest Bonferroni correction. Statistical significance in Kaplan-Meier survival curves was assessed with the Mantel-Cox log-rank test. All P values were calculated using Prism 6 software (GraphPad).

II.3.Results

II.3.1. Expression of CD7 CAR precludes T-cell expansion

To test whether normal T cells can be redirected to recognize malignant T cells with a CD7-specific CAR, we created a CAR construct with the CD7-specific single-chain variable fragment derived from the antibody clone 3A1e (antigen-binding affinity 8.1 nM) [342] fused to a second-generation CAR backbone containing an IgG1 C_H3 spacer and cytoplasmic endodomains from CD28 and CD3ζ genes [219] (Figure II- 1A). The CD7 CAR lacking intracellular signaling domains (ΔCAR) was used as a control. Transduction of activated human T cells with the gammaretroviral vectors resulted in efficient expression of the CD7 CAR constructs in T cells (Figure II-1B).

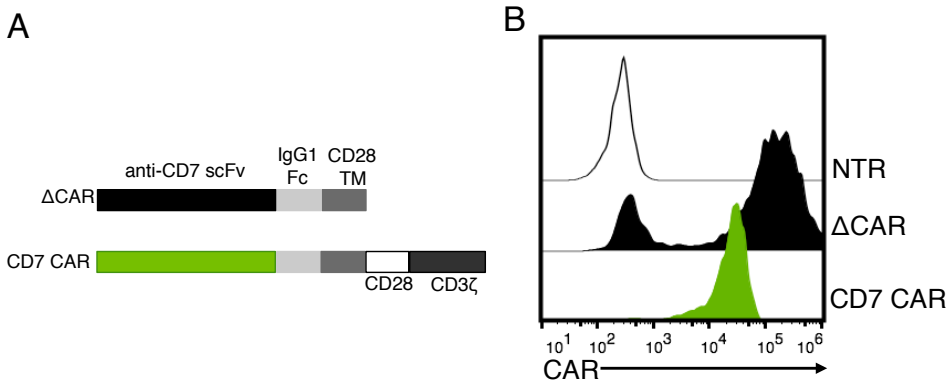


Figure II-1- Generation of CD7 CAR T cells (A) Schematic diagram of control (DCAR) and full-length CD7 CAR constructs used in the study. **(B)** Surface expression of the CD7 CAR constructs on retrovirally transduced T cells measured by flow cytometry using anti-human IgG Fc antibodies on day 6 posttransduction.

However, in contrast to control T cells expressing ΔCAR, the T cells expressing full-length CD7 CAR failed to expand (Figure II-2A). Lack of expansion was associated with poor viability of CD7 CAR T cells (Figure II-2B) and detectable residual expression of CD7 on the cell surface (Figure II-2C), suggesting antigen-driven fratricide.

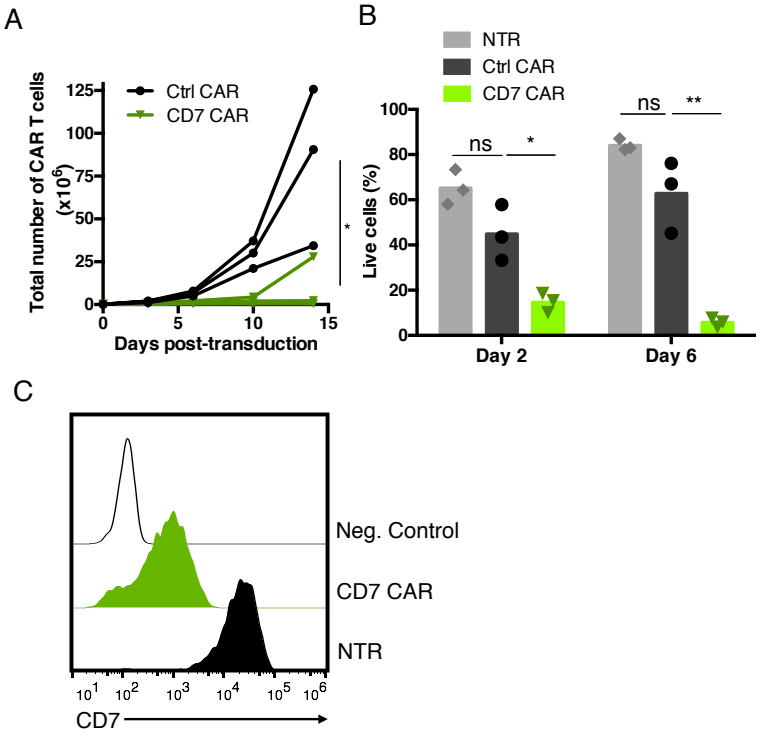


Figure II-2- T cells expressing CD7 CARs fail to expand. (A) Expansion of T cells transduced with truncated (ΔCAR) of full-length CD7 CAR in vitro for 14 days. **(B)** Viability of CD7 CAR T cells at days 2 and 6 posttransduction measured by flow cytometry. **(C)** Expression of CD7 in nontransduced or CD7 CAR-transduced activated T cells. A CD7-negative cell line Raji was used as a negative control. Data represent 2 to 5 independent experiments with n=3 donors in each. Ctrl, control; Neg., negative; ns, not significant; NTR, nontransduced. (*P<0.05; **P<0.001).

To prove that such phenomenon occurred as a consequence of recognition of CD7 by the CAR, we generated two new variants of the CD7 CAR using two distinct scFvs (3A1f and TH-69), which recognize different epitopes in the CD7 molecule. All three scFvs produced equal toxicity, preventing normal expansion of CD7 CAR T cells (Figure II-3) despite differences in epitope recognition and affinities.

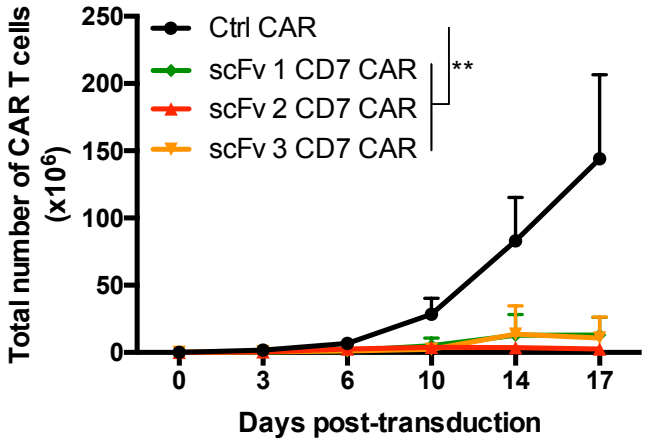


Figure II-3- CD7 CAR toxicity is independent different scFvs that recognize CD7. Expansion of T cells transduced with three different CD7 CARs differing only in the scFvs during 17 days. A truncated CAR was used as control. Ctrl Control **P<0.001.

Therefore, we hypothesized that ablating CD7 gene expression in T cells would prevent the fratricide and restore CD7 CAR T-cell expansion.

II.3.2. Genetic disruption of the CD7 gene enables expansion of CD7 CAR T cells

Sustained expansion and activity of CD7 CAR T cells would likely require permanent inhibition or disruption of CD7 gene expression in CAR-transduced T cells and their progeny. Therefore, we disrupted the CD7 gene in T cells prior to transduction by using CRISPR/Cas9. We used CRISPRScan [219] and COSMID [335] algorithms to select 2 guide RNAs (gRNA-72 and gRNA-85) targeting the CD7 gene with minimal predicted off-target effects (Table II-1). We chose to target exon 2 of the CD7 gene encoding the extracellular domain to reduce the probability of generating a truncated membrane-bound CD7 protein following nonhomologous end joining.

Table II-1- Sequence and locus of two gRNAs used to knockout CD7 in T cells

Name	Sequence	Locus
CD7gRNA-72	GGGGTCAATGTCTACGGCTC	17:82316686-82316709 (-)
CD7gRNA-85	GGAGCAGGTGATGTTGACGG	17:82316913-82316936 (+)

Activated PBMCs were electroporated with CRISPR/Cas9 protein complex with either gRNA-72 or gRNA-85; both combinations resulted in efficient disruption of the CD7 gene, reflected by the loss of surface CD7 expression 3 days after electroporation (Figure II-4A). After electroporation with CRISPR/Cas9 and a CD7-specific gRNA, T cells only gradually lost cell-surface expression of CD7 protein over 72 hours (Figure II-4B), and transducing T cells with CD7 CAR only 24 hours after the gene editing still resulted in impaired expansion of CD7 CAR T cells (Figure II-4C). Therefore, we transduced the cells with CD7 CAR-encoding retrovirus 3 days after electroporation. This method allowed us to consistently obtain a T-cell product containing 85% CD7-knockout ($CD7^{KO}$) CD7 CAR T cells (Figure II-4D).

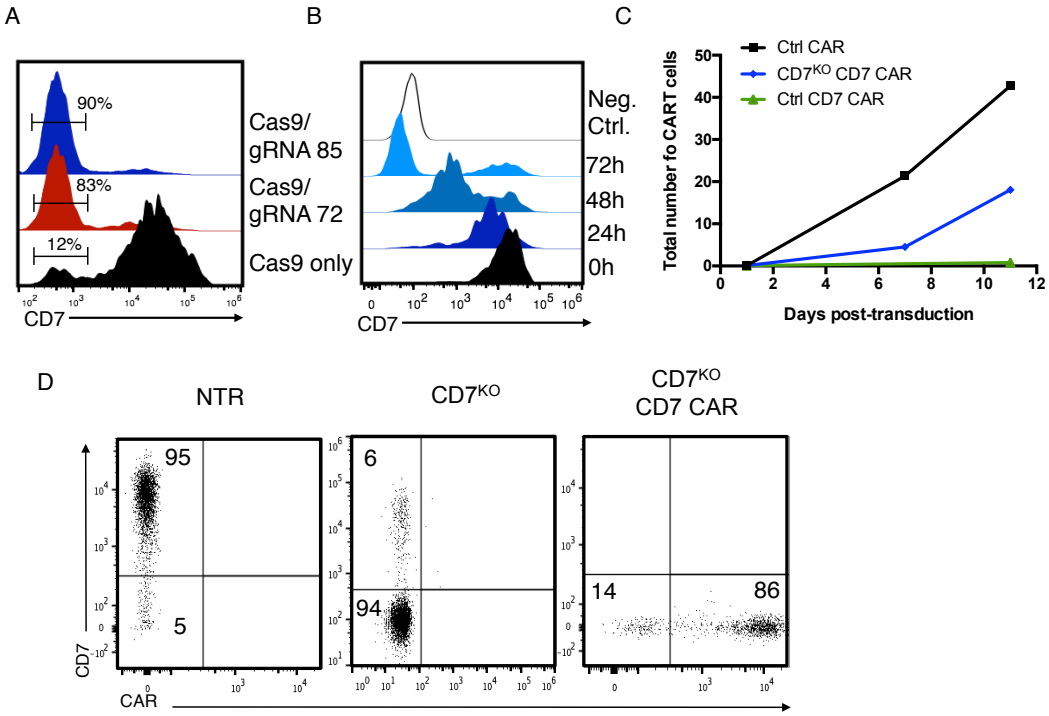


Figure II-4 Disruption of CD7 expression (A) Representative histogram showing ablation of CD7 expression in T cells after electroporation with CRISPR/cas9 and CD7-specific gRNAs 3 days after electroporation. Numbers denote frequency of CD7-negative cells. T cells electroporated with Cas9 only were used as a negative control. **(B)** Downregulation of surface CD7 expression in T cells after electroporation with CRISPR/cas9 and gRNA-85. A CD7-negative cell line Raji was used as a negative control. **(C)** Expansion of T cells generated with knockout of CD7 24h after transduction or without knockout **(D)** Representative dot plots showing expression of CD7 and CD7 CAR in T cells generated with the optimized protocol. Numbers indicate percentage of cells in each quadrant. h, hour Neg. Ctrl, Negative Control; NTR nontreated

Most important, the removal of CD7 expression promoted robust expansion of CD7 CAR T cells (Figure II-5A) and significantly improved T cell viability (Figure II-5B).

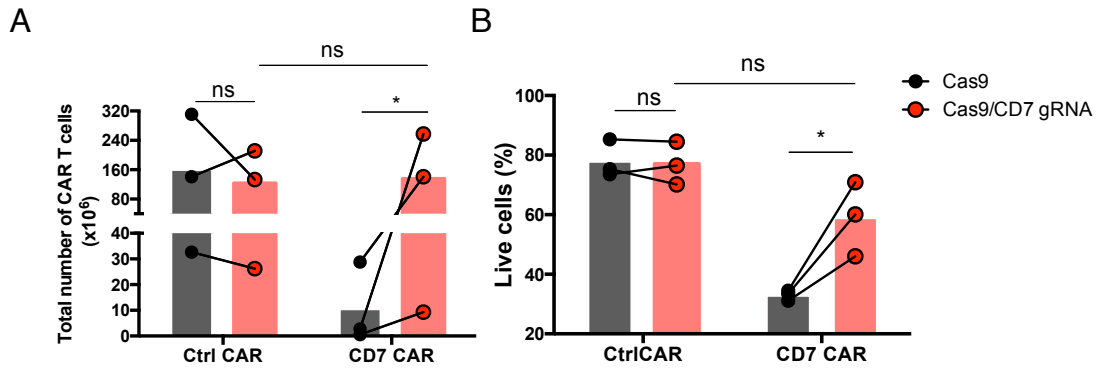
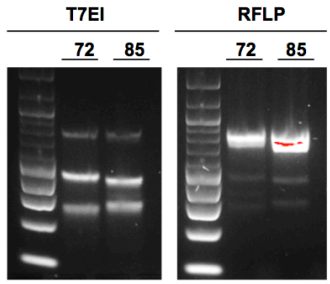


Figure II-5- Disruption of CD7 expression with CRISPR/Cas9 restores expansion of CD7 CAR T cells. (A) Total expansion of CD7 CAR T cells with and without CD7 knockout after 14 days of in vitro culture. **(B)** Viability of CD7 CAR T cells with and without CD7 gene disruption measured at day 6 after transduction by flow cytometry. Lines denote individual donors. Data represent 3 independent experiments with 3 donors in each. *P<0.05. not significant

To verify off-target sites for both gRNAs, we performed unbiased whole-genome screening with the GUIDE-Seq method and validated the identified sites in primary CD7^{KO} T cells (Figure II-6).

A

gRNA	# of sites	Screened
CD7_72	21	17
CD7_85	51	20



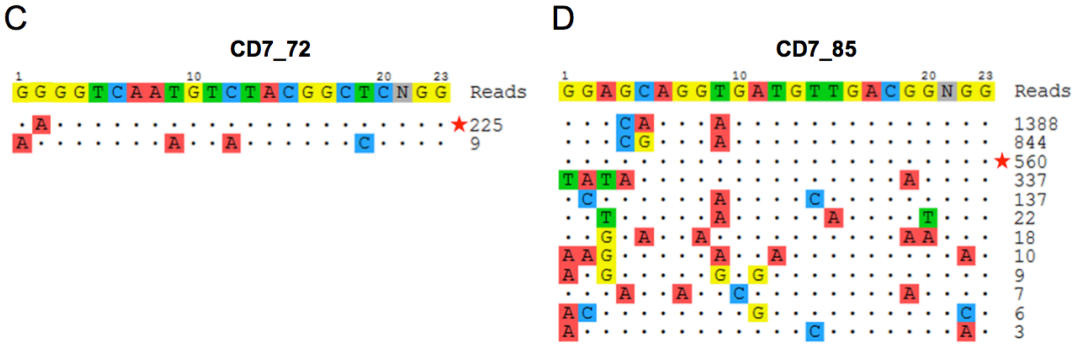


Figure II-6 Identification of gRNA off-target sites. (A) Number of potential off-target sites identified by the bioinformatic tool COSMID. **(B)** T7EI assay confirming gRNA activity in U2OS cells and RFLP analysis confirming capture of a short dsDNA tag at the gRNA target site. **(C)** GUIDE-Seq identified off-target sites for CD7_72. **(D)** GUIDE-Seq identified off-target sites for CD7_85. For both gRNA_72 and gRNA_85 the CD7 target site is marked by a red star

We detected no off-target activity above the limit of detection (0.1%) for gRNA-72. For gRNA-85, although 5 off-target sites had activity above 0.1%, none were significantly different from mock-treated samples (P< 0.18-0.3; Supplemental Table I). These results indicate that the genomic disruption of the CD7 gene was specific and prevented T-cell fratricide to enable the expansion of CD7 CAR T cells.

II.3.3. Genetic disruption of CD7 does not impair cytotoxicity of CAR T cells

Although the CD7 protein has a costimulatory function in T cells, whether its expression is critical or not for the antitumor activity of CAR T cells is unknown. We therefore determined whether genomic disruption of the CD7 gene using the CRISPR/Cas9 system altered the effector function of CAR T cells. We electroporated T cells with CRISPR/Cas9 and CD7-specific or control gRNA and transduced them with CD19 CAR (Figure II-7A), because the activity of this CAR is well documented. Removal of CD7 did not evidently alter the phenotype of CD19 CAR T cells (Figure II- 7B) and preserved the CD4:CD8 ratio (Figure II-7C).

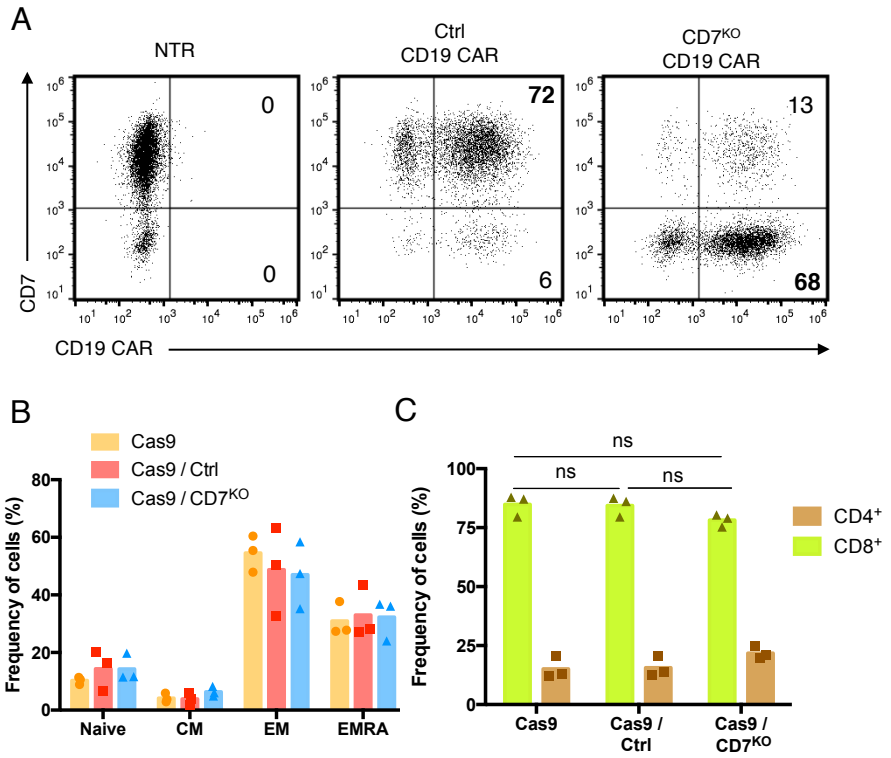


Figure II-7- Loss of CD7 does not alter phenotype or effector function of CAR T cells. (A) T cells were electroporated with Cas9 complexed with CD7-specific or control (CD19 specific) gRNA and transduced with CD19 CAR. Representative dot plots show expression of CD7 and CD19 CAR in T cells electroporated with Cas9+gRNA 7 days posttransduction. Nontreated activated T cells were used as control. Numbers denote frequency of cells in corresponding quadrants. (B) Frequency of naive-like cells (naive; $CCR7^+CD45RA^+$), central memory (CM; $CCR7^+CD45RA^+$), effector memory (EM; $CCR7^+CD45R^+$), and effector memory RA (EMRA; $CCR7^+CD45RA^+$) in CD19 CAR T cells assessed by flow cytometry on day 7 posttransduction. (C) Frequency of $CD4^+$ and $CD8^+$ CD19 CAR T cells 7 days posttransduction. ns not significant

Moreover, lack of CD7 did not compromise the ability of $CD4^+$ CD19 CAR T cells to produce the cytokines TNFα and IFNγ on coculture with $CD19^+$NALM6 cells (Figure II-8 A,B). We then measured the cytotoxicity of $CD7^+$ and $CD7^{KO}$ CD19 CAR T cells against a $CD19^+$ cell line, Raji. Both groups of CD19 CAR T cells efficiently eliminated tumor cells after 72 hours of coculture (Figure II-8 C,D).

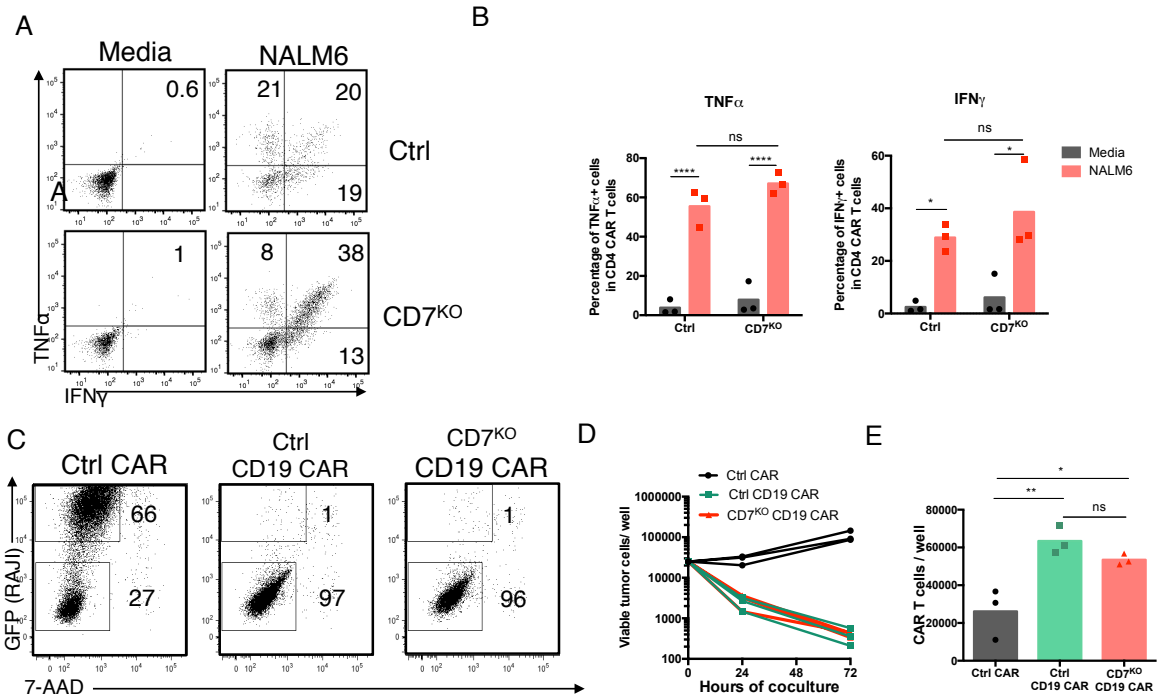


Figure II-8- Loss of CD7 does not effector function of CAR T cells. (A) CD19 CAR T cells were incubated with $CD19^+$ NALM6 cells, and production of TNFa and IFNg in $CD4^+$ cells was assessed by intracellular cytokine staining. Dot plots represent cytokine production in CD19 CAR T cells in the presence of NALM6 or in media alone. **(B)** Summarized data from 3 donors are shown on the right. **(C)** Control or $CD7^{KO}$ CD19 CAR T cells or control nontransduced T cells were cocultured with GFP^+ Raji cells at the effector-to-target ratio 1:1 for 72 hours. Dot plots show representative frequency of gated CAR T cells and GFP^+ tumor cells at the end of coculture. Total numbers of live tumor cells **(D)** and CD19 CAR T cells **(E)** were counted by flow cytometry at 72 hours using counting beads. Lines denote individual donors. Data represent 2 independent experiments with n 5 3 donors in each. *<P 0,05; **<P 0,01; ****<P 0,0001.

Loss of CD7 did not reduce overall expansion of CD19 CAR T cells (Figure II-8 E) and did not affect proliferation of $CD4^+$ and $CD8^+$ subsets (Figure II-9 A,B). Therefore, loss of CD7 did not abrogate the short-term survival or cytotoxicity of CAR T cells.

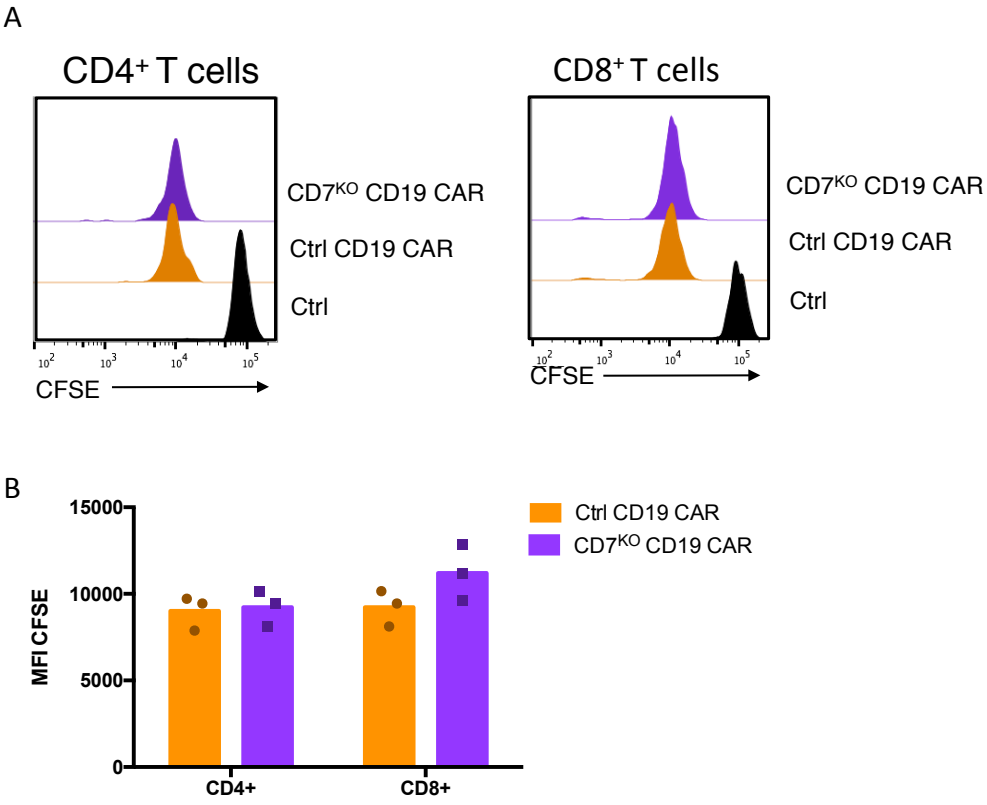


Figure II-9- Loss of CD7 does not compromise proliferation of CAR T cells. (A) Representative histograms showing CFSE intensity of $CD4^+$ (left) and $CD8^+$ (right) CD19 CAR T cells electroporated with control gRNA or CD7 gRNA ($CD7^{KO}$) before (Day 0) and after (day 3) incubation with RAJI tumor cell line. Histogram of the left represents T cells gated on $CD4^+$ population and on the right $CD8^+$ population. **(B)** Mean fluorescence intensity of CFSE in CD19 CAR T cells after coculture with Raji cells. Data from 3 donors are shown.

II.3.4. $CD7^{KO}$ CD7 CAR T cells eradicate malignant T-cell lines in vitro

Because CD7 is highly expressed in T-cell malignancies, we assessed the capacity of $CD7^{KO}$ CD7 CAR T cells (from this point on called CD7 CAR T cells) to kill the T-ALL cell lines Jurkat, CCRF, MOLT-4, and Sup-T1 and a T-cell lymphoma line Hut78, all of which were CD7-positive (Figure II 10-A). In comparison with control CAR T cells, $CD7^{KO}$ CD7 CAR T cells had robust cytotoxicity against eFluor670-labeled tumor cell lines, with 95% decrease in counts of viable tumor cells after 3 days of coculture at a 1:4 initial effector-to-target (E:T) ratio (Figures II 10-B,C). By contrast, CD7 CAR T cells were not cytotoxic against the CD7-negative cell line NALM6 (Figure II-10C), indicating that the cytolysis was CD7-specific.

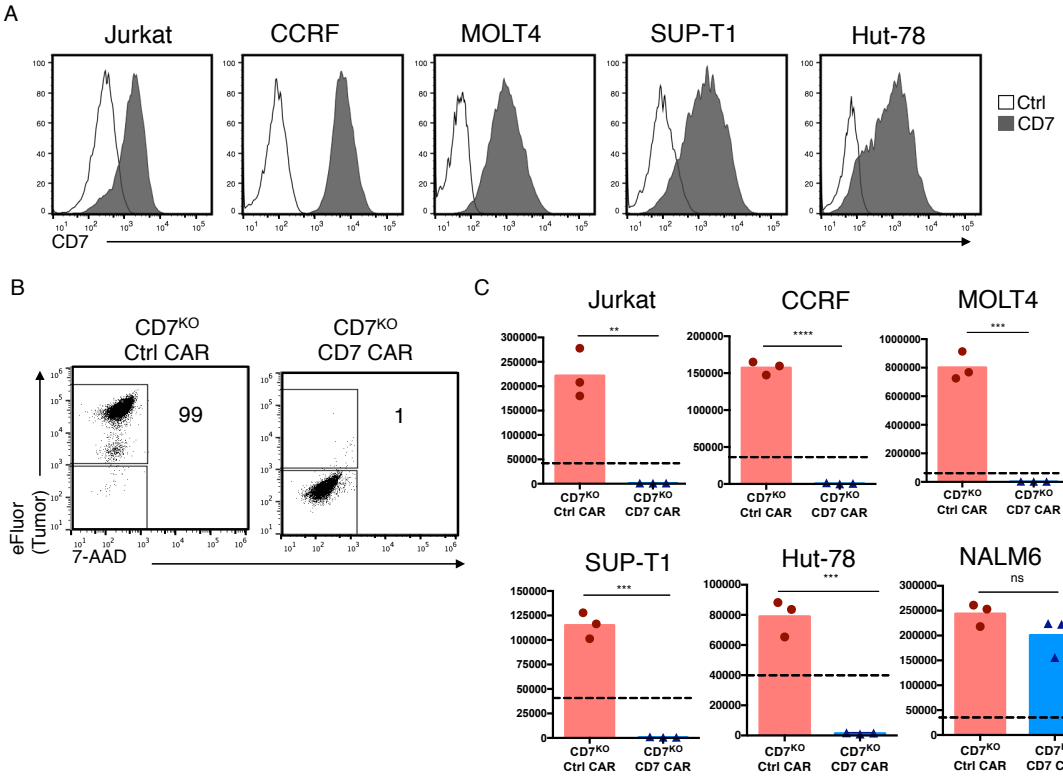


Figure II-10- Expanded CD7^{KO} CD7 CAR T cells eradicate T-ALL and T lymphoma cell lines. (A) Surface expression of CD7 (solid histograms) in T-ALL and T-lymphoma cell lines measured by flow cytometry in comparison with isotype control (open histograms). **(B)** Tumor cell lines were labeled with eFluor670 and cocultured with CD7 CAR T cells at the effector-to-target ratio 1:4 for 3 days. Dot plots show frequency of gated live tumor cells (CCRF) at the end of coculture. **(C)** Absolute counts of live tumor cells were measured by flow cytometry using counting beads at the end of coculture with CD7 CAR T cells. CD7^{+} cell line NALM6 was used as a negative control. Dashed lines represent the initial number of tumor cells on plating. . *<P 0,05; ***<P 0,001; ****<P 0,0001

Coculture with tumor cells also induced proliferation of CD7 CAR T cells (Figure II-11).

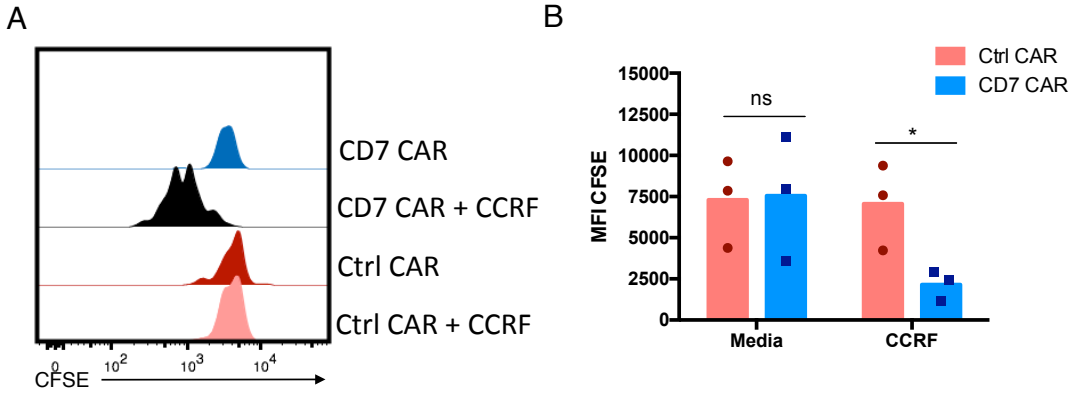


Figure II-11- CD7 CAR T cells proliferate in response to tumor stimulation. (A) Representative histograms showing CFSE dilution in CD7^{KO} control or CD7 CAR T cells in the presence or absence of CCRF tumor cells for three days. **(B)** Mean fluorescence intensity of CFSE in CD7 CAR T cells at the end of coculture. Data is representative of 3 donors (*, P<0.05; ns, not significant).

Since we generated 3 different CD7 CARs and different scFvs can change antitumor performance of T cells [229] we compared the ability of all scFvs against a T-cell leukemic line in a similar killing assay (Figure II-12). Although we did not observed substantial differences between all scFvs, scFv number one (clone 3A1) eliminated more tumor cells (Figure II-12 A) and expanded more (Figure II-12-B) during 3 days. Therefore and because this scFv demonstrated always a slightly better performance in all killing assays and expansions we decided to use exclusively the CD7 CAR with this scFv for further studies.

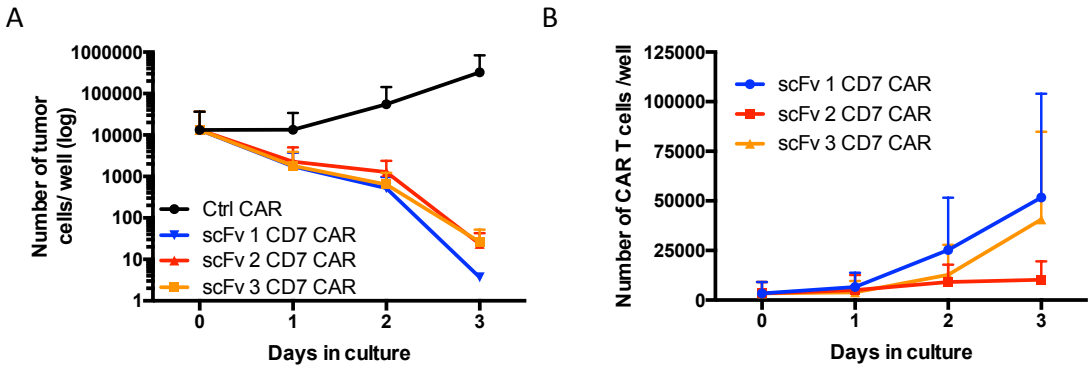


Figure II-12- CD7 CAR with three different scFvs have minimal differences in antitumor effect against a T-cell leukemic line. Killing assay of T cells transduced with CD7 CAR three different scFvs cells in coculture with CCRF at 1:4 E:T ratio for 3 days. **(A)** Number of remaining live tumor cells and **(B)** number of live T cells during the 3 days coculure

Moreover, CD7 CAR T cells also effectively eliminated tumor cells at lower E:T ratios 1:5 to 1:20 (Figure II-13).

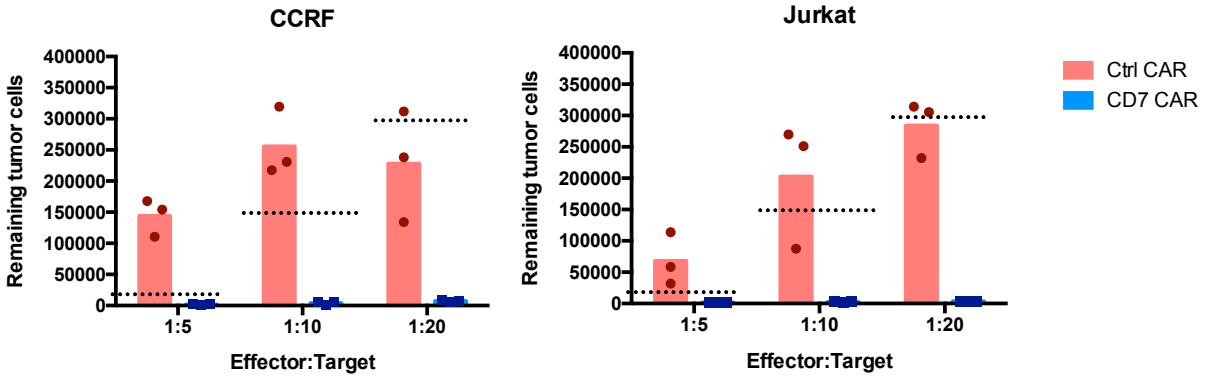


Figure II-13- CD7 CAR T cells eliminate tumor cells at low E:T ratios. CD7 CAR T cells were cocultured with GFP$^+$ tumor cell lines at indicated effector to target ratios 3 days. Live tumor cells were counted by flow cytometry using counting beads at the end of coculture. Dashed lines represent the initial number of tumor cells upon plating. (*, P<0.05; **, P<0.01).

Both CD4$^+$ and CD8$^+$ CD7 CAR T cells produced TNFα and IFNγ on coculture with T-ALL cell lines CCRF, and SupT1 and a lymphoma line Hut78 (Figure II-14). Therefore, CD7 CAR T cells demonstrate potent and specific antitumor activity against malignant T cells.

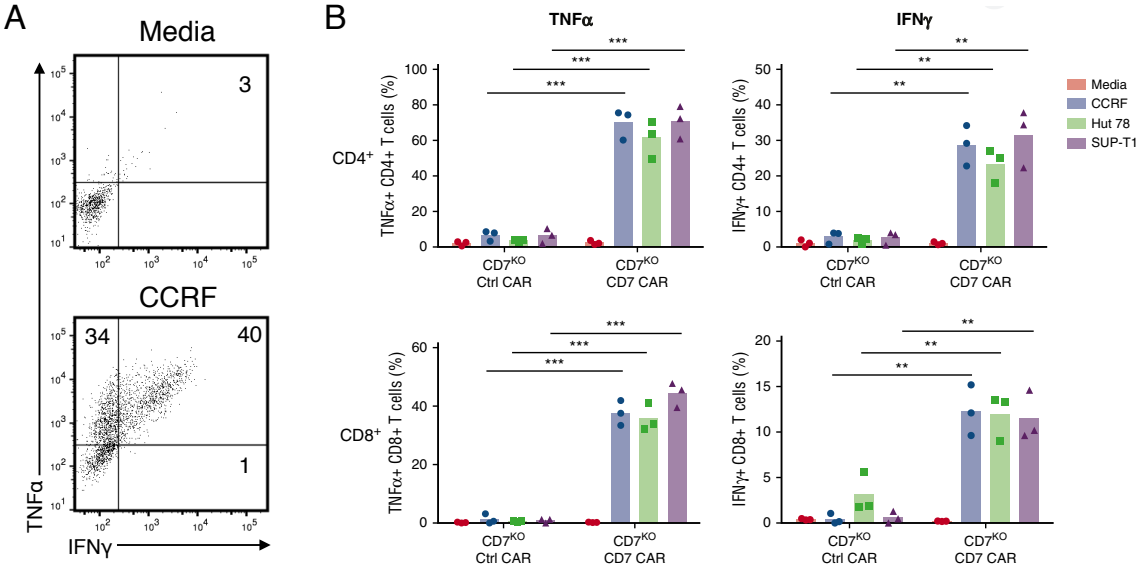


Figure II-14- CD7 CAR T cells produce cytokines upon culture with T-ALL and T lymphoma cell lines. (A) Representative dot plots showing intracellular cytokine staining for TNFα and IFNγ in CD7 CAR T cells upon coculture with CCRF cells or media alone. **(B)** Mean frequencies of cytokine-positive CD4$^+$ (top) and CD8$^+$ (bottom) CD7 CAR T cells on coculture with indicated CD7$^+$ cell lines or media alone. Data represent 2 independent experiments with n=3 donors in each. **P< 0,01; ***P< 0,001.

II.3.5. CD7 CAR T cells recognize and eliminate primary T-ALL cells

To further evaluate the capacity of CD7 CAR T cells to respond to primary tumors, we measured cytokine production on coculture with freshly thawed apheresis samples from T-ALL patients, whose blast cells had a range of CD7 expression (Figure II-15A). Both CD4$^+$ and CD8$^+$ CD7 CAR T cells produced increased levels of IFNγ and TNFα when cocultured with the primary cells (Figure II-15B,C). Similarly, we observed robust cytotoxicity of CD7 CAR T cells against freshly isolated primary T-ALL cells, resulting in >97% elimination of malignant T cells after 48 hours of coculture (Figure II-15 D-E) and concomitant expansion of CD7 CAR T cells (Figure II-15 E). These results indicate that CD7 CAR can efficiently redirect CD7^{KO} T cells to eradicate primary T-blast cells.

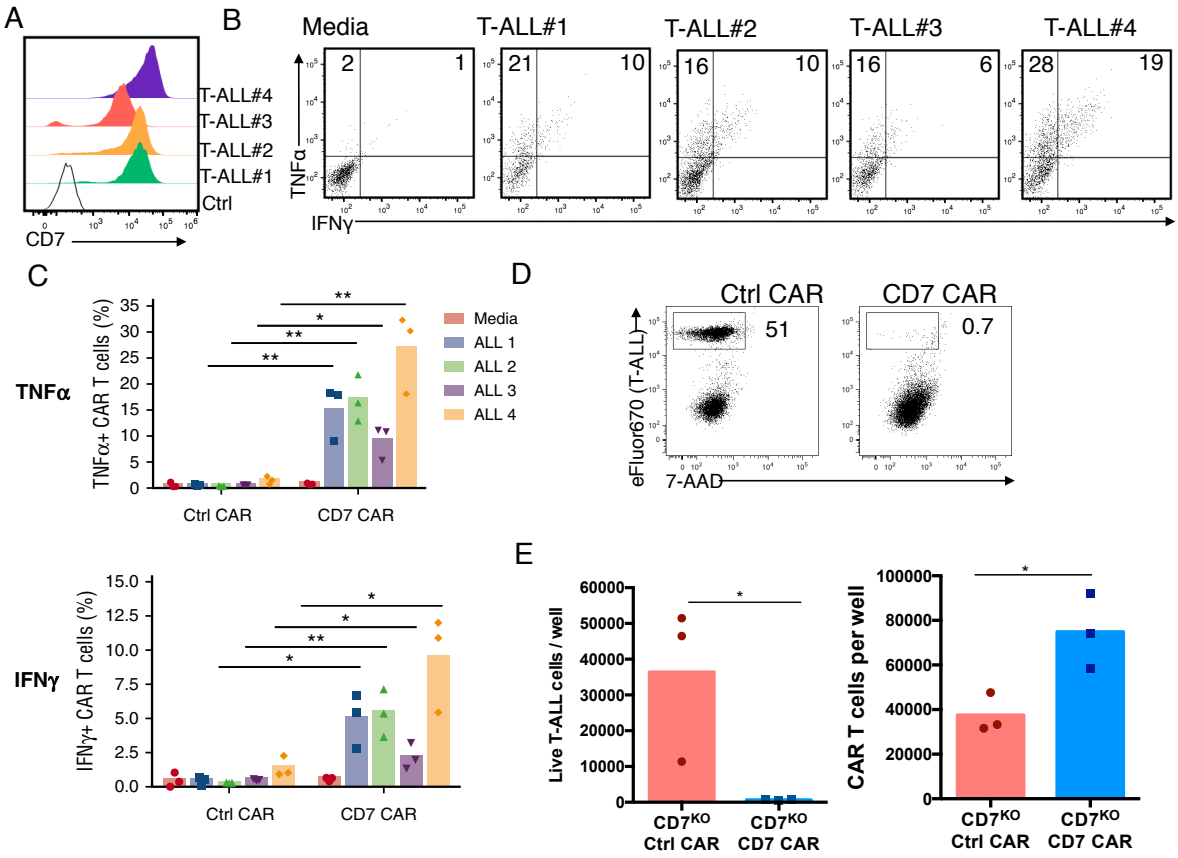


Figure II-15- Antitumor activity of CD7^{KO}CD7 CAR T cells against primary T-ALL blasts. (A) Surface expression of CD7 in peripheral blasts in 4 T-ALL patient samples. **(B)** Representative dot plots showing production of TNFα and IFNγ by CD7 CAR T cells on coculture with allogeneic primary T-ALL blasts. **(C)** Mean frequencies of cytokine-positive CD7 CAR T cells on coculture with individual T-ALL samples. **(D)** Peripheral blasts were freshly isolated from a T-ALL patient by leukapheresis, labeled with eFluor670 and cocultured with control or CD7 CAR T cells for 48 hours. Dot plots show the frequency of live tumor cells at the end of coculture. **(E)** Absolute counts of live tumor cells and CD7 CAR T cells quantified by flow cytometry at the end of coculture. Data represent 1 to 2 independent with n= 3 donors in each. *P <0,05; **P <0,01.

II.3.6. On-target off-tumor effects of CD7 CAR T cells

Apart from malignant T cells, CD7 is expressed by the majority of normal T and NK cells, raising the possibility that CD7 CAR T cells may produce T- and NK-cell aplasia following infusion. Indeed, we observed a significant reduction in numbers of live T and NK cells on coculture of CD7 CAR T cells with autologous PBMC for 24 hours (Figure II-16 A,B).

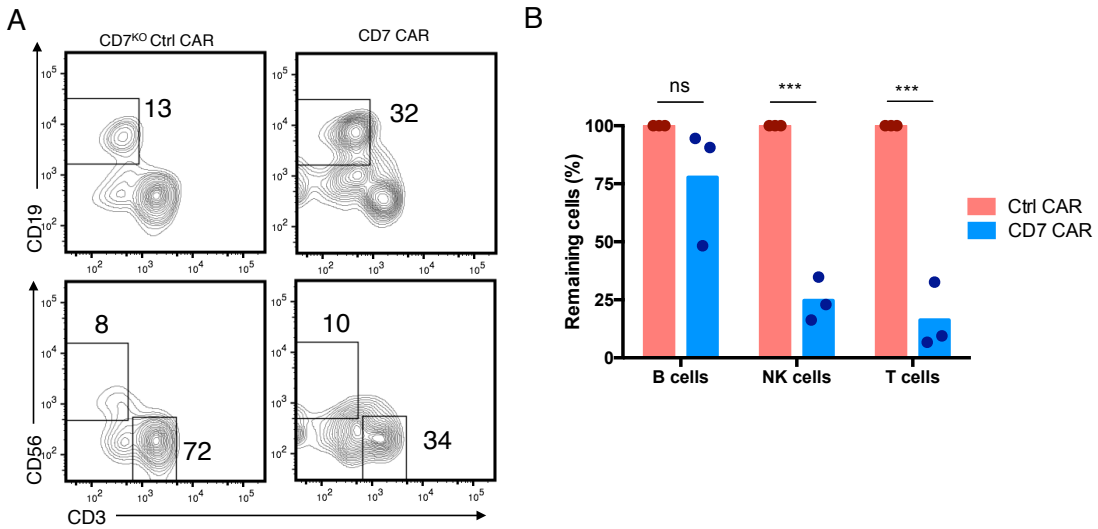


Figure II-16- CD7 CAR T cells are cytotoxic against normal T cells and NK cells. (A) Control or full-length CD7 CAR T cells were cocultured with eFluor670-labeled autologous PBMC for 24 hours, and the frequency of T and NK cells was measured by flow cytometry. Dot plots show the frequency of gated $CD19^+$ B cells (top) and $CD56^+$ $CD3^-$ NK cells and $CD3^+$ T cells (bottom) at the end of coculture. **(B)** Total numbers of autologous B cells, NK cells, and T cells at the end of coculture with autologous CD7 CAR T cells was quantified by flow cytometry using counting beads. Data represent 2 independent experiments with n= 3 donors in each. ***P <0,001

Although expected, to test if this toxicity could be mitigated using any of the 3 CD7 CARs that we generated using different scFvs, we repeated these assays with all scFvs (Figure II-17), especially considering that all performed equally well against tumor cells. Minimal differences were noticeable among all 3 variants of CD7 CAR, demonstrating that such toxicity cannot be avoided by changing the scFvs.

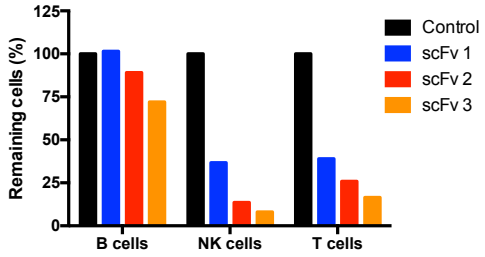


Figure II-17- Different scFvs do not avoid "on-target off-tumor effect". Total numbers of autologous B cells, NK cells, and T cells at the end a coculture with eFluor670-labeled autologous PBMC for 24 hours and autologous T cells transduced with CD7 CARs with different scFvs quantified by flow cytometry using counting beads.

Depletion of these lymphocytes may result in potentially life-threatening immunodeficiency. However, because lack of CD7 did not affect T-cell effector function (Figure II-7-9), we sought to determine whether $CD7^{KO}$ T cells themselves could protect the host by targeting pathogens through their native receptors. Because the infused product will contain both nontransduced and CD7 CAR-transduced $CD7^{KO}$ T cells, we tested the reactivity of these subsets to viral antigens by incubating cells with a peptide mix derived

from CMV, EBV, and adenovirus—the most common causes of viremia in highly immunosuppressed patients—and measured spot-forming units by IFNγ ELISPOT. We found that both nontransduced and CD7 CAR- transduced CD7^{KO} T cells responded to stimulation with viral peptides (Figure II-18A), although the response of CD7^{KO} T cells was attenuated in 1 donor. Notably, both CD4^{+} and CD8^{+} CD7^{KO} T cells retained their viral reactivity (Figure II-18 B) suggesting that although CD7 CAR T cells can reduce normal T-cell numbers, the infused CAR T-cell product itself retains antiviral activity.

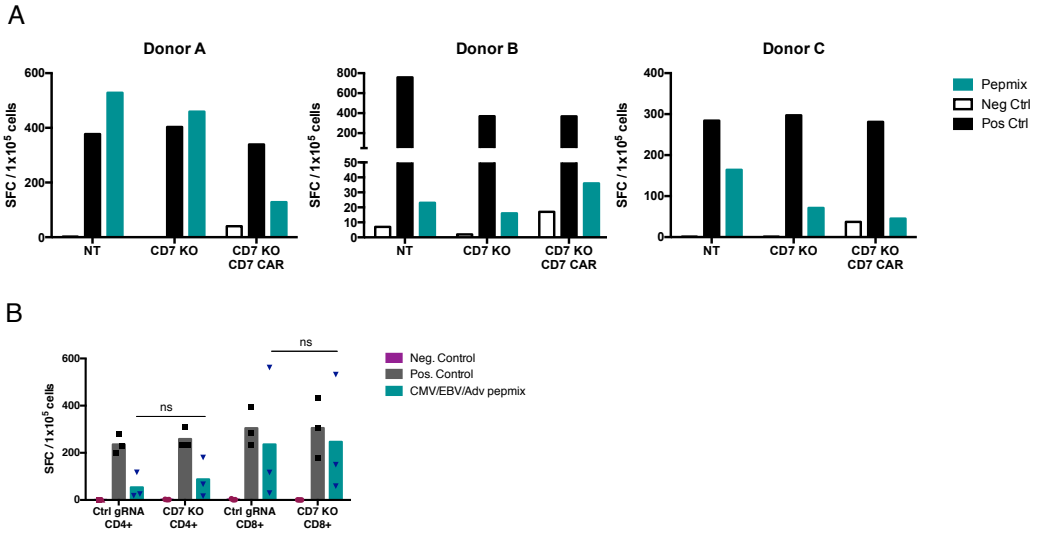


Figure II-18- CD7^{KO} T cells and CD7 CAR T cells can respond to viral antigens. (A) Nontransduced, CD7^{KO} and CD7^{KO} CD7 CAR T cells were stimulated with pepmixes from cytomegalovirus, Epstein-Barr virus, and adenovirus, and the number of IFNγ^{+} spot-forming cells was measured by ELISPOT. Individual data from 3 donors are shown as a means of triplicate determinations. **(B)** CD4^{+} and CD8^{+} T cells were MACS-purified and separately assayed for IFNγ production in response to pepmixes as described above. AdV, adenovirus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; Neg., negative; Pos., positive; SFC, spot-forming cells.

II.3.7. CD7 CAR T cells are protective in mouse xenograft models of T-ALL

We used a mouse xenograft model of disseminated T-ALL [219] by engrafting NOD–severe combined immunodeficiency (SCID) γ chain-deficient (NSG) mice intravenously with CCRF-CEM cells engineered to express GFP-firefly luciferase (Figure 19A). Three days after tumor engraftment, we injected a single dose of CD7 CAR T cells or control nontransduced T cells and followed the tumor burden by IVIS imaging. CD7 CAR T cells conferred robust protection against leukemia progression (Figure 19B-C) and significantly extended median survival of the mice (16 days in the control group vs 58 days in the CAR T group; P< 0.0026 by Mantel-Cox log-rank test) (Figure 19D). In some mice, tumor relapses originated in the periodontal region, a known tumor sanctuary with limited accessibility by human CAR T cells [219], [250].

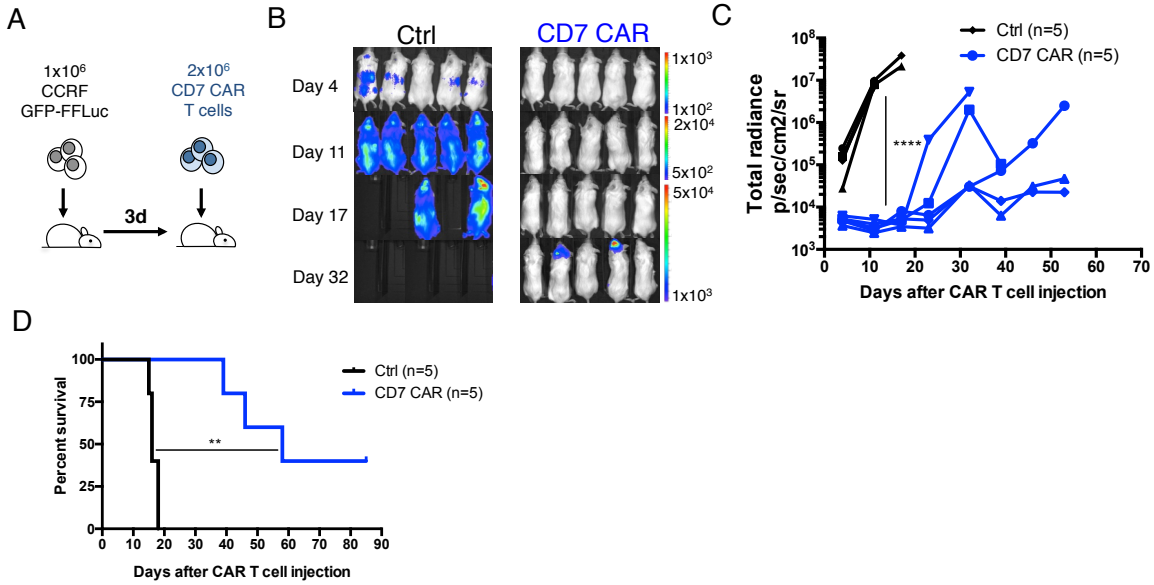


Figure II-19- CD7^{KO} CD7 CAR T cells control the progression of systemic T-ALL in the mouse xenograft model. (A) Schematic outline of the experiment. NSG mice (n=5 per group) were injected intravenously with 1x10^6 GFP-FFluc CCRF cells followed by a single intravenous injection of 2x10^6 of control or CD7 CAR T cells 3 days later. **(B)** Tumor burden was monitored weekly by measuring luminescence using IVIS imaging. **(C)** Overall kinetics of systemic tumor progression in mice. Each line denotes an individual animal. **(D)** Kaplan-Meier survival curve of mice injected with control or CD7 CAR T cells.

Similarly, CD7 CAR T cells protected mice in a higher tumor burden leukemia model in which CAR T cells were injected 6 days after CCRF administration and systemic engraftment (Figure II-20).

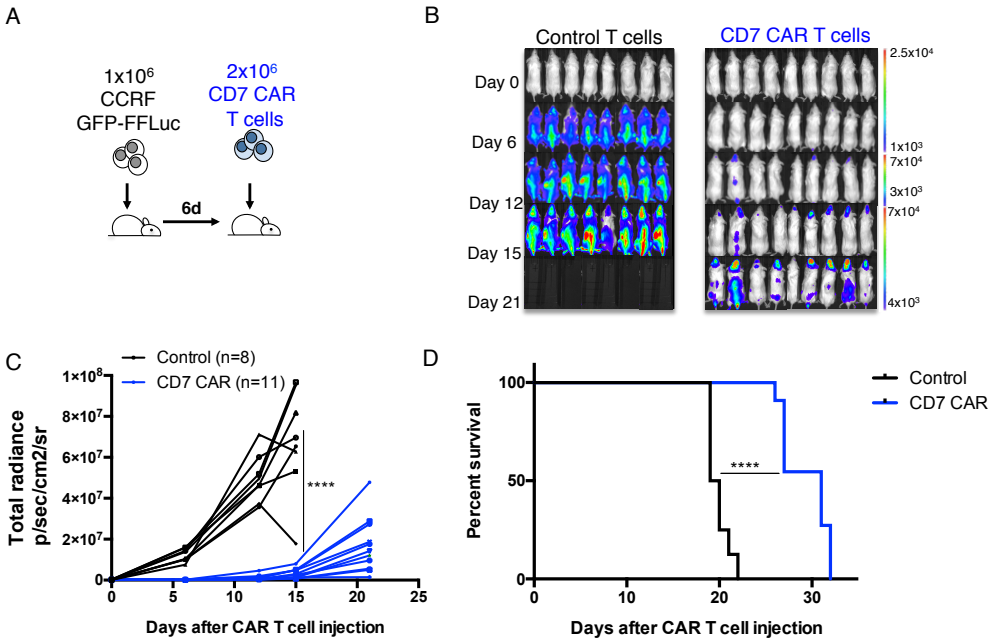


Figure II-20- CD7 CAR T cells are protective in a high tumor burden mouse model of T-ALL. (A) Schematic outline of the experiment. NSG mice (n=8 in control T cell group and 11 in CD7 CAR T cell group) were injected intravenously with 1x10^6 GFP-FFluc CCRF cells followed by a single intravenous injection of 2x10^6 of control or CD7 CAR T cells 6 days later. **(B)** Tumor burden was monitored weekly by measuring luminescence using IVIS imaging. (C) Overall kinetics of systemic tumor progression in mice. Each line denotes an individual animal. (D) Kaplan–Meier survival curve of mice injected with control or CD7 CAR T cells. (****, P<0.0001 by log- rank Mantel-Cox test).

Moreover, mice with early tumor relapses had a lower frequency of CD7 CAR T cells in peripheral blood at day 32 (Figure II-21A), although relapsed tumors retained expression of CD7, albeit displaying moderate downregulation of CD7 surface density (Figure II-21B). This downregulation, however, was insufficient to compromise the ability of CD7 CAR T cells to recognize and eliminate the relapsed tumor (Figure II-21C), indicating that a lack of CAR T-cell persistence rather than antigen escape was the primary culprit for the relapses.

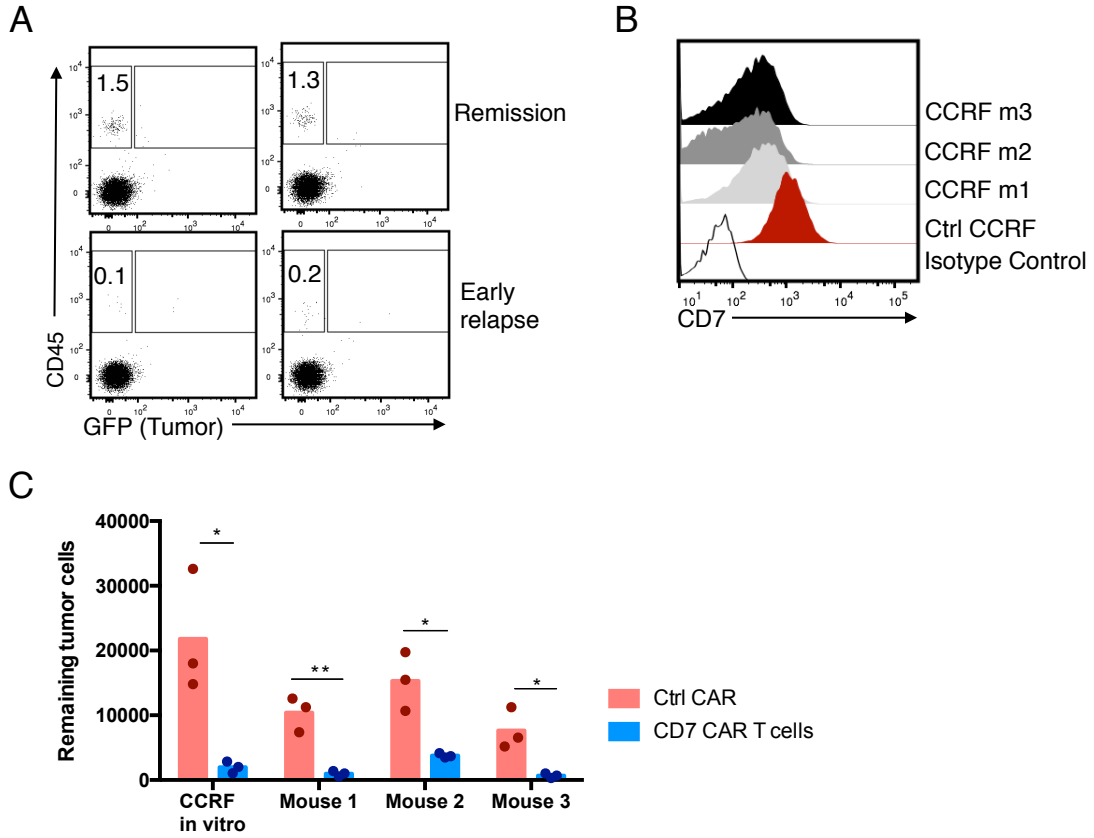


Figure II-21- Lack of persistence of CD7 CAR T cells in vivo (A) Relative frequency of CAR T cells ($hCD45^+$ GFP^+) in peripheral blood of mice in stable remission (top) or during early stages of relapse (bottom) on day 34 after CAR T-cell injection. **(B)** Surface expression of CD7 on relapsed CCRF tumor cells in peripheral blood of 3 relapsed mice (CCRF m1-m3, grey histograms) in comparison with control in vitro propagated cells (red histogram). Open histogram denotes CD7-negative cell line Raji. Data represent 2 independent experiments. **(C)** CCRF GFP^+ blasts were isolated from spleens of 3 relapsed mice and cocultured with $CD7^{KO}$ control or CD7 CAR T cells from 3 donors for 24 hours at a 1:1 E:T ratio. The numbers of viable tumor cells were counted at the end of coculture by flow cytometry using counting beads. *P< 0,05; **P < 0.01.

II.4.Discussion

Although CD7 is an attractive therapeutic target for T-cell malignancies, effector T cells modified with CD7-specific CARs fail to substantially downregulate CD7 expression, resulting in extensive fratricide and precluding T-cell expansion. Here we show that targeted

genomic disruption of the CD7 gene renders CD7 CAR T cells resistant to fratricide, permitting robust expansion without compromising T-cell antigen recognition through their native or chimeric receptors. This toxicity was observed with 3 different scFvs against CD7.

Genome editing, however, is not the only way to conceal CD7 from the cell surface. A recent report showed that the CD7 protein can be “trapped” inside the T cell when bound to an engineered CD7-specific scFv anchored in the endoplasmic reticulum of the cell [343].This interaction prevents CD7 from trafficking to the cell surface and effectively reduces fratricide of CD7 CAR T cells. While such method would obviate the need for genome editing in T cells, the long-term efficacy and feasibility of both methods to generate fratricide-resistant CD7 CAR T cells need to be compared in clinical studies.

$CD7^{KO}$ CD7 CAR T cells eliminated malignant T-cell lines and primary tumor cells and protected mice against systemic leukemia progression in a xenograft model. Although CD7 CAR T cells were cytotoxic against normal peripheral T and NK cells, the $CD7^{KO}$ T cells retained their response to virus-derived peptides through their native receptors, which may ameliorate in vivo immunosuppression.

It was previously demonstrated that the expression of a CD5-specific CAR on the surface of T cells did not cause complete self-elimination of T cells, because CAR expression coincided with the downregulation of CD5 from the T-cell surface, likely by internalization of CD5 following CD5 CAR engagement [219]. In contrast, we found that expression of CD7 CARs promoted only limited downregulation of CD7 expression that was insufficient to mitigate fratricide of CD7 CAR T cells without additional knockout of the CD7 gene. Understanding the mechanisms regulating the differential downregulation of CD5 and CD7 antigens may help extend opportunities for using CAR T cells targeting other T- lineage antigens that internalize upon ligation (eg, CD3- discussed in chapter III) [330].

The clinical feasibility of using targeted genome editing in T cells prior to their adoptive transfer has been well demonstrated. Genomic disruption of the HIV coreceptor CCR5 in $CD4^{+}$ T cells using zinc-finger nucleases rendered those cells resistant to infection and enabled CCR5-negative T cells to engraft and persist in HIV-infected patients [344]. Ablation of the T-cell receptor (TCR) gene with TALENs in CD19 CAR T cells enabled the use of third-party T cells to successfully induce remission in a patient with B-cell leukemia [345], an encouraging milestone on the way to “universal” off-the-shelf T-cell products with greatly diminished risk of graft-versus-host disease. In the latter study, the CD52 gene was also mutated in the transferred CAR T cells to enable resistance to alemtuzumab. In this study, we used the CRISPR/Cas9 system to ablate CD7 expression, because it allows for fast and efficient disruption of the target gene in T cells.

Although all genome editing tools are associated with potential off-target activity and mutations outside of the target gene, the off-target activity of CRISPR/Cas9 and other

editing systems can in part be predicted by using in silico methods and unbiased genome-wide screening [334], [335], [339]. We used these algorithms to select 2 gRNA with minimal predicted binding sites outside the target CD7 gene and assessed off-target activity by unbiased methods of identification of double-strand breaks in vitro [339] and verified in the intended target cells. Further in vivo validation can be achieved using deep sequencing to detect emerging oligoclonality or clonal dominance in patients and thereby identifying mutations that lead to a proliferative or survival advantage of T cells. Although these risks may vary according to the type of genome editing tool used [346], the consequences of off-target activity of genome editing tools should resemble the risks of random insertional mutagenesis by gammaretroviral or lentiviral vectors in T cells [347]. Despite the widespread use of these viral vectors over 20 years in more than 2000 patients in clinical trials, no evidence of malignant transformation has been observed when mature T cells are the targets for transduction, although exposure of hematopoietic stem cells to gammaretroviral vectors has led to malignant change following expression of some (but not all) transgenes [348]. At the moment, the use of CRISPR/Cas9 for a good manufacturing practice (GMP)–compliant production of therapeutic T cells is hampered by limited availability of GMP-grade Cas9 reagents. However, Cas9 can also be delivered as (cGMP) messenger RNA, which may circumvent the need for a GMP-grade recombinant protein; the efficiency of this approach for CD7 gene disruption in T cells remains to be tested.

The direct consequences of CD7 knockdown for residual immune function appear modest. Although CD7 is present in most peripheral T and NK cells, evidence that its expression is critical for mature T-cell function is lacking. CD7-knockout mice display unimpaired development and homeostasis of mature T cells and mount normal T cell-dependent immune responses [328], [329]. CD7 provides costimulation in T cells by activating PI(3)K and PI(4)K pathways [326], [327], [342] on binding its ligand K12/SECTM, which is expressed by myeloid cells and thymic epithelial and stromal cells [349]–[351]. In contrast, binding of galectin-1 by CD7 can trigger apoptosis [352], suggesting that CD7 may regulate T-cell proliferation and survival in a context-dependent manner. In our study, knocking out the CD7 gene in activated T cells did not affect their expansion or CAR-mediated cytotoxicity, suggesting that loss of CD7 is unlikely to impair the antitumor activity of CAR T cells—at least in the short term—and that costimulatory endodomains embedded in the CAR may compensate for the lack of CD7-derived costimulation. Finally, $CD7^{KO}$ T cells were able to respond to viral antigens through their native receptors in vitro, further demonstrating their maintained functionality.

Because CD7 is normally present on mature T and NK cells and their precursors, administration of CD7 CAR T cells may cause long-term ablation of these critical lymphocyte populations. This on-target off-tumor effect may have only limited impact on host immunity.

First, CD7 is not expressed by all peripheral T cells [353], [354] and therefore those CD7-negative subsets may be spared, limiting the extent of T-cell aplasia. Second, the infused CD7 CAR T cells are polyclonal and contain both nontransduced and CAR-transduced T cells. If these cells replenish the immune system, they may provide protection to viral (and likely other) pathogens via their native TCRs. Indeed, CD7 CARs could be expressed in multivirus-specific $CD7^{KO}$ T cells to further augment protection [355]. Furthermore, $CD7^{KO}$ CD7 CAR T cells could be used as a temporary "bridge-to-transplant" therapy, enabling subsequent stem cell transplant that would in turn terminate CAR T-cell activity. Finally, incorporating a clinically validated suicide switch system to eliminate CAR T cells if persistent toxicity occurs may provide an additional exit strategy [356]. One or several of these approaches could be used in combination to mitigate the risk of permanent T-cell aplasia.

In summary, this study demonstrates the feasibility and efficacy of $CD7^{KO}$ CD7 CAR T cells for the targeted therapy of T-cell tumors. Because $CD7^{KO}$ T cells retain activity against viral antigens through their native receptors, it should be feasible to use this approach to eradicate $CD7^{+}$ malignancies while retaining a functional T-cell immune system. Additionally, our genetic editing approach may enable the generation of CAR T cells to be redirected to other T-lineage antigens to broaden the range of targetable tumors or to target other tumors, which have aberrant of expression of this lymphoid marker such as Acute Myeloid Leukemia.

Chapter III.CD3 CAR as a treatment for T cell lymphomas

Development of chimeric antigen receptors allowed the ex vivo generation of T cells capable of recognizing antigens to which immune cells are considered to be tolerant. While self-tolerance is a key characteristic of the immune system to avoid auto-reactivity, CAR therapy could be designed to induce controlled, short-term immune responses against particular cell-surface antigens that are present in tumor cells and avoid extensive damage to the host. The search for more and better tumor markers is under intense research, and existing monoclonal antibody libraries facilitate the process of CAR design. However, earlier clinical results and safety profiles of such antibodies in humans do not guarantee the feasibility of CARs containing the corresponding scFvs. CARs and antibodies activate different immune cells and different pathways. Thus, while some CARs built on the success of monoclonal antibodies, others may not follow same path…

III.1.Introduction

Adoptive transfer of T cells engineered to express a chimeric antigen receptor that is activated by recognition of CD19 in B-cell blasts led to complete remissions in several patients with relapsed or refractory B-cell leukemia treated among different clinical trials [181], [195], [203], [357], increasing the number of available therapeutic options for such patients. This success was facilitated, among other reasons, by targeting a B-cell lineage-specific antigen that is not expressed by hematopoietic stem cells and by the limited toxicities resulting from on-target/off-tumor effects. Few are the cancers with antigens with such characteristics. Although T cells have pan-T-cell markers, few are truly T-cell specific, as these are also often expressed by other immune cells. Most importantly, on-target/off-tumor effects can potentially be severe, and not amenable to simple replacement therapies.

Targeting of the T-cell markers CD5 [219] or CD7 [358] using CAR-T cell therapy has been previously demonstrated by our group. Although precise targeting of a lineage specific antigen is associated with clinical responses, antigen loss, until recently considered a rare phenomenon for some antigens, is now viewed as one of the main reasons for relapses observed in clinical trials using CD19 CAR T cells [296]. This effect is particularly important when considering tumors such as T-cell malignancies, since loss of at least one pan-T-cell marker is observed in up to 70% of patients [323], even without targeted therapies. Therefore, targeting multiple antigens may be a desired approach to avoid tumor escape due to antigen loss as well as CAR-T cell inability to eliminate antigen-negative tumor T-cells.

CD3 is a pan-T-cell marker that is exclusively expressed by T cells. CD3 has several subunits (ε,γ,δ and ζ) that, together with the α and β subunits of the TCR, form a complex responsible for activation of T cells. While the ζ subunit has only internal domains, ε, γ and δ have small extracellular domains [359]. CD3 antibodies, particularly against the ε subunit, have been extensively tested and even used in the clinic. Muromonab (OKT3), an antibody recognizing CD3ε, was the first therapeutic monoclonal antibody approved by a regulatory agency. Initially used to reduce acute rejection after organ transplantation due to its immunosuppressant effect [360], its use as a potential therapeutic drug against T-cell acute lymphoblastic leukemia has been considered [361]. Despite its less frequent use in the clinic nowadays, muromonab and similar CD3 antibodies are still extensively used in research as an unspecific T cell stimulus, due to their ability to cross-link CD3 molecules and directly activate T cells, bypassing the need for TCR activation. Indeed, although clinically used to induce immunosuppression, OKT is acutely associated with transient T cell activation.

Although CD3 is only found in a small subset of immature T-cell malignancies, such as T-cell ALL, its expression is uniformly observed in the majority of patients with mature T-cell lymphomas [130]. T-cell lymphomas are an aggressive and difficult to treat type of hematological malignancies, with high rates of relapse and low long-term survival [362]. Currently, there are few treatment options for patients with relapsed or refractory disease.

Here, we investigate the feasibility of targeting CD3 using a chimeric antigen receptor derived from the OKT3 antibody clone as a treatment for T-cell lymphomas. We found that, similar to CD7 CAR, CD3 CAR induces extensive fratricide, preventing T-cell expansion and survival. Removal of CD3 using CRISPR/cas9 can, as demonstrated for CD7 CAR, avoid fratricide in this setting. Unexpectedly, although CD3 CAR-T cells can recognize $CD3^+$ leukemia/lymphoma cell lines, they were unable to eliminate normal T cells. In fact, by mimicking the activation effect observed with OKT3, CD3 CAR T cells activate normal T cells, which in turn can eliminate CD3 CAR T cells in vitro. This inability of CD3 CAR T cells to survive and proliferate in the presence of normal T cells may prevent their use in the clinic.

III.2.Material and Methods

III.2.1. CAR Design and Transduction

Three CD3-specific chimeric antigen receptors were created from OKT3, 6EHX and H1_L1.4 CD3 antibodies using commercial gene synthesis (Bio Basic, Inc) by cloning in a previously created CAR backbone containing CD28 and CD3ζ activating endodomains, and the C_H3 domain from IgG1 Fc as a spacer region. The OKT3 clone was used in all experiments except when stated otherwise. A CD19 CAR previously developed [363] with same backbone was used as a control. For transduction of all CARs, T cells were activated by plate-bound OKT3 and anti-CD28 antibodies, transduced with gammaretroviral vectors, and expanded in the presence of interleukin-7 (IL-7) and IL-15 in cytotoxic T-lymphocyte (CTL) media, as described before [219].

III.2.2. Genomic disruption of CD3 and FAS in T cells

Two guide RNAs (gRNAs) for the CD3 gene (gRNA-30: GGGCACTCA-CTGGAGAGTTC and gRNA-37: GGCACTCACTGGAGAGTTCT) and one for Fas (gRNA-60: GGAGTTGATGTCAGTCACTT) were designed using CRISPRscan and COSMID algorithms [334], [335]. Both genes were disrupted in T cells according to the published

protocol [336]. Briefly, the 20-nt sequence complementary to the specific gene locus was incorporated into an oligonucleotide primer and used to amplify the gRNA scaffold from PX458 plasmid (a gift from Feng Zhang; Addgene 48138). gRNAs were generated through in vitro transcription with High-Yield RNA Synthesis Kit (NEB Bio Labs) from the DNA template following the manufacturer's instructions, and purified using the RNA Clean & Concentrator-25 kit (Zymo Research). We electroporated 0.6 µg of gRNA and 1 µg of cas9 protein (PNA Bio) into 0.25×10^6 activated T cells using the Neon Transfection System (Thermo Fisher Scientific) in 10 µL of buffer T and using 3 1600-V 10-ms pulses. Following electroporation, T cells were incubated overnight in CTL media supplemented with 20% fetal bovine serum in the presence of IL-7 and IL-15. T cells were then expanded in normal media.

III.2.3. Flow cytometry

Anti-human CD3, TCR and Nerve Growth Factor Receptor - NGFR- (BD Biosciences) were used to stain cells in phosphate-buffered saline or CTL medium. Anti-IgG Fc (Jackson ImmunoResearch) was used for CAR detection in all assays. All flow cytometry data were obtained in BD fluorescence-activated cell sorter (FACS) Canto II (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC).

III.2.4. Cytotoxicity assay

Jurkat (ATCC TIB-152), Loucy (ATCC CRL-2629) and Hut 78 (ATCC TIB-161) cell lines were purchased from ATCC and expanded according to ATCC recommendations. KG1a (ATCC-CCL246.1) cell line was used as negative control. Tumor cells were stained with eFluor670 and incubated with CD3 CAR T cells for 3 days without exogenous cytokines, unless stated otherwise. Cells from individual wells were collected at indicated time points. We added 7-AAD to discriminate dead cells, and we obtained absolute cell counts with CountBright Absolute Counting Beads (Thermo Fisher Scientific) using flow cytometry.

III.2.5. Intracellular staining assay

Tumor cells were stained with eFluor670 (Thermo Fisher Scientific), according to the manufacturer's instructions. CD3 CAR T cells were cultured with stained tumor cells for 5 hours at a 1:4 effector to target ratio. Brefeldin A (BD GolgiPlug) was added 1 hour after plating. At the end of coculture, cells were incubated with antibodies for surface antigens and permeabilized for 10 minutes using BD FACS Permeabilizing Solution 2, followed by incubation with IFN-γ antibodies.

III.3.Results

III.3.1. Expression of CD3 CAR in T cells leads to T cell elimination

CD3 is ubiquitously expressed in T cells but not in other cell lineages in the body. Formation of complexes between CD3 and TCR molecules allows the activation of T cells, starting with binding of TCR to MHC molecules and activation of ITAMs present in several CD3 subunits. Since phosphorylation of CD3 is the initial intracellular activation event, this mechanism has been explored to activate T cells in a TCR-independent fashion: for example, through the incorporation of CD3 subunits' domains in CARs, or by using crosslinking CD3 antibodies. In this work, we used the well-known OKT3 clone to design a second generation CD3-specifc CAR with a IgG1 spacer (with C_H3 domain only) and CD28 as the co-stimulatory domain (Figure III-1A). The CAR vector also encoded a truncated form of the NGFR to allow easy detection. After introduction in T cells via a gamma-retroviral vector, however, CD3 CAR expression was not maintained, disappearing a few days after transduction (Figure III-1B). This loss of CAR expression was accompanied by a dramatic reduction in cell viability (Figure III-1C), which was attributed to fratricide, a phenomenon previously observed in CD7 CAR-T cells (Chapter II). Based on our experience generating CD7 CAR T cells, we decided to use CRISPR/Cas9 to remove CD3 from the cell surface as a way to avoid fratricide.

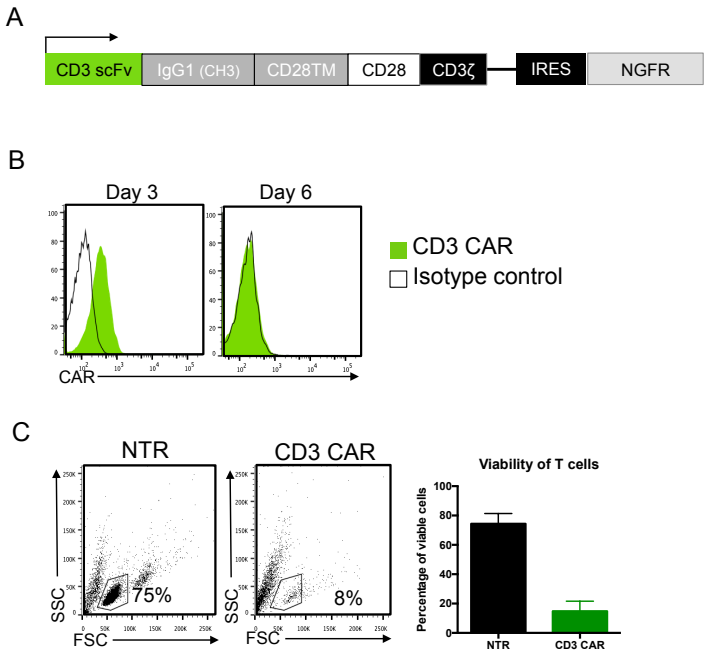


Figure III-1- Expression of a CD3 CAR leads to T–cell fratricide. (A) Schematic representation of second generation CD3 CAR with IgG1 spacer, CD28 transmembrane and co-stimulatory domains and CD3 zeta endodomain. **(B)** CD3 CAR expression 3 and 6 days after transduction of T cells with a γ-retrovirus. **(C)** Viable cells quantified by flow cytometry at day 4 after transduction using normal FSC and SSC properties. Bar graph represents the quantification for 3 donors.

III.3.2. Knockout of CD3 in T cells prevents fratricide induced by CD3 CAR

To generate CD3 CAR T cells without inducing fratricide, we used the same protocol developed to make CD7^{-} CD7-specific CAR-T cells using CRISPR/cas9 (Section II.3.2). T cells were first genetically modified to disrupt CD3, preventing its expression, and after 3 days CD3 CAR was introduced. After screening different gRNAs for CD3 disruption, two demonstrated high efficiency, proving it to be feasible to generate CD3^{-} T cells (Figure III-2A). Since the majority of the resulting T cells were CD3 negative, their transduction with CD3 CAR resulted in an efficient and stable CAR expression after several days in culture (Figure III-2B), with comparable viability to non-transduced T cells (Figure III-2C).

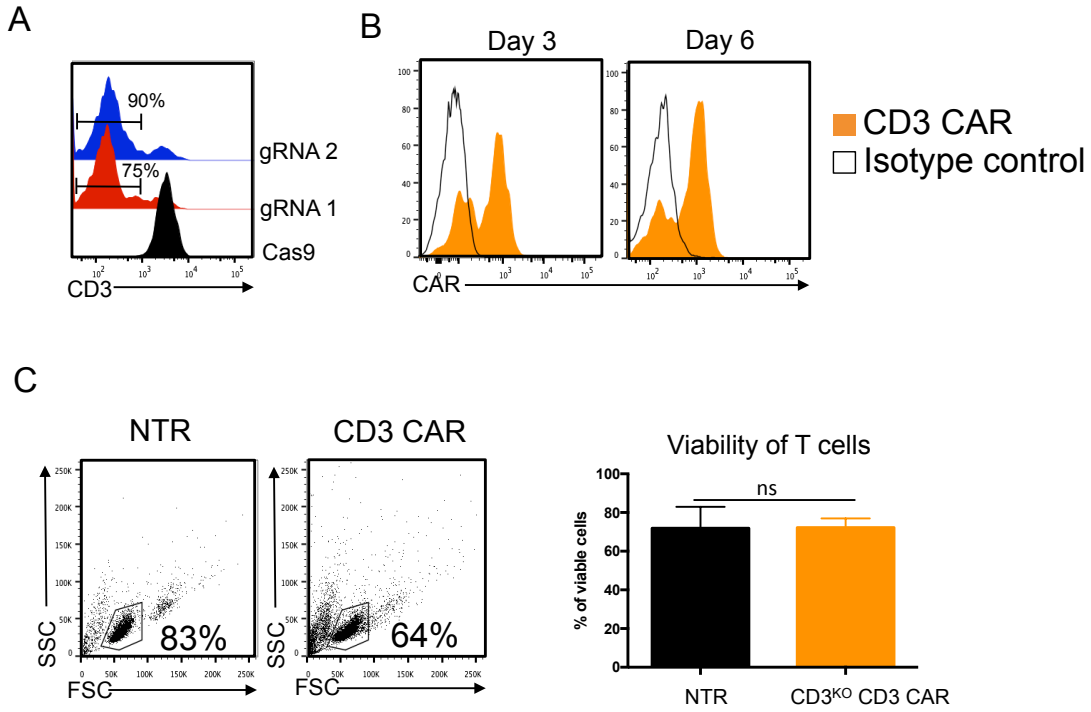


Figure III-2- Knockout of CD3 in CD3 CAR-T cells avoids fratricide. (A) Knockout of CD3 in normal T cells using two gRNAs that bind to CD3 gene. Cas9 only was used as control. **(B)** CD3 CAR expression 3 and 6 days after transduction of CD3^{KO} T cells with a γ-retrovirus. **(C)** Viable cells quantified by flow cytometry at day 4 after transduction using normal FSC and SSC properties in NTR and CD3^{KO} CD3 CAR. Bar graph represents the quantification for 3 donors. ns-not significant

III.3.3. CD3^{KO} T cells have similar cytotoxicity as unedited T cells in vitro

Since CD3 is an important molecule in T cell activation, we tested the performance of CD3-negative T cells transfected with a CD19 CAR versus a CD3-positive counterpart. A CD19 CAR was chosen for this comparison because of the inability to generate CD3-

positive CD3 CAR-T cells. CD19 CAR-T cells, with or without CD3 expression, were cocultured with the CD19^{+} Raji tumor cell line at 1:4 effector to target ratio for 3 days. At the end of culture, tumor cells were quantified by flow cytometry (Figure III-3A). Disruption of CD3 in CD19 CAR-T cells did not affect the ability of T cells to be activated through the CAR and to eliminate target cells (Figure III-3B), demonstrating that endogenous CD3ε is not required for CAR-T cell cytotoxicity. Notably, detection of IFN-γ production using intracellular staining demonstrated a higher percentage of IFN-γ^{+} cells in unedited (CD3^{+}) CD19 CAR-T cells compared to CD3^{-} CD19 CAR-T cells. This suggests that the presence of CD3ε and/or proper assembly of TCR/CD3 complexes can lead to higher production of cytokines, a characteristic associated with better CAR-T cells performance and persistence in vivo. Nonetheless, CD3^{-} CAR-T cells were still able to produce IFN-γ, but to a lesser extent.

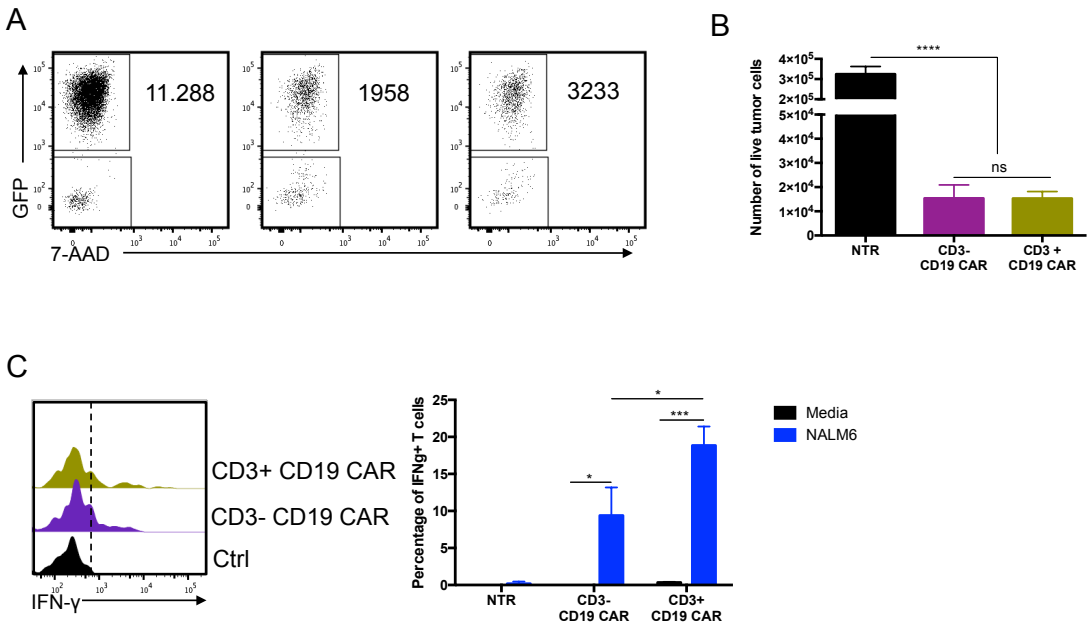


Figure III-3- Comparison of cytotoxicity and cytokine production between CD3+ and CD3KO CD19 CAR-T cells. (A) Representative dot plots and **(B)** quantification of remaining live tumor cells in 3 donors after a of coculture assay with RAJI tumor cells and CD19 CAR T cells with or without CD3 for 3 days at 1:4 effector:target. Numbers denote total number of live tumor cells per well. **(C)** Representative histogram of intracellular staining for IFN-γ of CD19 CAR T cells after 4h coculture with RAJi cells. **(D)** Total percentage of IFN-γ+ cells for 3 donors. NTR non-transduced; Ctrl- Control (non-transduced cells without tumor cells). (*P>0.05 **P>0.001 *P>0.0001)

III.3.4. CD3 disruption prevents TCR expression of cell surface.

All CD3 and TCR subunits are required for proper assembly of the TCR/CD3 complex, which then migrates to the cell membrane. Disruption of this process prevents the TCR from being expressed on the membrane. Indeed, CD3ε gene disruption using CRISPR/cas9 not only abolished CD3 expression but also prevented the expression of the TCR on the T cell surface (Figure III-4A). It is not conceivable that TCR genes were disrupted during the

process and, thus, absence of the ε subunit of CD3 likely led to TCR retention in the cytoplasm. Without their TCR, T cells can in theory be collected from a third party and injected in an HLA-mismatched patient without any risk of causing GvHD. To test this hypothesis in vitro, activation of normal T cells or CD3 CAR T cells was assessed by incubating them with peptides derived from staphylococcus or a combination of three viruses (CMV, EBV and adenovirus), all capable of activating T cells through the TCR (Figure III-4B). While normal T cells were activated by these different stimuli (detected by IFN-γ production), CD3 CAR T cells, because of the lack of TCR, did not respond to any of the bacterial and viral peptides.

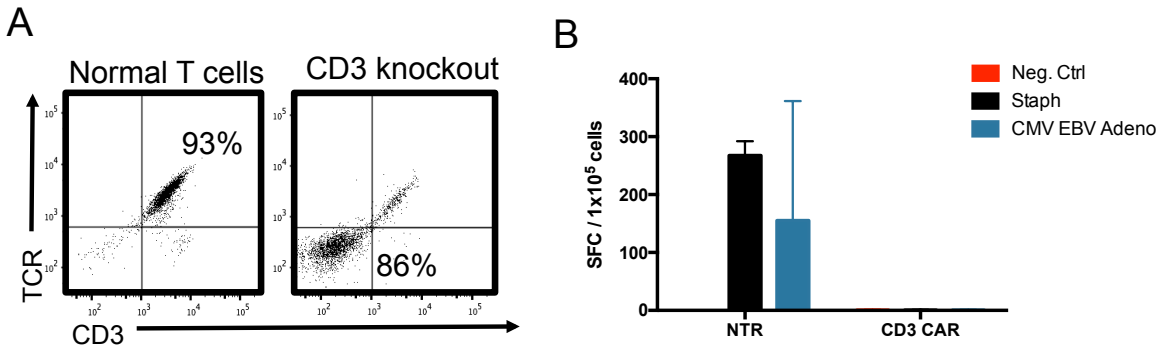


Figure III-4- Lack of activation of CD3^{KO} T cells due to lack of TCR in cell surface. (A) Expression of TCR and CD3 in normal T cells (left) and after CD3^{KO} (right). **(B)** Results of ELISPOT to detect IFN-γ in T cells previously incubated in bacterial peptide from staphylococcus and a mixture of viral peptides (CMV, EBV and adenovirus)

III.3.5. CD3 CAR T cells eliminate tumor cell lines in vitro

Although it is expressed in all normal T cells and in the majority of patients with T-cell lymphoma, CD3 is present only in a small subset of patients with other T-cell malignancies [323]. To test CD3 CAR T cell cytotoxicity, we cocultured these cells with CD3^{+} cell lines derived from T-cell leukemias (Jurkat and Loucy) and lymphomas (Hut-78) (Figure III-5A). Of note, while Jurkat is an αβ TCR+ cell line, Loucy expresses the less common γδ TCR. CD3 CAR T cells were cocultured with all three cell lines at 1:10 effector to target ratio for 3 days. Remaining live tumor cells were quantified by flow cytometry (Figure III-5B). While non-transduced T cells failed to eliminate tumor cells, CD3 CAR T cells were able to recognize and eliminate all CD3^{+} tumor cell lines (Figure III-5C).

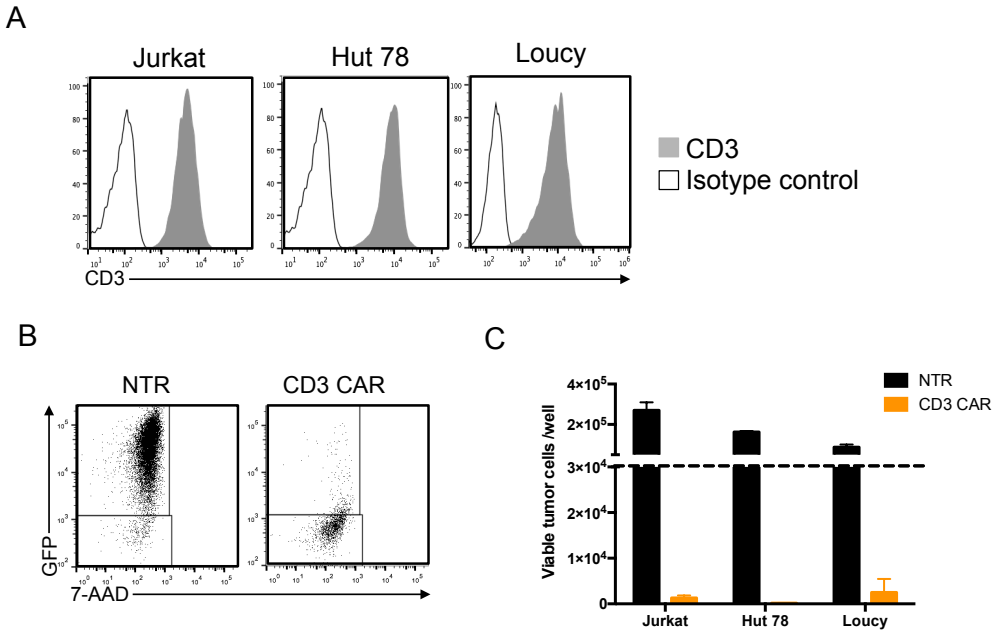


Figure III-5- Elimination of tumor cell lines in vitro by CD3 CAR T cells. (A) Expression of CD3 in Jurkat, Loucy (T cell leukemias) and Hut-78 (T-cell lymphoma) cell lines compared with isotype control. **(B)** Representative dot pots of a killing assay where CD3 CAR T cells or nontransduced (NTR) cells were coculture for 3 days with tumor cell lines at 1:10 effector:target ratio. **(C)** Total number of live tumor cells remaining after the killing assay.

III.3.6. CD3 CAR T cells are eliminated by normal T cells

Since CD3 expression in healthy cells is limited to T cells, on-target/off-tumor effect is expected to be limited to elimination of this specific type of lymphocytes. To test the ability of CD3 CAR-T cells to eliminate normal T cells, we performed a killing assay at 1:10 effector to target ratio. Unexpectedly, however, after quantification of live normal T cells and CD3 CAR T cells (Figure III-6A), not only did we observe that normal T cells were spared by CD3 CAR-T cells (Figure III-6B), but also that CD3 CAR-T cells were completely eradicated (Figure III-6C). To demonstrate that this unanticipated observation resulted from coculture of CAR-T cells with healthy CD3+ T cells, we performed a parallel coculture with a CD3 negative tumor cell line, which did not eliminate nor was eliminated by CD3 CAR-T cells as expected. This demonstrates that CD3 CAR can activate CD3 in normal target T cells and that this mechanism is stronger or faster that T cells activation through the CAR, leading to elimination of the intended effector cells by the intended target cells. To circumvent this deleterious effect, we employed several modifications in CAR design to try to avoid the elimination of CAR-T cells by normal T cells.

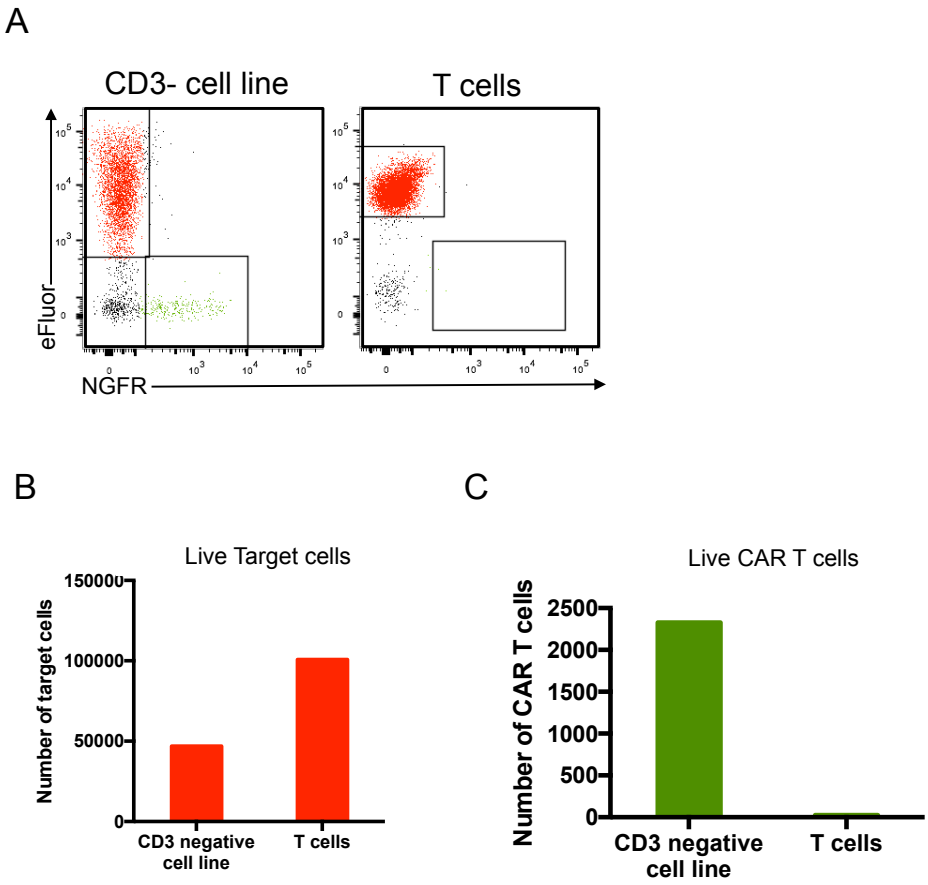


Figure III-6- Elimination of CD3 CAR T cells in culture with normal T cells. (A) Representative dot plot of a killing assay of CD3 CAR T cells with KG1a (CD3- myeloid leukemic line) used as control and normal T cells at 1:10 effector:target ratio. **(B)** Number of live target cells and **(C)** live CAR T cells at the end of the killing assay after 3 days. In both dot plots and bar graphs, red is used for target cells and green for CAR T cells.

III.3.7. Different scFvs do not prevent normal T cell-induced CD3 CAR-T cell fratricide

CD3 activation to expand T cells is commonly achieved in vitro by using OKT3 antibody, which crosslinks CD3 molecules on the cell surface. Since different antibodies may have distinct properties, we decided to test two new CARs with scFvs derived from two alternative monoclonal antibodies (clones 6EHX and H1_L1.4). CD3 CAR expression with these new scFvs was similar to that of the first CAR (Figure III-7A). However, coculture of the new CD3 CAR T cells with normal T cells (Figure III-7B) did not prevent the elimination of CAR T cells (Figure III-7C,D). This demonstrates that this undesired elimination is not dependent on the type of scFv used in the CAR and that different scFvs can equally activate CD3 in normal T cells.

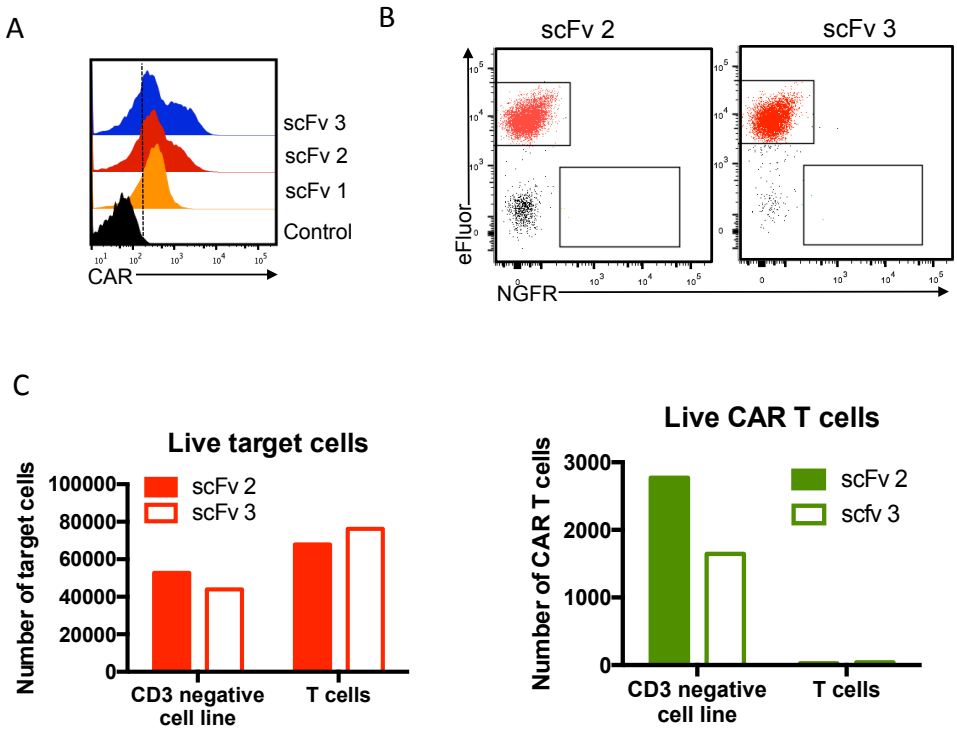


Figure III-7- Use of different scFvs to avoid CD3 CAR T cell elimination. **(A)** Expression of CD3 CAR created from different scFvs in T-cell surface detected by flow cytometry. **(B)** Representative dot plots of a killing assay with two new CD3 CAR T cells with normal T cells at 1:10 effector:target ratio. **(C)** Number of live target cells and **(C)** live CAR T cells at the end of the killing assay after 3 days. In both dot plots and bar graphs, red is used for target cells and green for CAR T cells.

III.3.8. Changes in spacer of CD3 CAR do not prevent CD3 activation in normal T cells

Because CARs are created using sequences from other human receptors, some of their original properties may be present in the CAR molecules as well. Because most of these human receptors are folded as dimers, CARs are also likely to exist as dimers [364], [365] . As an attempt to avoid or reduce CD3ε crosslink in normal cells, we tried to create a CD3 CAR that would be expressed as a monomer. Since we used an IgG sequence to make the spacer in the CAR, we removed both cysteines in the hinge part of the CAR and replaced them by two serines (Figure III-8A), preventing CAR molecules from forming the disulfide bonds that usually attaches two IgG molecules. The rationale for doing this is that we assumed that binding of a dimeric CAR to CD3 would automatically crosslink two CD3 molecules. If the CARs were expressed as monomers, there would be reduced likelihood of CD3 crosslinking. However, once more, in a similar killing assay in coculture with normal T cells (Figure III-8B), even when expressed as monomers, CD3-specific CARs activated target cells, which in turn were capable of eliminating the engineered T cells (Figure III-8C).

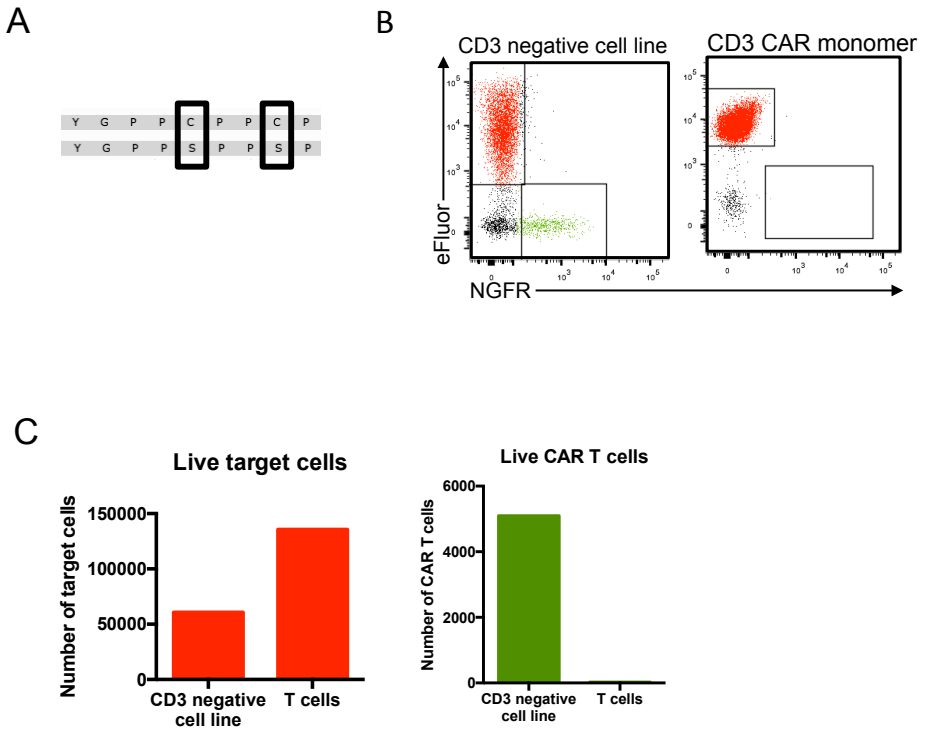


Figure III-8- Creation of CD3 CAR monomer to avoid CD3 crosslink in T cells. (A) Representative schematic of the modification in the sequence of the small hinge present in the CAR to avoid its dimerization. **(B)** Representative dot plots of a killing assay of CD3 CAR T cells with KG1a (CD3- myeloid leukemic line) used as control and normal T cells at 1:10 effector:target ratio. **(C)** Number of live target cells and **(D)** live CAR T cells at the end of the killing assay after 3 days. In both dot plots and bar graphs, red is used for target cells and green for CAR T cells.

III.3.9. Arming CD3 CAR T cells to avoid elimination by normal T cells

Since several approaches to modify the CD3-specific CAR did not prevent CD3 crosslinking, we attempted next to avoid elimination of CAR T cells by arming them with genes that confer protection against T-cell cytotoxicity. We over-expressed PD-L1 (to activate PD-1 in normal T cells, which is an inhibitory receptor), cathepsin B and PI-9 proteins (intracellular inhibitors of granzyme B and perforin, respectively), by including one of the corresponding genes them in the viral cassette together with CD3 CAR (Figure III-9A). Because of the small cargo size that γ-retroviral vectors can deliver to cells, these new constructs precluded the inclusion of NGFR as a detection system for CAR expression. However, staining for PD-L1 (the only cell surface protein) demonstrated a transduction efficiency similar to that observed with the original CD3 CAR (Figure III-9B). Despite our inability to detect CAR expression directly, these armed T cells were able to efficiently eliminate the $CD3^+$ Hut-78 line in a killing assay (Figure III-9C,D), demonstrating that the CAR was being adequately expressed. Since we observed that the transduced T cells can eliminate tumor cells despite lack of direct CAR detection, we decided to test their activity against normal T cells. Unfortunately, in a coculture with normal cells, overexpression of PD-

L-1, cathepsin B or PI-9 by CAR-T cells was unable to produce elimination of the target cells (Figure III-9E,F). Although we could not directly detect CAR^{+} T cells, because tumor cells were eliminated by the transduced cells but normal cells were not, we were forced to assume that CAR-T cells were still being eliminated by normal T cells and that none of these 3 gene products conferred any protection against killing.

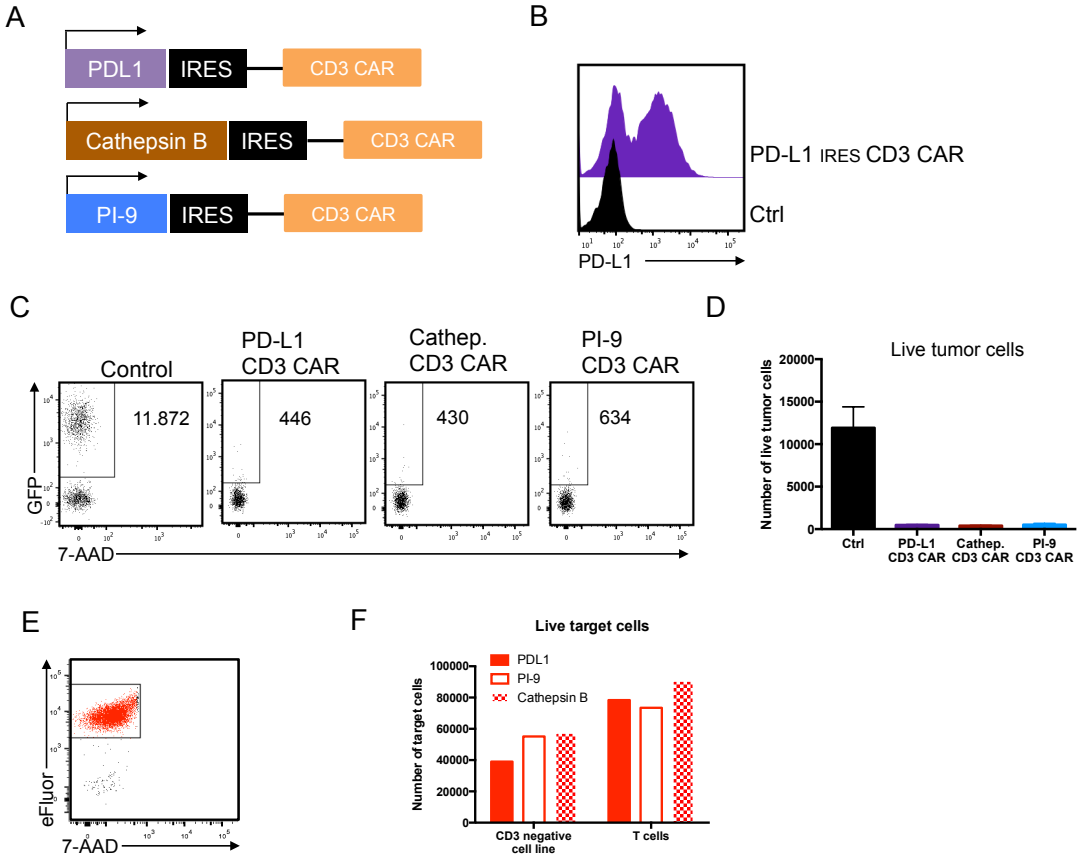


Figure III-9- Armed CD3 CAR T cells to inhibit degranulation of normal T cells when in contact in CD3 CAR T cells. (A) Schematic representation of new constructs to overexpress PD-L1, Cathepsin B and PI-9 in CD3 CAR T cells. PD-L1 expression in PD-L1 CD3 CAR T cells detected by flow cytometry 3 days after transduction. **(C)** Representative dot plots of killing assay of CD3 CAR T cells with Hut-78 tumor cell line at 1:4 effector:target ratio for 3 days and **(D)** the total number of live tumor cells after the killing assay. **(E)** Representative dot plot of a killing assay of CD3 CAR T cells with normal T cells at 1:10 effector:target ratio for 3 days. **(F)** Number of live target cells at the end of the killing assay after 3 days.

III.3.10.Removal of FAS to prevent CD3 CAR T cell elimination

Since we were unable to prevent the activation of normal T cells due to CD3 crosslink by PD1 ligation or granzyme B/perforin inhibition, we tried an alternative approach. It has previously been demonstrated that CD3 crosslinking activates expression of Fas in the cell surface [366], leading to T-cell apoptosis. We confirmed an increase in Fas expression in T cells (Figure III-10A), but also the upregulation of Fas ligand. Thus, we hypothesized that elimination of CD3 CAR-T cells could be due to Fas activation in CAR-T cells by Fas ligand expressed in normal T cells. So we disrupted Fas and CD3 expression at the same time in

CD3 CAR T cells (Figure III-10B). Interestingly, we observed that Fas$^-$ CD3 CAR-T cells were only slightly less efficient in eliminating the Hut-78 lymphoma line in a short-term killing assay (Figure III-10C).

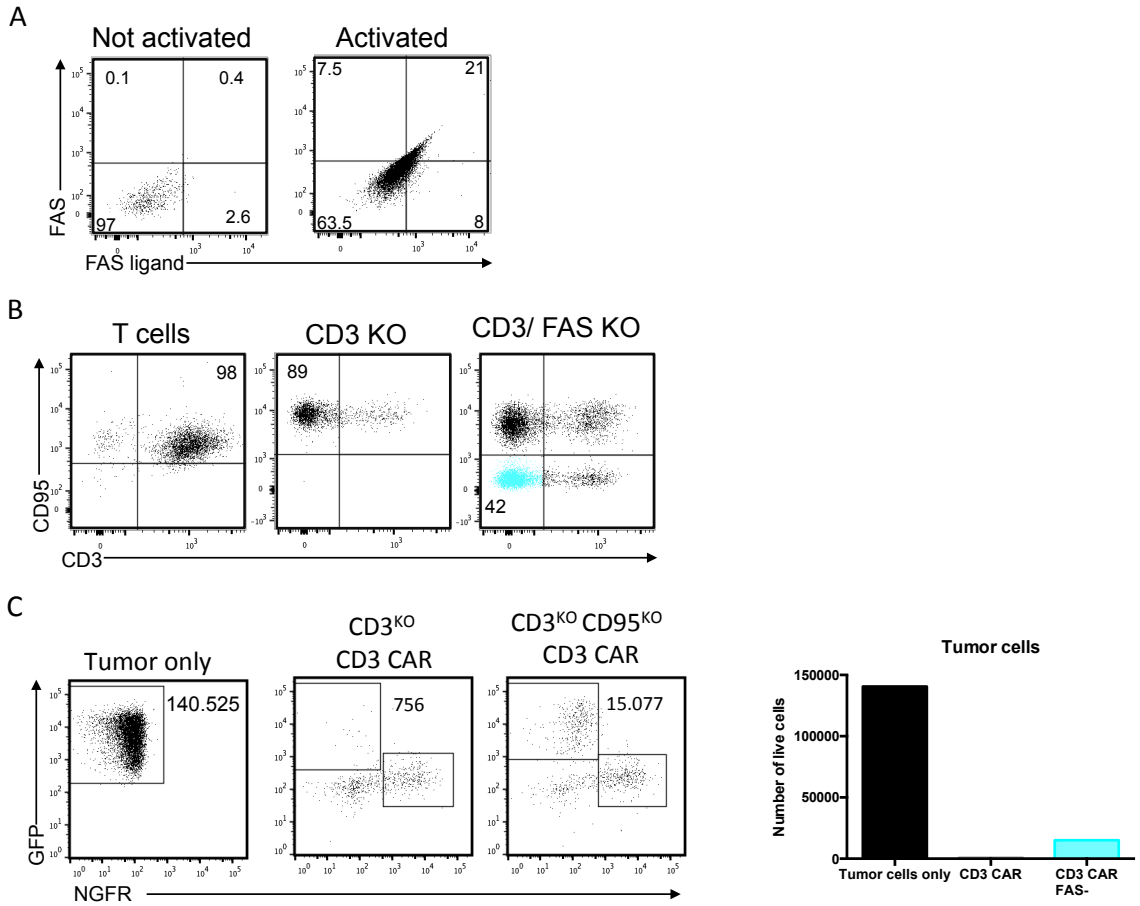


Figure III-10- Knockout of Fas in CD3 CAR T cells. (A) Expression of Fas and Fas Ligand before and after activation of T cells with CD3 antibody. **(B)** Expression of Fas and CD3 before (left) and after knockout of CD3 only (middle) and CD3 and CD95 using CRISPR/cas9 with gRNAs specifically designed to bind in CD3 and CD95 genes. **(C)** Representative dot plots of killing assay of CD3 CAR T cells with Hut-78 tumor cell line at 1:4 effector:target ratio for 3 days. Bar graph shows the total number of live tumor cells after the killing assay.

Roughly 40% of the T cells used to generate CD3 CAR-T cells, were double negative (Fas$^-$ CD3$^-$). These cells were then used in a coculture with normal T cells. If the activation of the Fas pathway was indeed responsible for elimination of CAR+ cells, Fas$^-$ CD3$^-$ CD3-CAR$^+$ T cells would be spared apoptosis and should be able to eliminate the target cells (Figure III-11A). However, once more, CD3 CAR-T cells were equally eliminated regardless of Fas expression (Figure III-11B), demonstrating that Fas-related apoptosis is not primarily responsible for elimination of CD3 CAR-T cells.

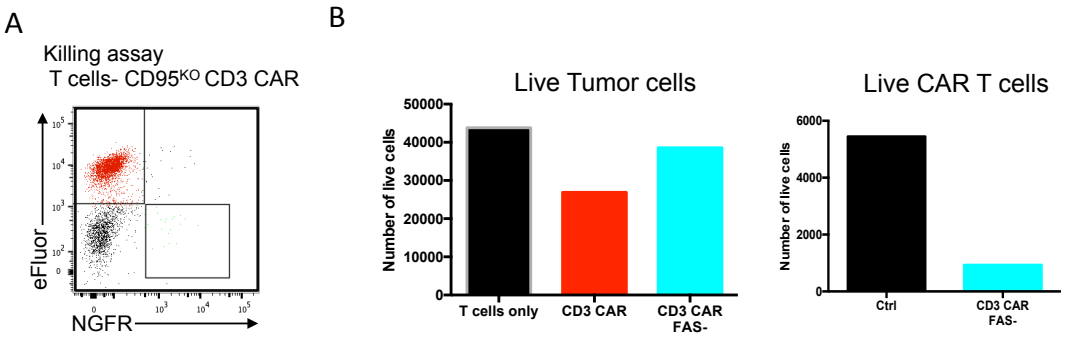


Figure III-11- Killing assay of Fas- CD3 CAR T cells with normal T cells. (A) Representative dot plots of a killing assay with CD3 CAR T cells without FAS with normal T cells at 1:10 effector:target ratio. **(B)** Number of live target cells and live CAR T cells at the end of the killing assay after 3 days.

All these attempts at preventing elimination of CD3 CAR-T cells when cocultured with normal CD3+ T cells failed, indicating that upon injection into patients CD3 CAR-T cells will likely be prematurely eliminated by T cells, decreasing their persistence and anti-tumor effect.

III.4.Discussion

Genetic modification of T cells with CARs allows redirecting lymphocytes against a particular antigen associated with a specific disease. CD3 is expressed in tumor cells of several patients with T-cell malignancies. Thus, the creation of a CD3 CAR could potentially make any T cell able to eliminate $CD3^+$ cells. Our work showed that CD3 CAR T cells are capable of eliminating different malignant T-cell lines. However, CD3 is also expressed in normal T cells and, in contrast to what has been demonstrated for other CARs, even when the target antigen is knocked out in CD3-specific CAR T cells, not only were they unable to eliminate normal T cells, but they were themselves killed, possibly due to crosslinking of CD3 in native T cells by the CAR. Therefore, this work suggests that targeting CD3 with a CAR may not be a feasible therapeutic approach.

Oncologic treatment has been shifting to targeted therapies. Such approach allows a more personalized treatment, with potential less side effects, since it is directed against a specific tumor marker. Despite being a targeted therapy, tumor cells can still avoid their elimination by downregulating that particular target. Indeed, downregulation of target antigens for antibodies and CAR T cells has already been demonstrated. Although CD3 has a more limited expression than CD5 or CD7 in T cell malignancies, it is still commonly found in patients with T-cell lymphoma and thus could still be an interesting target for CAR-T cell therapy, especially in combination with other T-cell-specific CARs, so as to avoid relapses due to antigen loss. Because normal T cells also express CD3, self-elimination of T cells

(fratricide) immediately after CAR transduction was observed as expected. Since the same phenomenon was observed with a CD7-specific CAR, we employed the same strategy described in chapter II to avoid fratricide: disruption of CD3 before introduction of the CD3 CAR avoided the toxicity. Interestingly, both CD3 and CD7 required 3 days for complete disappearance from cell surface, so no further optimization of the protocol was required to create CD3 CAR-T cells.

Lack of the CD3ε subunit completely abolishes proper assembly of TCR/CD3. Consequently, after CD3 disruption, edited T cells do not express TCR, which should prevent these edited cells from being activated by pepMHC presentation. Although these TCR^- are not able to recognize diseased cells using their native receptors, they should also not be able to recognize and eliminate normal cells bearing a mismatched HLA type. Thus, in theory, they could potentially be used in an allogeneic context without any risk of GvHD. In this work, we demonstrated that $CD3^-$ CD3 CAR-T cells do not respond to bacterial and viral antigens due to lack of TCR expression. Nonetheless, in vivo studies would have be performed to definitely conclude that these cells would not elicit GvHD. Ablation of TCR expression using gene editing tools is under intense research as a means to create off-the-shelf universal T cells [315], [316]. Although this step is not expected to improve their anti-tumor effect, it can potentially allow a wider use of CAR-T cell therapy, with improved manufacturing processes at eventually decreased costs. This approach has already been tested in the clinic [317]. Although no severe GvHD was observed, some mild alloreactive effects were still detected. Such effects were attributed to a small percentage (<0.1%) of residual TCR^+ T cells, which could not be effectively depleted by magnetic sorting. Interestingly, a purification step would not be required for CD3 CAR-T cells since any remaining $CD3^+$ T cells would be eliminated by the CD3 CAR-T cells. Although we observed this native T cell elimination by CD3 CAR T cells when just a small percentage of cells in culture were $CD3^+$, when we used a more physiological ratio to assess the effect of CD3 CAR T cells on endogenous T cells, CD3 CAR-T cells were themselves unexpectedly eliminated despite their efficient killing of $CD3^+$ tumor cell lines. Changes in CAR design did not prevent this phenomenon that we attributed to crosslinking and activation of TCR/CD3 on the target cells when the CAR binds to the CD3ε subunit. Although not an entirely unforeseen effect, considering the ability of CD3 antibodies to activate T cells, we predicted the CAR would be simultaneously fully activated since it leads not only to CD3 but also co-stimulatory (in this case, CD28 signaling). Indeed, when a lower effector to target ratio is used (e.g. 1:1) CD3 CAR T cells can eliminate normal T cells but are also concomitantly eliminated, demonstrating a similar potency of both cell populations despite the differences in their activating signals. Attempts to prevent CD3 CAR-T cell elimination by overexpression of genes important for T-cell activation/degranulation did not rescue CD3 CAR-T cell

elimination. CD3 CAR-T cells overexpressing PD-L1 (which prevents TCR activation by binding to PD-1 [367]), cathepsin B (which inhibits pore formation by perforin on the cell surface of lymphocytes [368]), or PI-9 (a granzyme B protease inhibitor that protects cells from apoptosis induction in target cells [369]) were similarly eliminated by normal T cells. Furthermore, disruption of Fas, an important receptor in T cell homeostasis [73] that is upregulated by CD3 monoclonal antibodies [366], did not change the negative outcome. It is possible that other innate death receptors, such as TRAIL, induce a similar effect but this was not demonstrated.

Interestingly, a CD3 CAR has been already reported using the NK-92 cell line [370]. Since NK cells do not express CD3, its knockout is not required. However, NK cells and the NK-92 cell line do not persist as long as T cells, and thus their anti-tumor activity has not been validated yet. Although the same work demonstrated the ability of CD3 CAR NK-92 cells to eliminate normal T cells, although at lower effector to target ratios that may not be similar to what transferred cells would encounter in vivo, the viability of CD3 NK-92 cells after meeting normal T cells was not demonstrated. It is conceivable, nonetheless, that NK-92, a leukemic cell line, can employ mechanisms similar to those of other tumor cells to avoid being killed. Moreover, apart from variations in the type of cells used, differences in CAR design (third generation with CD28 and 4-1BB) and transduction system (lentivirus) cannot be ruled out as reasons for this discrepant result.

An alternative approach to a CD3 CAR was demonstrated by Pule and collaborators, who instead of targeting CD3 designed a CAR specific for the constant region of the TCR β subunit [371]. The novelty of this CAR is that, like the kappa CAR for B-cell leukemias [372], it avoids complete T-cell aplasia by eliminating only the portion of T cells that express the TRBC1 constant region without affecting T cells whose TCR constant region is encoded by the TRBC2 gene. As in the CAR NK-92 model, the authors demonstrated elimination of normal T cells without establishing viability of the CAR T cells after killing. However, they did demonstrate in a mouse model engrafted with human PBMCs a statistically significant reduction of CAR^+ T cells and maintenance of normal T cells in the spleen comparing to controls. It is unclear at the moment if any of these two alternatives (CD3 CAR NK-92 cells or TRBC1 CAR T cells) will lead to low persistence in vivo, but this will be assessed in future clinical trials.

Based on these results, we conclude that targeting of CD3 with CAR-T cells may not be a feasible approach. Even if a solution to prevent CD3 CAR-T cell elimination is found, there may be a higher risk of cytokine release syndrome associated with this particular CAR. Cytokine release syndrome has been one of the side effects of CAR T cell therapy in the clinic that has raised more concerns [282]. High levels of pro-inflammatory cytokines are commonly detected in patients that receive CAR-T cells. Since CD3 CAR-T cells can

activate endogenous T cells, both transfected and native T cells can produce cytokines, further increasing the risk of developing this life-threatening syndrome. The capacity for CD3 CAR-T cells to eliminate normal T cells without activating them or to eliminate T-cells before they secrete cytokines must be proven before further clinical testing.

Chapter IV. CD7 CAR T Cells for the Therapy of Acute Myeloid Leukemia

An ideal targeted therapy is strictly directed against molecules that are exclusively expressed by malignant cells. However, true tumor specific antigens are scarce. While the search to find what could be ideal targets continues, the field has investigated antigens that, although not free of toxic effects, enable the destruction of malignant cells with minimal and manageable side effects. Among possibly targetable antigens, those aberrantly expressed by tumor cells have been exploited. Acute myeloid leukemia is a heterogeneous cancer often associated with aberrant phenotypic changes. Not uncommonly, these phenotypic changes are accompanied by the aberrant expression of lymphoid markers, such as CD56 or CD7, making some of them interesting targets for such directed therapies…

Part of this chapter was published as:

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IV.1.Introduction

Although the standard-of-care treatment for acute myeloid leukemia (AML) commonly induces complete remissions, in many patients the disease will relapse with poor prognosis and few options for targeted therapy. Chimeric antigen receptor (CAR) T cells targeting myeloid-lineage antigens such as CD33, CD123, Lewis Y, CLL-1, CD44v6, and folate receptor β have produced promising results in preclinical models of AML [220], [373]–[378]. However, evidence of sustained complete responses in patients treated with CD33 CAR, CD123 CAR, or Lewis Y CAR T cells has been lacking [178], [210], [379]. In addition, because T cells expressing these CARs do not discriminate between malignant cells and normal cells expressing the same antigen, clinical application is associated with toxicity against normal hematopoietic progenitor cells [379].

Alternatives to the above approach are to target antigens that are selectively expressed on leukemic and mature normal cell subpopulations but are absent on critical primitive progenitors (e.g., CLL-1 [376]) or to choose an antigen expressed by malignant myeloid cells but that is absent on the normal members of that lineage. Both these approaches mitigate on-target toxicity against critical myeloid precursors, but it is not yet known which is superior. In this study, we describe the targeting of a lineage-restricted antigen, CD7, that is absent on normal myeloid cells but which becomes aberrantly expressed by leukemic clones. This aberrant expression allows selective elimination of the malignant cells but spare normal myeloid cells and their precursors

CD7 is a transmembrane glycoprotein expressed by 30% of AML, and its expression is associated with more aggressive disease and resistance to standard therapy [380]–[385]. Normal CD7 expression is largely limited to T and NK cells and their precursors, where it acts as a costimulatory receptor for T- and B-lymphocyte interactions during lymphoid development. The function of CD7 in mature T cells is likely redundant, as CD7-deficient mice demonstrate largely unperturbed peripheral T cell function. CD7 is also expressed in a subset of myeloid progenitors in cord blood, although its role in these cells has not yet been defined [386]–[388].

Although the expression pattern of CD7 suggests it can be used to target AML with high selectivity and limited toxicity against normal myeloid cells, its presence on normal T cells means that expression of a CD7 CAR on these cells would not be feasible, since it would prove fratricidal [343], [358], [389]. We therefore developed a means of editing the CD7 gene in T cells prior to CD7 CAR expression, and have showed that such edited T cells expand well and retain functionality through both their native and chimeric receptor (Chapter II) [358].

Here, we demonstrate that CD7-edited ($CD7^{KO}$) CD7 CAR T cells can selectively eliminate AML cell lines, primary leukemia blasts and AML precursor cells in vitro and in a xenograft model of the disease, without evident toxicity against normal myeloid cells and hematopoietic progenitors. These results support the feasibility of targeting AML with CD7 CAR T cells.

IV.2.Materials and Methods

IV.2.1. Generation of CD7 CAR T cells

A CD7-specific single-chain variable fragment derived from clone 3A1e of CD7-specific antibody was created using commercial gene synthesis (Bio Basic, Inc) and cloned into a second-generation CAR backbone containing CD28 and CD3ζ endodomains and the C_H3 domain from IgG1 Fc as a spacer region [219], [358]. A truncated CD7 CAR lacking signaling endodomains was used as a control. T cells were activated by plate-bound OKT3 and anti-CD28 antibodies and CD7 gene was removed using CRISPR/cas9 before transduction as previously described (section II.2.2) [336], [358]. Briefly, T cells were electroporated with 0.4 µg of gRNA and 1 µg of cas9 protein (Integrated DNA Technologies) with $0.25x10^6$ of activated T cells by using the Neon Transfection System (Thermo Fisher Scientific) in 10 µL of buffer T and using 3 1600-V 10-ms pulses. Following electroporation, T cells were incubated in CTL media supplemented with 20% fetal bovine serum (FBS) in the presence of IL-7 and IL-15 overnight after electroporation. T cells were then expanded in cytotoxic T-lymphocyte (CTL) media in the presence of interleukin-7 (IL-7) and IL-15 and 10% FBS for 3 days and transduced with gammaretroviral vectors encoding CD7 CARs.

IV.2.2. Flow cytometry

Anti-human CD7 (Biolegend); CD4, CD8, CD14, CD15, CD33, IFN-g (BD Biosciences); and CD45 (Beckman Coulter) were used to stain cells. Anti-IgG Fc (Jackson ImmunoResearch) was used for CAR detection in all assays. All flow cytometry data were obtained in BD fluorescence-activated cell sorter (FACS) Canto II (BD Biosciences) and Gallios (Beckman Coulter) and analyzed with FlowJo software (FlowJo, LLC).

IV.2.3. Cytotoxicity assay

Cell lines GDM-1 (ATCC CRL-2627) and Kasumi-3 (ATCC CRL-2725) were purchased from ATCC. KG1a cell line was a gift from Dr. Cliona Rooney. The cells were expanded according to ATCC recommendations. Tumor cell lines were stained with eFluor670

(Thermo Fisher Scientific) and incubated with CD7 CAR T cells for 3 days without exogenous cytokines, unless stated otherwise. Cells from individual wells were collected at indicated time points. 7-AAD was added to discriminate dead cells, and cells were quantified using CountBright Absolute Counting Beads (Thermo Fisher Scientific). For a sequential killing assay, after flow cytometry analysis, tumor cells were added to same CAR T cells using the original effector:target (E:T) ratio of 1:4 until CAR T cells. Frozen PBMCs from AML patients at Methodist Hospital and Texas Children's Hospital were used for killing cytotoxic assay at ratio 1:1 (E:T) for 48 hours. AML patients and healthy donors gave written informed consent to be entered on protocols approved by the Baylor College of Medicine Institutional Review Board, in accordance with the Declaration of Helsinki.

IV.2.4. Intracellular staining assay

CD7 CAR T cells were cultured with tumor cells for 5 hours at a 1:4 E:T ratio. Brefeldin A (BD GolgiPlug) was added 1 hour after plating. At the end of coculture, cells were incubated with antibodies for surface antigens and permeabilized for 10 minutes using BD FACS Permeabilizing Solution 2, followed by incubation with anti-IFN-γ antibody and analyzed by flow cytometry.

IV.2.5. AML mouse xenograft model

Five- to seven-week-old nonobese diabetic (NOD)–Cg-^{Prkdcscid} Il2$^{rgtm1Wjl/SzJ}$ (NSG) mice were purchased from the Jackson Laboratory and maintained at the Baylor College of Medicine Animal Facility. All procedures were done in compliance with the Institutional Animal Care and Usage Committee of Baylor College of Medicine. Mice were sublethally irradiated (200 cGy) and injected intravenously 24h later with $1x10^6$ KG-1a-FFluc cells followed by a single injection of $2x10^6$ of CD7 CAR T cells 5 days later. Tumor burden was monitored using an IVIS Imaging system (Caliper Life Sciences) by recording bioluminescence from mice injected with 150 mg/kg of D- luciferin intraperitoneally at indicated time points. Living Image software (PerkinElmer) was used to visualize and calculate total luminescence. For analysis of tumor cells in peripheral blood, 100 µL of blood was collected by tail vein bleeding. After red blood cell lysis, cells were incubated with anti-human CD45 and CD3, and CD7 antibodies for subsequent flow cytometric analysis.

IV.2.6. Colony formation assay

CD7 CAR T cells or control CAR were incubated with mononuclear cells from cord blood (CB) of healthy donors (E:T ratio of 10:1) or PBMCs from patients with AML (E:T ratio of 5:1) for 5 hours and then plated in duplicate in methylcellulose-based medium supplemented with recombinant cytokines (MethoCult H4434 Classic; STEMCELL

Technologies). After 12 days of culture, the numbers of granulocytic, monocytic and erythrocytic colony-forming units in CB or leukemic colonies in AML samples were assessed using an inverted microscope.

IV.2.7. Co-culture with normal PBMC

Monocytes were isolated from PBMCs of healthy donors using $CD14^+$ magnetic beads (Miltenyi Biotec) according to manufacture instructions. Monocytes were stained with eFluor670 and co-cultured with CD7 CAR T cells or control CAR T cells at an E:T ratio of 1:1 overnight. Granulocytes were collected from peripheral blood of healthy donors. After red cell lysis, immune cells were incubated with CD7 CAR T cells at an E:T ratio of 1:5 overnight. Granulocytes were discriminated by SSC and FSC and CD15 staining. Absolute cell counts were obtained for both myeloid populations using CountBright Absolute Counting Beads by flow cytometry.

IV.2.8. Generation of $CD7^-$ virus-specific T cells (VSTs)

T cells from healthy donors were activated using a mixture of pepmixes (15-mer peptides overlapping by 11 amino acids) spanning antigens from CMV (PP65, IE1), Adenovirus (Hexon, Penton) and EBV (EBNA-1, LMP-1, LMP-2, EBNA-3a, 3b, 3c). Two days post stimulation, T cells were electroporated following the same protocol used to knock out CD7 in CD7 CAR T cells. T cells electroporated only with cas9 were used as control. Cells were expanded in CTL media (10% FBS) with IL-7 (10 ng/ml) and IL-15(10 ng/ml) for 7 days post electroporation. A second activation was performed by co-culturing the expanded VSTs with an irradiated K562 cell line (K562cs) genetically modified to express CD80, CD83, CD86 and 4-1BBL (kindly gifted by Cliona Rooney) together with irradiated autologous activated T cells (aATCs) pulsed with the same pepmixes in 1:1:5 ratio (VST:aATC:K562cs) in presence of IL-7 and IL-15. Cells were expanded for 7 days and then used for assays.

IV.2.9. Co-culture VSTs with CD7 CAR and monocytes

To confirm antiviral capacity of $CD7^-$ VSTs, monocytes pulsed with peptides derived from CMV, EBV and adenovirus were cocultured for 24h at a 1:1 E:T ratio. The number of $CD14^+$ cells was assessed by flow cytometry with CountBright Absolute Counting Beads. To confirm that $CD7^-$ VSTs are protected against CD7 CAR T cells, VSTs were stained with eFluor670 and both populations of T cells were initially cocultured for 48 hours (E:T ratio of

2:1). After flow cytometric analysis of the remaining VSTs, peptide-pulsed autologous monocytes were added to the same well and incubated overnight (at an E:T ratio of 2:1). The number of live monocytes was then quantified by flow cytometry.

IV.2.10.Statistical analysis

Data points from individual donors are shown in all figures. Statistical significance in pairwise comparisons was determined by an unpaired two- tailed Student t test and in multiple comparisons by a one-way analysis of variance with posttest Bonferroni correction. Statistical significance in Kaplan-Meier survival curves was assessed with the Mantel-Cox log-rank test. All P values were calculated using Prism 6 software (GraphPad).

IV.3.Results

IV.3.1. CD7 is expressed by AML blasts but is absent on normal myeloid cells in peripheral blood

CD7 is stably expressed in T- and NK-cell precursors and is maintained in most of their peripheral progeny, but is absent from most B-cell and myeloid subsets. We detected no expression of CD7 in peripheral monocytes, granulocytes, or B cells, though most T and NK cells were CD7-positive (Figure IV-1A). We then analyzed CD7 expression in 20 primary AML samples collected from patients at Texas Children's Hospital and Houston Methodist Hospital. We detected surface expression of CD7 in 6 out of 20 samples (Table IV-1), albeit with varying intensities (Figure IV-1B). CD7 expression was also detected in AML cell lines KG-1a, Kasumi-3, and GDM-1 (Figure IV-1C). These data indicate that CD7 is expressed in leukemic, but not normal, myeloid cells and may be suited for the selective targeting of AML.

Table IV-1- AML patient characteristics. Samples from these 6 patients were used in in vitro assays.

AML sample ID	Age (years)	Sex	Cytogenetics
1	12	M	46, XY
2	44	F	inv(16)(p13.1q22)
3	13	M	46, XY
4	8	M	t(9;11), MLL-R
5	13	M	t(6:11), MLL-R
6	16 mo	M	Trisomy8, MLL-R

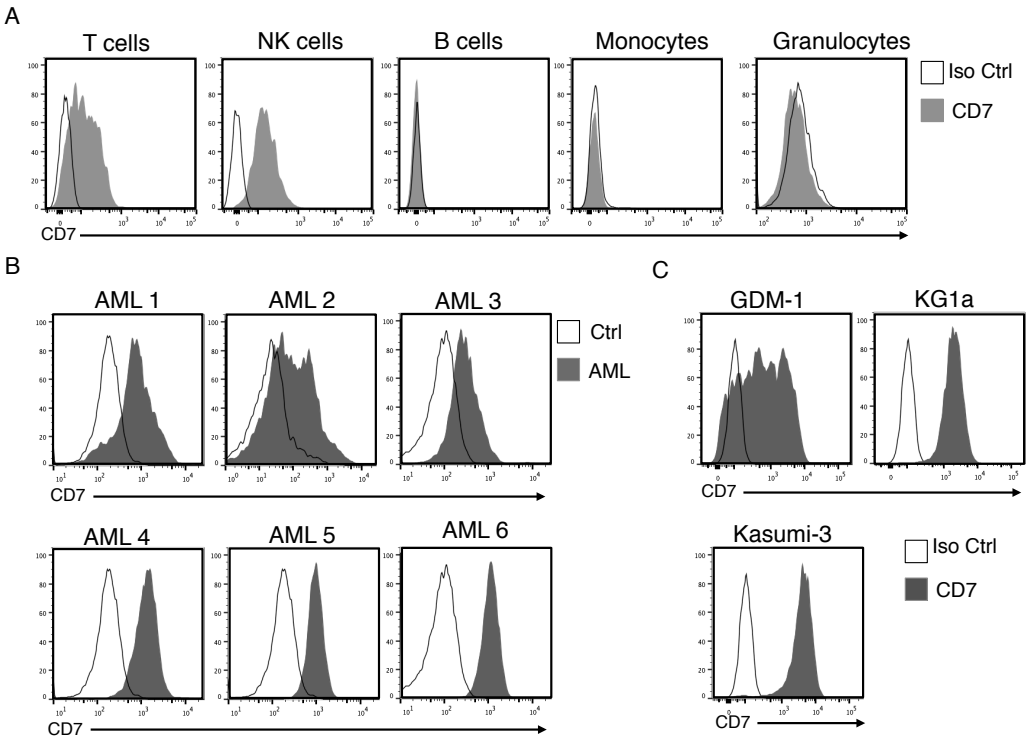

Figure IV-1- CD7 expression in normal and malignant cells. (A) Representative histograms of CD7 expression in immune subsets from peripheral blood of healthy donors. **(B)** Surface expression of CD7 measured by flow cytometry in primary AML samples collected from pediatric and adult patients; **(C)** CD7 expression in AML cell lines. Iso Ctrl, Isotype Control.

IV.3.2. CD7 CAR T cells are highly cytotoxic against CD7⁺ AML cell lines

Generation and expansion of functional CD7 CAR T cells requires CD7 removal prior to CAR expression to minimize T-cell fratricide. We have previously shown that by CRISPR/Cas9-mediated disruption of the CD7 gene in primary activated T cells we could generate CD7 CAR T cells (hereafter CD7 CAR T cells) with specific cytolytic activity against $CD7^+$ T-lymphoblastic leukemia (Chapter II). We have used this approach to generate CD7-edited T cells expressing a second-generation CD7 CAR with the CD28 costimulatory endodomain. Following gammaretroviral transduction, the expanded T cells were uniformly CD7-negative and >80% were CD7 CAR-positive (Figure II-4D).

To assess the CD7-directed activity of the CAR T cells against AML blasts, we cocultured T cells expressing CD7 CAR or an irrelevant CAR with fluorescently labeled $CD7^+$ AML cell lines KG-1a, Kasumi-3, and GDM-1 for 3-5 days. To model physiologic conditions, we used a low effector-to-target ratio of 1:40 and analyzed the number of residual live tumor cells at the end of coculture. We observed a 2-3 log reduction in the counts of residual malignant cells upon coculture with CD7 CAR T cells but not with control T cells (Figure IV-2A,B). Indeed, CD7 CAR T cells retained cytotoxicity against AML cells

even at a 1:100 effector-to-target ratio (Figure IV-2C), indicating CD7 CAR T cells have high cytolytic potential. We also detected robust production of IFN-gamma by both $CD4^+$ and $CD8^+$ CD7 CAR T cells upon incubation with AML blasts (Figure IV-2D). To establish whether the CD7 CAR T cells were resistant to functional exhaustion, we used a sequential killing assay in which T cells were plated with KG-1a cells at a 1:4 effector-to-target ratio and replated every 3 days with fresh tumor cells to restore the initial ratio. Unlike control T cells, CD7 CAR T cells repeatedly eliminated AML tumor cells for at least 6 rounds (Figure IV-2E). Collectively, CD7 CAR T cells demonstrate robust and sustained cytotoxicity against AML cell lines in vitro.

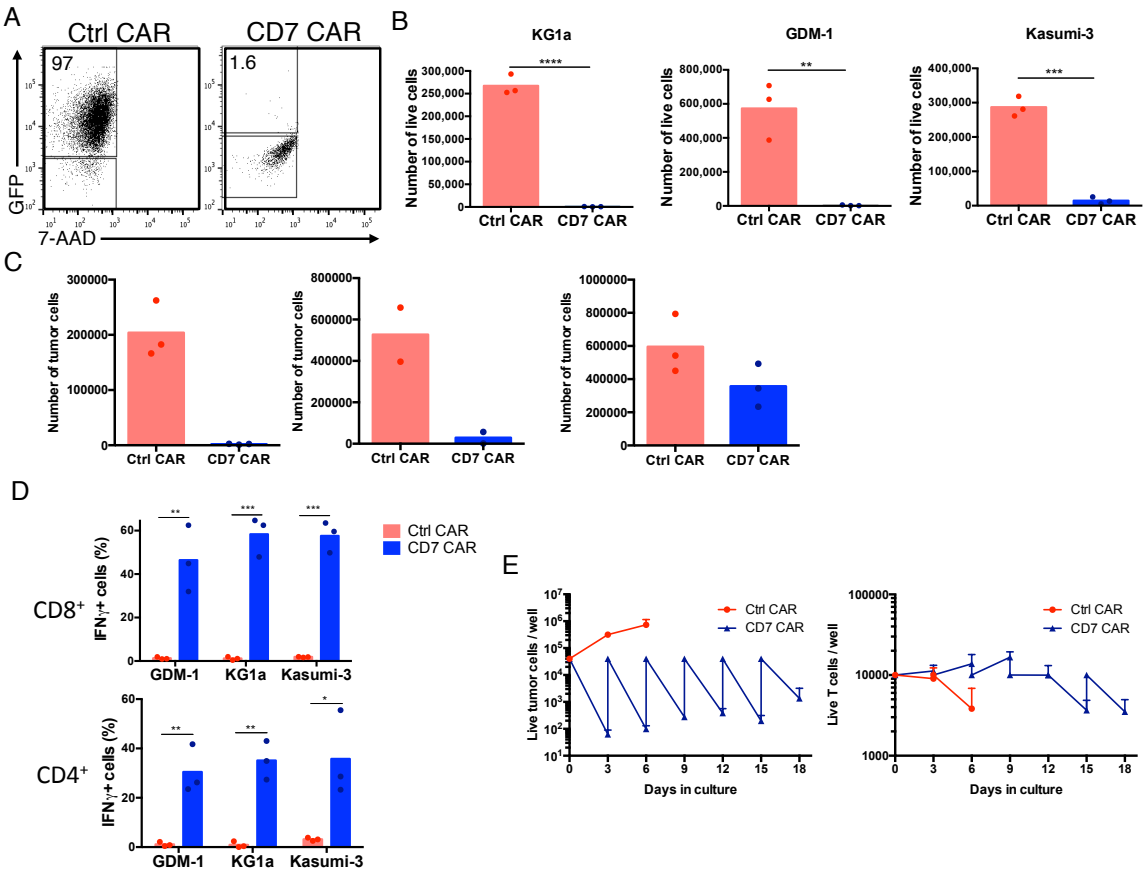

Figure IV-2- CD7-edited CD7 CAR T cells are cytotoxic against AML cell lines. (A) Frequency of residual live GFP^+ KG-1a AML cells upon coculture with control or CD7 CAR T cells for 3 days at a 1:40 effector-to-target (E:T) ratio. Absolute counts of live AML cells at the end of the coculture are plotted on the bar graphs **(B)**. **(C)** Absolute counts of residual live KG-1a cells after coculture with CD7 CAR T cells at the indicated E:T ratios. **(D)** IFN-gamma production by CD8+ and CD4+ CD7 CAR T cells upon 4-hr coculture with indicated AML cell lines by intracellular cytokine staining and flow cytometry. Individual data points for each donor are shown. **(E)** Repeated cytotoxicity against KG-1a AML cells in a sequential killing assay. CD7 CAR T cells were plated with KG-1a AML cells at a 1:4 E:T ratio and the ratio was restored every 3-4 days after quantification of residual live tumor cells (left) and CAR T cells (right). Data are shown as mean±SD. Data represent 2-3 independent experiments. *P<0.05; **P<0.01; *** P<0.001; ****P<0.0001 by unpaired Student's *t* test.

IV.3.3. CD7 CAR T cells eradicate primary AML blasts and leukemia colony-forming cells

To evaluate the activity of CD7 CAR T cells against primary AML, we measured cytotoxicity against several primary AML samples expressing varying levels of CD7 (Figure IV-3A). We observed between 3- and 30-fold reductions in the counts of residual live AML samples after 48h of coculture with CD7 CAR T cells at a 1:2 effector-to-target ratio (Figure IV-3B, C).

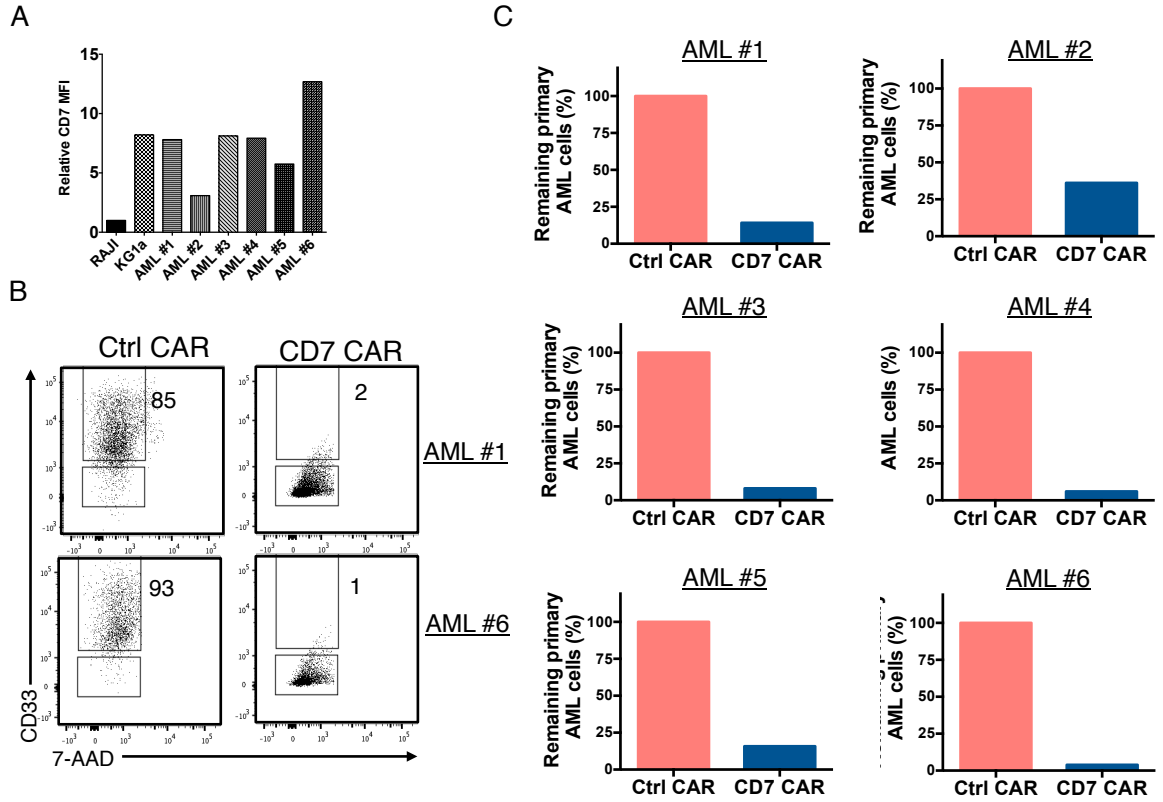

Figure IV-3- Elimination of primary AML blasts by CD7 CAR T cells. (A) Normalized MFI of CD7 in primary AML blasts compared with KG-1a (positive control) and Raji (negative control). **(B)** Representative dot plots showing frequencies of live primary AML blasts after 48h coculture with control or CD7 CAR T cells at a 1:1 E:T ratio. **(C)** Absolute counts of live AML cells from individual patients at the end of coculture with control or CD7 CAR T cells.

Quiescent chemoresistant AML stem cells (LSC) are associated with AML relapses and thus should be effectively targeted by CAR T cells to achieve durable remission. To assess the LSC-directed activity, we co-cultured CD7 CAR T cells with AML cells for 5hr and plated the surviving cells on a methylcellulose medium that supports long-term expansion of LSC-derived colonies. We observed decreased numbers of colonies after a coculture with CD7 CAR T cells compared with control T cells (Figure IV-4), indicating the cytotoxic activity of CD7 CAR T cells extends to leukemic colony-forming cells.

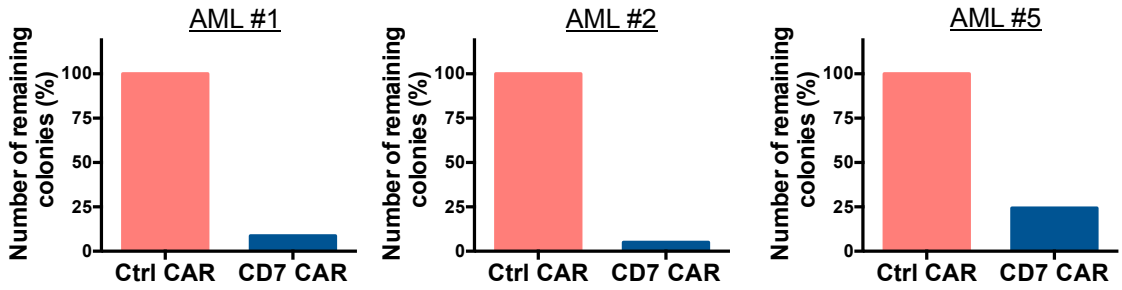

Figure IV-4- Elimination of primary LSC by CD7 CAR T cells. Control or CD7 CAR T cells were cocultured with AML blasts for 5h at a 5:1 E:T ratio and plated on MethoCult media. The bar graphs show relative numbers of leukemic colonies formed after 14 days of culture.

IV.3.4. CD7 CAR T cells protect against systemic AML in vivo

Next, we assessed the ability of CD7 CAR T cells to control the progression of AML in vivo. We used a mouse xenograft model of AML in which sub-lethally irradiated NOD.SCID.IL2rg$^{-/-}$ (NSG) mice were engrafted via intravenous injection of 1x10^{6} KG-1a AML cells expressing firefly luciferase (FFluc). After 5 days, we injected a single dose of non-transduced or CD7 CAR T cells intravenously (Figure IV-5A). Tumor burden was monitored weekly by in vivo luminescence imaging, and surviving animals were euthanized 100 days after T cell injection. Mice receiving control T cells developed systemic leukemia (Figure IV-5B,C) and all succumbed to the disease with median survival of 56 days (Figure IV-5D). In contrast, injection of CD7 CAR T cells significantly impeded leukemia progression and prolonged the median survival to 97 days. No xenogeneic graft-versus-host disease (GvHD) was observed in mice receiving CD7 CAR T cells. In mice with residual AML the blasts maintained CD7 expression (Figure IV-5E), indicating the activity of human CD28ζ CD7 CAR T cells is self-limiting in this xenogeneic system.

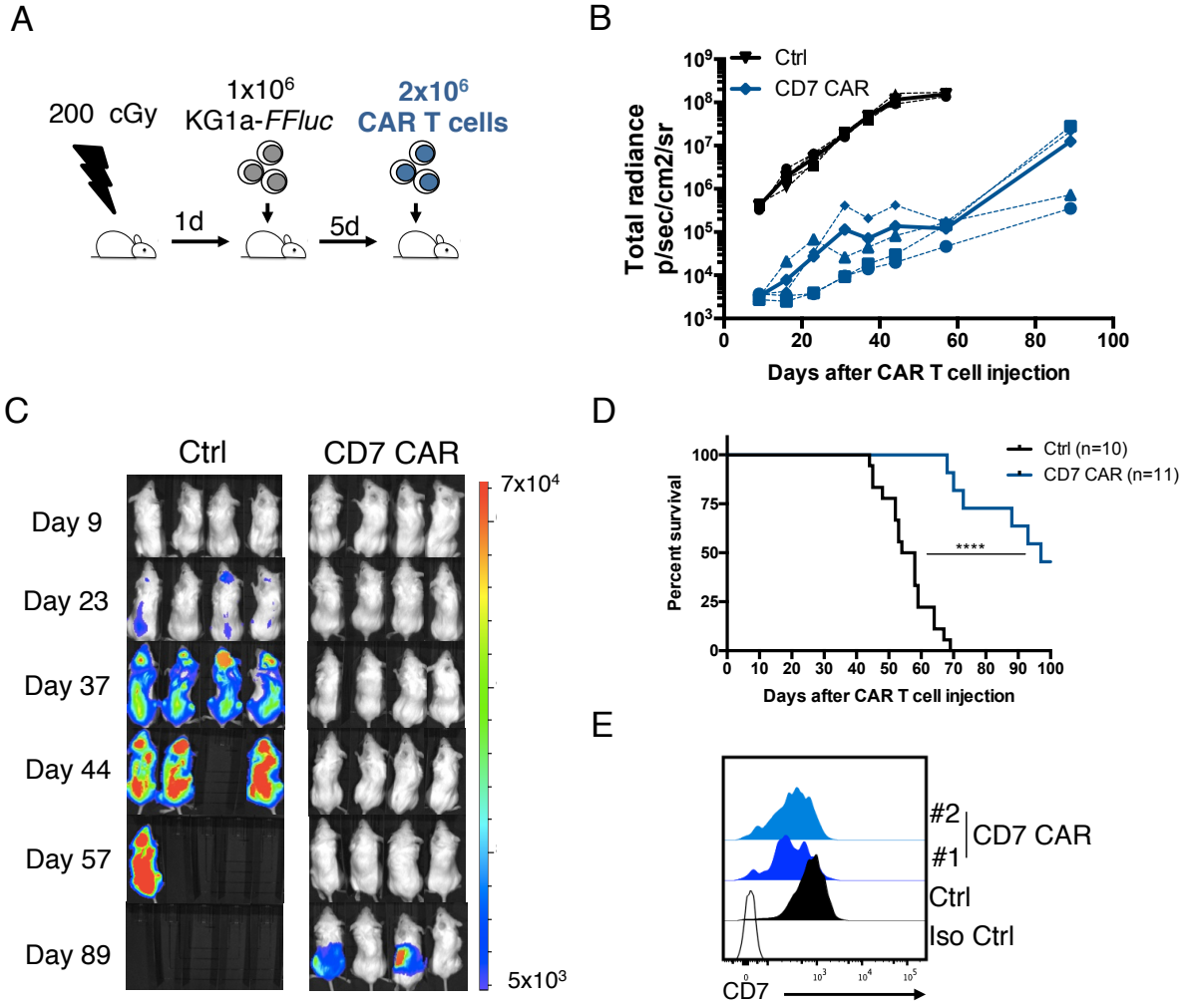

Figure IV-5- CD7 CAR T cells are protective in a mouse xenograft model of AML. (A) General outline of the experiment. NSG mice received FFluc-expressing KG-1a cells 24h after sublethal irradiation with 200 cGy. Five days later, mice received a single injection of control or CD7 CAR T cells intravenously and were monitored for tumor progression. **(B)** Kinetics of leukemia progression in individual mice that received either control or CD7 CAR T cells by IVIS imaging. **(C)** Representative images showing leukemia progression in individual mice. **(D)** Kaplan-Meier curves showing survival of mice in each experimental group. ****, P<0.0001 by Mantel-Cox log rank test. **(E)** Expression of CD7 in residual KG-1a AML isolated from mice treated with CD7 CAR T cells. In vitro cultured KG-1a cells served as a positive control.

IV.3.5. Optimization of CD7 CAR

As demonstrated, mice injected with CD7 CAR T cells were protected against T-cell (Figure II-19) and myeloid (Figure IV-5) leukemic progression, although tumors were not completely eliminated. Therefore, we changed the CAR structure in an attempt to improve its antitumor affect. CARs are designed empirically and, despite many clinical trials, it is not clear if there is an ideal design. Hence, we started by making a new CD7 CAR with the C_H3

domain removed, leaving only a short hinge (SH) derived from the IgG4 sequence. (Figure IV-6A). We tested both CD7 CARs with intermediate and short hinge in a coculture with KG1a and Kasumi-3 at 1:50 E:T ratio. We did not observe any statistical difference between these two variations of CD7 CAR (Figure IV-6B). Next, we tested both CARs in the same AML xenograft mouse model as used before (Figure IV-5A). Although mice who received CD7 CAR with short hinge had higher tumor burden, this difference was also not statistically significant and did not affect the survival of the groups that received CD7 CAR T cells (Figure IV-6C,D). This demonstrates that CD7 CAR anti-tumor effect is not improved by using a shorter spacer.

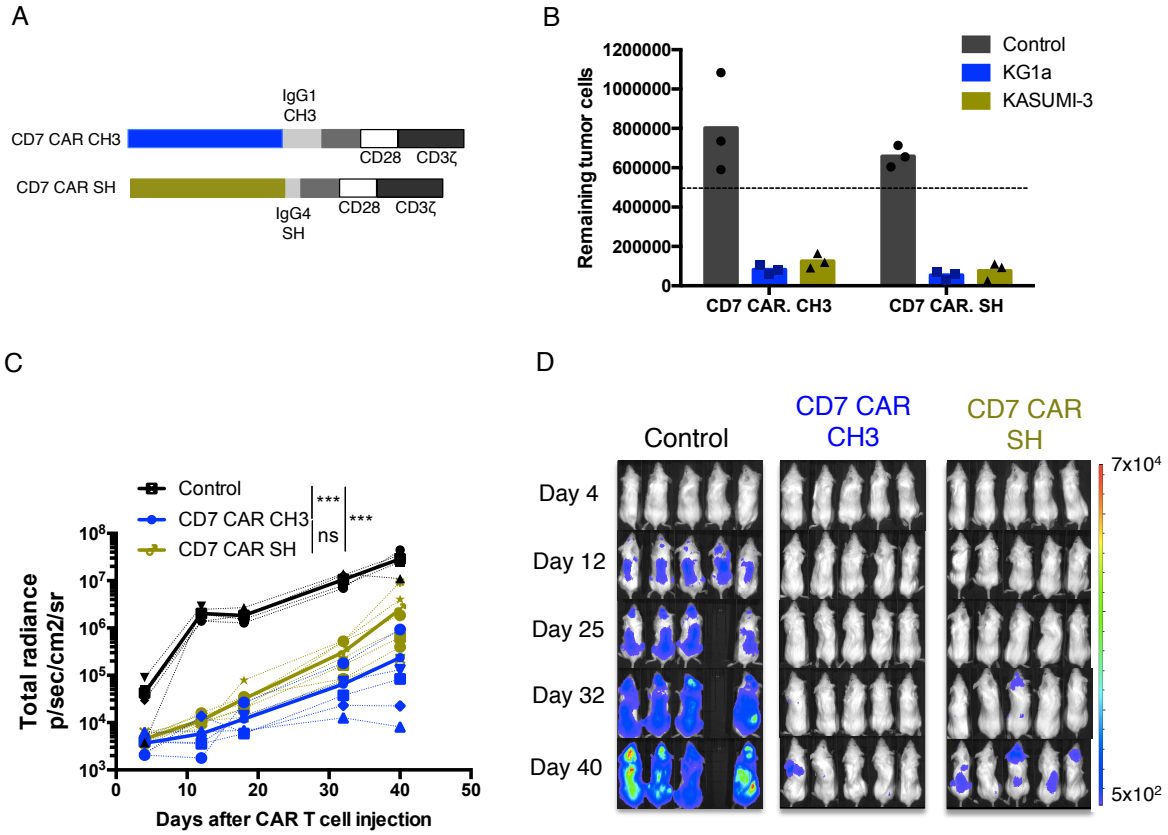

Figure IV-6- Use of CD7 CAR T cells with shorter spacer. (A) Schematic representation of CD7 CARs tested. From the previous CD7 CAR used C_H3 domain was removed leaving only the short hinge domain **(B)** Killing assay of CD7 CAR T cells with KG1a and Kasumi-3. CAR T cells and tumor cells were cocultured for 3 days at 1:50 E:T ratio. **(C)** Kinetics of leukemia progression in individual mice that received either control, CD7 CAR T cells with C_H3 domain or short hinge by IVIS imaging. **(D)** Representative images showing leukemia progression in individual mice.

Different co-stimulatory domains have been used in clinical trials with but if one in particular is better than other is unknown. Traditionally, second generation CARs tested in the clinic, have CD28 or 4-1BB although other endodomains are also commonly tested in the preclinical studies. Recent data demonstrates that CARs with co-stimulatory endodomains from the TNF receptor family increase persistence of T cells in vivo [226].

However retroviral transduction of $CD7^{KO}$ T cells with CD7 CAR with 4-1BB prevented normal expansion of T cells. Even the use of the other two scFvs had similar effect (Figure IV-7A). Furthermore, CD7 CAR T cells with 4-1BB in opposite to those who express a CAR with CD28 endodomain had low viability after transduction (Figure IV-7B).

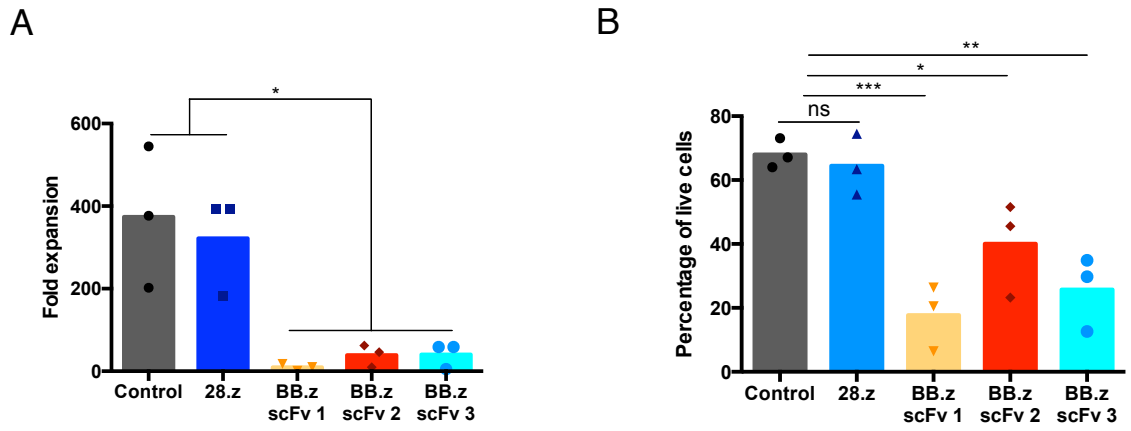

Figure IV-7- CD7 CAR T cells with 4-1BB failed to expand due to increased number of dead cells. **(A)** Fold expansion of CD7 CAR T cells with CD28 or 4-1BB co-stimulatory domains compared with truncated (control) CAR T cells during 14 days. Three CARs with different scFvs were tested in the 4-1BB group. (B) Percentage of live cells detected by flow cytometry at day 3 after retroviral transduction.

This suggests that a CD7 CAR with a 4-1BB endodomain can have toxic effects in CAR T cells and that replacing the scFv does not mitigate this. Because this phenomenon has also been observed with other CARs, an attempt to understand and solve this problem will be the goal of chapter V. Due to the inability to generate viable CD7 CAR T cells with the 4-1BB endodomain, we did not pursue further attempts at improving the anti-tumor effect by replacing the CAR endodomain. Instead, we decided to keep the CD28 co-stimulatory domain.

IV.3.6. Normal myeloid progenitor and mature cells are spared by CD7 CAR T

CD7 is absent on most normal mature myeloid and erythroid cells, and we observed no toxicity of CD7 CAR T cells against peripheral monocytes (Figure IV-8A,B) or granulocytes (Figure IV-8C) after in vitro coculture.

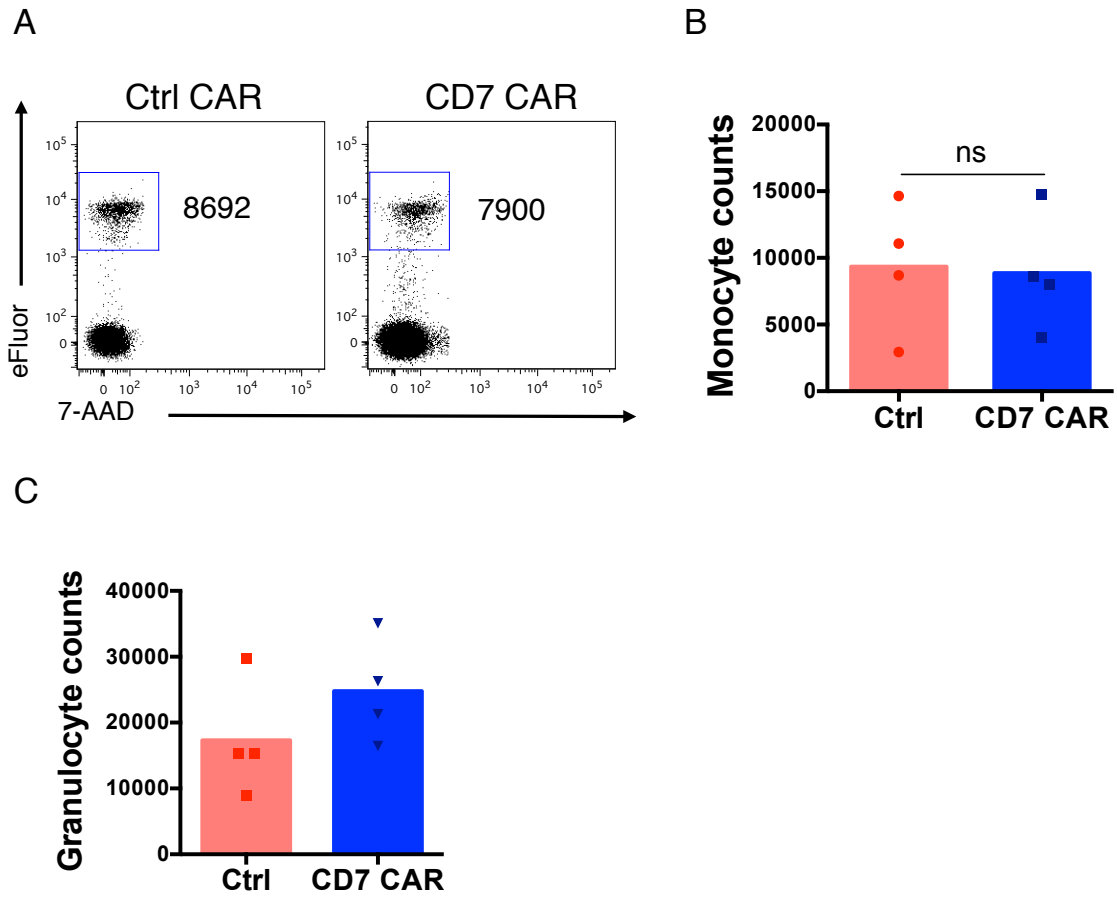

Figure IV-8- Lack of reactivity of CD7 CAR T cells against mature myeloid cells. (A) $CD14^{+}$ monocytes were purified from PBMC using magnetic beads and labeled with eFluor 670 prior to coculture with control or CD7 CAR T cells at a 1:1 ratio. Representative dot plots show the numbers of residual live monocytes after 24h of coculture. **(B)** Data from 4 donors are summarized in a bar graph **(B)**. **(C)** Total blood cells after RBC lysis were cocultured with autologous CD7 CAR T cells for 24h. Live granulocytes were quantified at the end of coculture by flow cytometry.

Since CD7 may be transiently upregulated on some hematopoietic progenitors that are enriched in cord blood, we measured the cytolytic activity of CD7 CAR T cells against hematopoietic progenitor cells from cord blood. We cocultured CD7 CAR T cells with cord blood cells for 5hr followed by a 12-day expansion on a methylcellulose medium supporting both myeloid and erythroid differentiation. CD7 CAR T cells had no impact on monocytic, granulocytic, and erythrocytic colony counts compared to co-culture with control T cells. These data indicate primitive myeloid and erythroid progenitors are not inhibited by CD7 CAR T cells (Figure IV-9A,B).

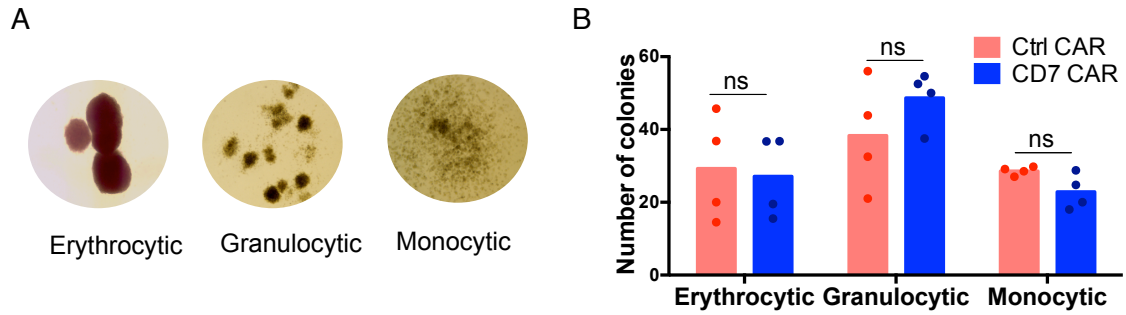

Figure IV-9- Lack of reactivity of CD7 CAR T cells against cord blood precursors. Cord blood cells were cocultured with control or CD7 CAR T cells at a 10:1 E:T ratio for 5hr and plated on the MethoCult media. Numbers of erythrocytic, granulocytic, and monocytic colonies were quantified 14 days later based on their typical morphology **(A)** observed by optical microscopy. **(B)** Data from individual donors are shown in the bar graph. Data represent two independent experiments. ns, not significant by unpaired Student's t test.

IV.3.7. CD7-edited virus-specific T cells are functional and are protected from cytolysis by CD7 CAR T cells

CD7 expression is limited to the T- and NK-lineage cells, and we have previously demonstrated that these types of immune cells can be targeted by CD7 CAR T cells. This on-target off-tumor activity has the potential to suppress anti-viral immunity and predispose the host to viral infections, similar to the immunosuppression seen after a hematopoietic stem cell transplant. Infusion of donor-derived multivirus-specific T cells (VSTs) shortly after transplant can effectively protect patients from viral infections and reactivation of endogenous viruses. However, most VSTs are CD7-positive and thus would likely be targeted by CD7 CAR T cells. We have previously demonstrated that disruption of the CD7 gene in T cells does not affect their immediate effector function and protects them against CD7 CAR-mediated cytotoxicity. Thus, we hypothesized that knocking out the CD7 gene in VSTs ($CD7^{KO}$ VSTs) will protect these cells from cytolysis by CD7 CAR T cells. These cells could then be infused into patients, in case immunosuppression following CAR T cell treatment were to develop, restoring anti-viral immunity. However, activation and expansion of VSTs differ from that of polyclonally stimulated T cells, and successful gene editing in human VSTs has not yet been demonstrated. Using a CRISPR/Cas9-mediated gene editing method in VST, we were able to disrupt the CD7 gene consistently in >85% of polyclonal EBV-, CMV-, and adenovirus-specific T cells (Figure IV-10A). Loss of CD7 did not affect the cytotoxicity of VSTs against autologous monocytes pulsed with a viral peptide mix (Figure IV-10B).

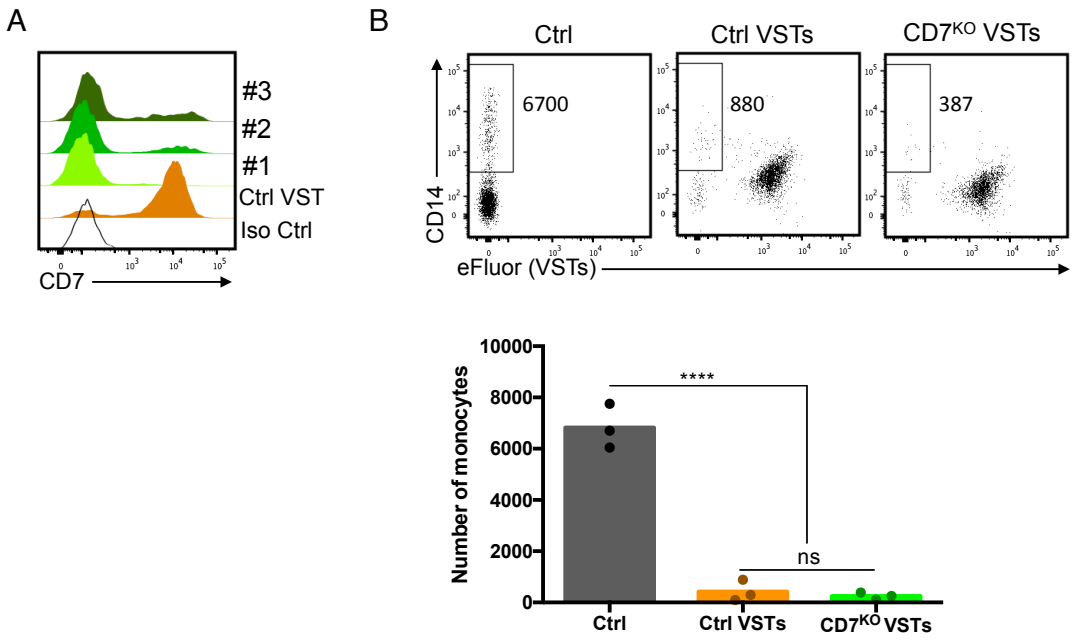

Figure IV-10- Knockout of CD7 in VSTs does not impair cytotoxic function against infected cells. **(A)** Representative histogram of CD7 expression in unedited VSTs and 3 different donors after knockout using CRISPR/cas9. **(B)** Cytotoxic assay of normal VSTs and CD7KO VSTs against monocytes pulsed with peptides derived from CMV, EBV and adenovirus. Upper figure represents the coculture of monocytes with normal T cells (Ctrl) or VSTs. Monocytes were magnetically sorted (CD14+) and incubated overnight with VSTs with or without CD7 stained with eFluor 647 at effector:target ratio 1:1. Numbers denote total number of monocytes per well. Bar graph shows total quantification of monocytes at the end of coculture.

Notably, CD7^{KO} VSTs were protected against cytolysis by CD7 CAR T cells in a 48-hour coculture assay. To assess the anti-viral function of CD7^{KO} VST in the presence of CD7 CAR T cells, we cocultured CD7^{KO} or unmodified VST with CD7 CAR T cells (Figure IV-8A) and measured the number of remaining live VSTs (Figure IV-8B,C). Later, we added autologous monocytes pulsed with viral pepmixes to the remaining live VSTs and counted the number of monocytes that were not eliminated. Prior incubation of normal VSTs with CD7 CAR T cells significantly impaired the ability of unmodified VSTs to eliminate peptide-pulsed monocytes, while the activity of CD7^{KO} VST was preserved (Figure IV-8D). Since edited VSTs were not eliminated by CD7 CAR T cells, they were as efficient at killing peptide-pulsed monocytes as VSTs alone. In contrast, unmodified VSTs were eliminated by the initial incubation with CD7 CAR T cells (Figure IV-B,C), preventing them from eliminating monocytes in the triple coculture step. These data indicate that disruption of the CD7 gene in VST is feasible and preserves their antiviral activity in the presence of CD7 CAR T cells, offering a solution to offset the risk of T-cell aplasia after their administration.

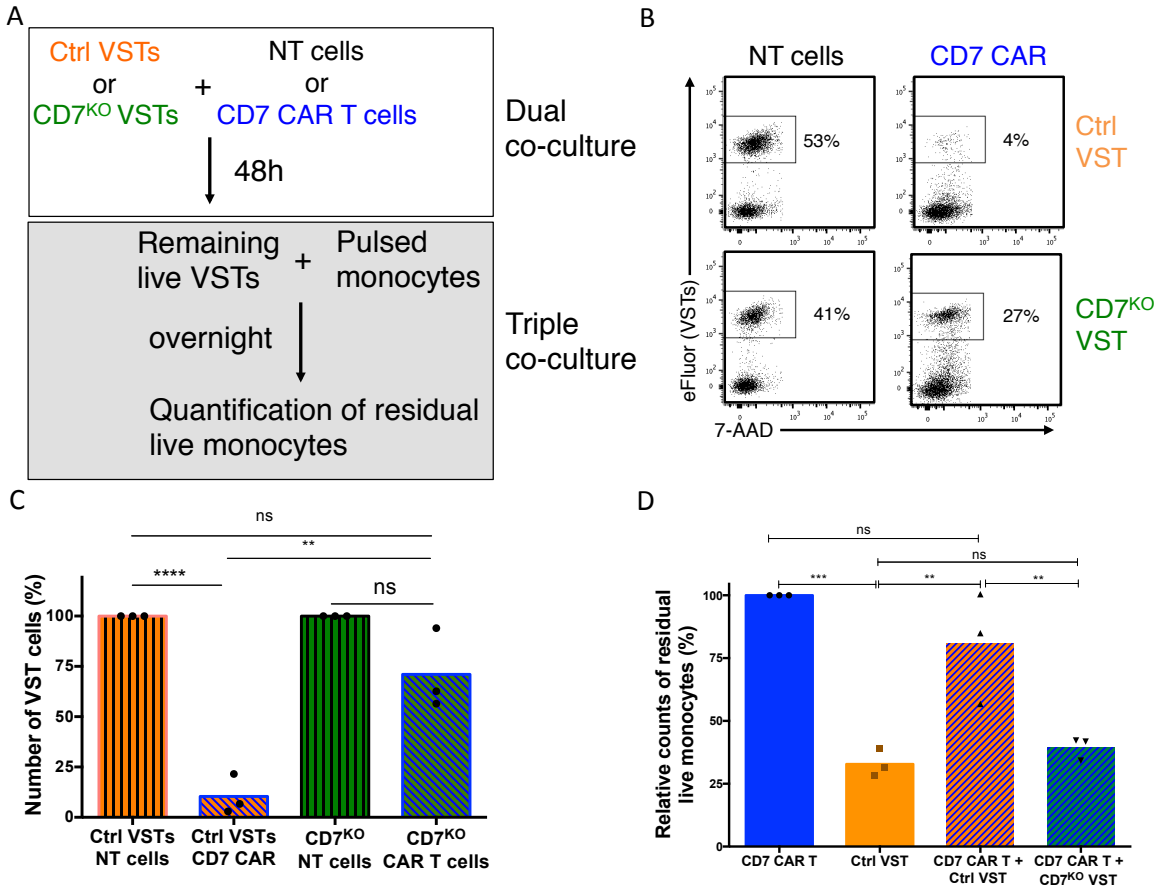

Figure IV-11- CD7^{KO} VSTs are protected against fratricide from CD7 CAR and can eliminate viral-infected cells. **(A)** Schematic representation of the experiment to test the capacity of CD7^{KO} VSTs to eliminated viral-infected monocytes and at the same time avoid the toxicity from CD7 CAR T cells. **(B)** Histograms show edited and unedited VSTs stained with eFluor 647 at the end of coculture with CD7 CAR T cells or control at effector:target 2:1 for two days and **(C)** the total number of live VSTs was counted by flow cytometry. **(D)** In a second step, the same VSTs cocultured with CD7 CAR T cells were combined with previously pulsed monocytes with peptides from CMV, EBV and adenovirus at effector:target ratio 2:1 for 4h and total number of remaining cells were counted by flow cytometry. Data represent 2 to 3 independent experiments with n=3 donors each. **P<0.01; **** P<0.0001

IV.4.Discussion

The risk of unwanted myeloablation complicates the development of safe targeted therapies of AML, as few surface antigens are selectively expressed in malignant cells but absent in critical non-malignant myeloid and erythroid cells. Here, we demonstrate that T cells expressing a CD7-specific CAR can effectively target a subset of aggressive AML expressing CD7 without cytotoxicity against normal myeloid and erythroid cells. Therefore, the CD7-directed targeting can be used for non-myeloablative therapy of AML. As the off-tumor activity of CD7 CAR T cells may lead to the ablation of CD7^{+} T- and NK-cells, we demonstrate a potential solution to offset potential immunosuppression by generating CD7-

edited multivirus-specific T cells that are fully functional against viral targets but are protected from cytolysis by CD7 CAR T cells. Therefore, the CD7-directed targeting coupled with the CD7-edited VST can potentially be used for safe, non-myeloablative therapy of AML.

CD7 is expressed in ~30% of AML cases, most of which are intermediate-to-high risk disease by cytogenetics [384], [385]. Some, but not all, studies reported CD7 positivity correlates with chemoresistance and higher incidence of relapse post stem cell transplant [380]–[383] and is associated with poor prognosis in myelodysplastic syndromes [390], [391]. CD7 expression in malignant myeloid cells is an indicator of their immaturity [392] and a more "primitive" $CD34^+$ phenotype that is often associated with chemoresistant leukemia-initiating cells [105], [393], [394]. In cord blood, a fraction of Lin^- $CD34^+$ $CD38^-$ hematopoietic cells co-expresses CD7; these cells constitute multilymphoid and common lymphoid progenitors [386]–[388]. In the bone marrow, however, CD7 expression on these lymphoid progenitors has not been detected [387], [388], [395]; although CD7 can be expressed at a low level on a small fraction of $CD38^+$ $CD34^+$ Lin^- cells with an unknown function [396], [397]. Later in hematopoiesis, CD7 expression is restricted only to T- and NK-cell progenitors and their descendants [322], [398]. Thus, the cytotoxic activity of CD7 CAR T cells within the myeloid compartment should be limited primarily to leukemic cells whereas normal myeloid and erythroid lineage cells will be largely spared. Although the predicted effect of CD7 CAR T cells on non-malignant myeloid and erythroid cells is minimal, elimination of normal $CD7^+$ T- and NK-cells can be expected [358]. Prolonged aplasia of these critical immune cells can result in dangerous immunosuppression with weakened responses against external pathogens and, most importantly, endogenous latent viruses, such as EBV and CMV, which can re-activate in the absence of immune control. Viral infections are a significant cause of morbidity and mortality in post-stem cell transplant patients, who have minimal peripheral T-cell levels for several weeks after transplant due to the delayed thymic output. Prior reports demonstrated that infusions of donor-derived virus-specific T cells protect these patients against developing potentially fatal viremias [399], [400]. A similar approach can, in theory, be used to offset T-cell aplasia in patients receiving CD7 CAR T cells; however, these VSTs must be CD7-edited to avoid cytotoxicity from the CD7 CAR T cells themselves.

Ultimately, CD7 CAR T cells may best be used as a transient transplant- enabling therapy in patients with refractory or relapsed disease. In this scenario, the transient activity of CAR T cells would minimize the tumor burden and serve as a bridge to the curative stem cell transplant, which would terminate CAR T cell activity and reset normal T-/NK-lymphopoiesis. To facilitate robust yet transient activity of CD7 CAR T cells, we have used a second-generation CAR backbone with CD28 costimulation, which has been shown in clinical studies to promote rapid expansion and high cytotoxicity yet transient persistence of

CD19 CAR T cells [203], [318], [401], [402]. Our optimization attempt using the 4-1BB endodomain to prolong persistence was not successful since these cells did not expand well and had reduced viability. Although increased persistence of CD7 CAR T cells will most likely prolong T- and NK-cell aplasia, this effect could be mitigated with a suicide gene, which upon activation would cause elimination of these cells as desired, without affecting the viability of endogenous T cells. Alternatively, if used as a bridge to transplant, CD7 CAR T cells would be eliminated by the conditioning regimen used for transplant. Nevertheless, use of any endodomain besides CD28 would be valuable only if it were to to increase even further the CAR-T cell cytotoxic effects in a short period of time, rather than just increase their persistence (an effect that is not entirely clear when endodomains from the TNF receptor family, like 4-1BB, are used). Although the CD28 endodomain creates a T cell profile that suits this application, the lack of expansion observed with 4-1BB raises concerns of toxicity, a topic that will be explored in chapter V.

Removal of CD7 expression from the surface of T cells is essential to mitigate fratricide of CD7 CAR T cells and enable their expansion [358], [389]. As we demonstrated in a chapter II, CRISPR/Cas9-mediated disruption of the CD7 gene in T cells prior to CD7 CAR transduction results in permanent removal of the CD7 gene in 85-90% of T cells with no detected off-target activity; the remaining $CD7^+$ T cells are eliminated by fratricide after CAR transduction [358].

Xenogeneic graft-versus-host disease (GvHD) driven by xenoreactive human T cells is a common complication of mouse xenograft models of human tumors [403], [404]. However, we observed no signs of xenogeneic GvHD upon infusion of CD7 CAR T cells into leukemia-bearing mice, likely reflecting their transient persistence and potential interference between CAR and TCR signaling. Indeed, administration of allogeneic T cells expressing CD28.zeta CD19 CARs produces only minimal GvHD in both clinical studies and preclinical models, a phenomenon potentially attributed to the interaction between CAR-derived CD28 and CD3zeta signaling and TCR signaling within the same cell [405], [406]. Therefore, administration of allogeneic third party-derived closely HLA-matched T cells expressing the CD28.zeta CD7 CAR T cells may produce desired anti-tumor activity with low potential for GvHD in the transient, “bridge-to-transplant” setting, thus obviating the need to generate an autologous product from patients generating an autologous product from patient’s peripheral blood mononuclear cells that may contain malignant blasts and often hypofunctional T cells weakened by numerous rounds of chemotherapy.

Although adoptive cell therapy approaches for AML have been under active development for many years, clinical benefit has been only modest and largely dwarfed by the resounding success of CARs directed to B-cell antigens, such as CD19. As many of the targetable antigens are also expressed by critical hematopoietic precursors (CD33, CD123,

etc.), the activity of CAR T cells directed to those antigens has to be carefully dosed to avoid systemic myeloablation. The approach presented in this study is directed at CD7, which is not known to be expressed on critical myeloid and erythroid progenitors and mature cells, and thus CD7 CAR T cells may benefit patients with $CD7^+$ disease.

Chapter V.Tonic 4-1BB Costimulation in Chimeric Antigen Receptors Impedes T Cell Survival and Is Vector-Dependent

Despite the tremendous success of CD19 CAR T cells in human trials, which led to their commercial approval, CAR development is empirical and several key aspects are not fully understood. The search for the best CAR molecule characteristics is an ongoing endeavor the evidence so far suggests that each CAR may need to be independently developed. While the differential effects of the type and number of endodomains used start to be unveiled, the complexity of CAR biology demonstrates that other key aspects of their design should be taken into account to achieve maximum performance. It is clear that tonic signaling – a constant, antigen-independent CAR activation – can result in different CARs with the same backbone behaving differently. Several solutions may be employed to limit tonic signaling, though with different rates of success depending on the CAR…

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V.1.Introduction

Ligation of chimeric antigen receptors (CARs) in T cells by surface tumor-associated antigens mimics the natural engagement of T cell receptors (TCRs), leading to activation and degranulation of transgenic T cells. Incorporation of costimulatory endodomains in second-generation CARs has increased proliferation and survival of CAR T cells by providing an additional Signal 2 [190], [203], [204], [318]. The most widely used costimulatory endodomains are derived from *CD28* or *TNFRSF9* (4-1BB) genes. Despite stark differences in T cell kinetics, correlating with the distinct signaling cascades triggered by these endodomains, CARs incorporating either 4-1BB or CD28 have led to dramatic CAR T cell expansion in patients with B cell malignancies and the complete elimination of otherwise refractory tumors [202]–[204], [318].

In addition to antigen-driven stimulation, CARs frequently produce antigen-independent tonic signaling in T cells. This constitutive signaling is commonly enhanced by the high surface density and self-aggregating properties of CARs, although the contribution of this signaling to regulating persistence and function of transgenic T cells has been debated. Tonic CAR stimulation could maintain T cell expansion by mimicking signals that promote peripheral expansion of memory T cells specific for persistent pathogens [407]. However, recent independent studies using c-Met and GD2-specific CARs expressed from lenti- and gammaretroviral vectors, respectively, indicated that, while tonic signaling from CARs harboring the CD28 costimulatory endodomains indeed promoted antigen-independent expansion of T cells in vitro, the expanded CAR T cells had inferior anti-tumor properties and limited persistence in vivo [226], [408]. Reducing surface levels of CD28.zeta CD19 CAR by expressing it from the endogenous TCR alpha (*TRAC*) gene locus prevented in vivo exhaustion and improved the anti-tumor function of CAR T cells [274]. Moreover, replacing CD28 with 4-1BB costimulation reversed exhaustion in GD2 CAR T cells [226]. However, whether and under which circumstances tonic 4-1BB signaling can have similar adverse ramifications in T cells has not yet been studied.

Here, we model tonic CAR-derived 4-1BB signaling in T cells and demonstrate a mechanism by which it impairs CAR T cell expansion and cytotoxic function. We show that tonic 4-1BB signaling is amplified in gammaretroviral vectors, and attenuating CAR expression in alternative expression systems decreases tonic signaling-associated toxicities and augments anti-tumor activity. These results highlight potential inhibitory properties of 4-1BB costimulation and have direct implications for adoptive T cell therapy.

V.2.Materials and Methods

V.2.1. Chimeric Antigen Receptor Constructs

To model tonic 4-1BB signaling, we used a chimeric antigen receptor (CAR) backbone containing 4-1BB and CD3zeta endodomains and the transmembrane and stalk region of CD8a. We used anti-CD19 (FMC63), and anti-kappa light chain single-chain variable fragments (scFv) to create 2nd generation CARs harboring either 4-1BB or CD28 costimulatory domains. CAR constructs were subcloned into SFG gammaretroviral vectors. An EMCV-derived internal ribosomal entry site (IRES) was cloned immediately upstream of the CAR in IRES BB.z constructs. A lentiviral vector REL was created by replacing the PGK-GFP cassette in the pRRLSIN.cPPT.PGK-GFP.WPRE vector (a gift from Didier Trono, Addgene plasmid #12252) with the full human EF-1a promoter containing intron 1. For the confocal microscopy studies, we generated CD19 CARs fused on the C terminus with Emerald GFP via a flexible linker. We verified the functionality of CD19 CAR-Emerald in cytotoxicity assays to ensure intact CAR signaling. Site-directed mutagenesis of the TRAF2 sites in the 4-1BB endodomain was performed using the InFusion cloning kit (Clontech). Final constructs were verified by Sanger DNA sequencing.

V.2.2. CRISPR/Cas9-Mediated Disruption of the FAS Gene

A FAS-directed sgRNA with zero predicted off-target sites was selected using CRISPRScan and COSMID algorithms [335], synthesized using HiScribe T7 High Yield RNA Synthesis kit (NEB), and purified with MegaClear Kit (Ambion). Purified RNA was mixed with recombinant Cas9 protein (PNA Bio) at a 0.35:1 (m/m) ratio and electroporated into T cells using Neon Transfection System (Invitrogen) as previously described [358]. Cells were subsequently transduced with CD19 CAR the following day and expanded for 7 days.

V.2.3. Mouse Xenograft Model

8- to 10-week-old female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (The Jackson Laboratory) were inoculated intravenously with 1x10^6 NALM-6 cells engineered to express FFluc-GFP fusion protein. Three days later, mice received a single intravenous injection of 1.0x10^6 CD19 CAR T cells. Tumor burden was monitored by recording luminescence with an IVIS Imaging system (Caliper Life Sciences). Mice were euthanized after the tumor burden reached luminescence level of 10^8 photons/s or after displaying signs of high tumor burden.

Peripheral blood was collected by tail vein bleeding. All animal experiments were conducted in compliance with the Baylor College of Medicine IACUC.

V.2.4. Cell culture

CD19+ Burkitt's lymphoma cell line Raji (a gift from Gianpietro Dotti) was modified to express GFP+ by stable retroviral transduction and sorted to obtain pure GFP+ cells. A pre-B-ALL cell line NALM-6 expressing GFP and Firefly Luciferase (NALM6-FFluc) was a gift from Stephen Gotschalk. Both cell lines were expanded and maintained in CTL medium (45% RPMI-1640, 45% Click's medium, 10% fetal bovine serum supplemented with 1% penicillin/streptomycin and 1% L-Glutamine), were STR fingerprinted and routinely checked for mycoplasma contamination. Buffy coats were purified using Ficoll and activated with plate-bound OKT3 and anti-CD28 antibodies (BD Biosciences) for 48 hours, after which cells were removed from the antibody-coated plate and used for retro- or lentiviral transduction. T cells were expanded in CTL medium supplemented with 10ng/ml IL-7 and 10ng/ml IL-15, and the media was replenished every 48-72 hours.

V.2.5. Generation of gammaretroviral and lentiviral vectors and T cell transduction

Retroviral transduction was performed as previously described [219]. Briefly, 293T cells were co-transfected with CAR-encoding retroviral plasmid SFG, and packaging plasmids RD114 and pEQ-PAM3. Retroviral supernatant was collected 48h and 72h later, filtered with 0.45 μM filters and frozen. Lentiviral vectors were generated by co-transfecting 293T cells with CAR-encoding REL plasmids and packaging plasmids pMD2.G and psPAX2 (both gifts from Didier Trono, Addgene plasmids #12259 and #12260, respectively) in the presence of 4mM of sodium butyrate (Sigma-Aldrich) and 4mM of caffeine (Sigma-Aldrich). Viral supernatants were collected 24h and 48h after transfection, filtered and concentrated with PEG-it^{TM} Virus Precipitation Solution (System Biosciences) at +4°C overnight.

Retro- and lentiviral supernatants were pre-adsorbed on a 24-well non-tissue culture treated plate (Falcon) coated with Retronectin (Takara) by centrifugation at 4600g +32°C for 1h. Excess viral supernatant was aspirated and activated T cells were added to the plate at 250,000 cells/well followed by a brief centrifugation at 1000g for 10min. Transduced T cells were transferred to a tissue culture plate 24h later and expanded in CTL media as described above. For most experiments, T cells were transduced once.

V.2.6. Flow cytometry

Mouse anti-human antibodies to CD3 (UCHT1), CD4 (SK3), CD8 (SK1), CD45RA (2H4), and Fas (UB2) (all Beckman Coulter), CCR7 (150503, BD Biosciences), FasL (NOK-1) and ICAM-1 (HA-58, BioLegend) and TRAIL (RIK-2, eBioscience) were used to evaluate T cell phenotype. Cell death was assessed by Annexin V staining (BD Biosciences). Activation of caspase 8 was measured with Vybrant FAM Caspase-8 Assay kit (Thermo Fisher). CARs were detected using anti-idiotype antibodies 136.20.1 (CD19 CAR), 1A7 (GD2 CAR) or goat anti-mouse Fab-specific polyclonal antibodies (Jackson Immunoresearch). Samples were acquired on BD Gallios and analyzed with FlowJo v.9.

V.2.7. Cytotoxicity assays

CD19 CAR T cells we co-cultured with Raji-GFP cells at a 1:4 effector-to-target ratio in a flat-bottom 96-well plate for 72h followed by FACS analysis in the presence of 7-AAD and CountBright counting beads (ThermoFisher).

V.2.8. Western blot analysis

Cells were dissociated with PBS + 3 mM EDTA and lysed in a buffer containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (all from Sigma-Aldrich), and protease inhibitors (Thermo Scientific). Protein concentrations were determined using a Bio-Rad protein assay (BioRad) with BSA as the standard. Samples were denatured in Laemmli buffer containing 5% of 2-Mercaptoethanol (all from BioRad) at 95°C for 10 minutes. Cell lysate (10 µg per lane) was run on a pre-made 10% SDS polyacrylamide gel (BioRad) and transferred to nitrocellulose membranes (BioRad). Membranes were blocked with 5% BSA or milk powder in Tris-buffered saline (TBS) + 0.1% Tween-20 (all from Sigma) and then probed with primary antibodies followed by a horseradish peroxidase (HRP) conjugated secondary antibodies. Blots were developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposed to GeneMate Blue Basic Autoradiography Film (BioExpress). ImageJ (National Institutes of Health) was used for Western blot quantification. Protein levels were normalized to loading controls (GAPDH).

Antibodies used in the study are as follows: anti-CD3.ζ (sc-1239, Santa Cruz Biotechnology), anti-CD247 (pY142) (558402, BDPharmigen), GAPDH (sc-47724, Santa Cruz Biotechnology) anti-IKKa/b (pS176/pS180) (16A6, Cell Signaling Technologies), anti-IKKa (3G12, Cell Signaling Technologies). Secondary antibodies: goat anti-mouse (sc-2005, Santa Cruz Biotechnology), goat anti-rabbit (111-035-003, Jackson ImmunoResearch Laboratories). For Western blotting, all antibodies were diluted 1:1000, except for secondary antibodies, which were diluted 1:10,000.

V.2.9. Real-Time PCR analysis

CD19 CAR T cells from 3 donors were pelleted 7d post-transduction. Genomic DNA was isolated using the DNeasy Blood & Tissue kit (QIAGEN). RNA was purified with the RNeasy Plus Mini kit (QIAgen) and cDNA was built using the Superscript III RT kit (ThermoFisher). Quantitative PCR was performed using Good Laboratory Practice (GLP)-validated TaqMAN primers specific for the cytoplasmic tails of 4-1BB.zeta and CD28.zeta CARs and GAPDH (Applied Biosystems) on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Absolute numbers of copies were calculated using standard dilution controls containing target sequences in plasmid DNA according to GLP standards. Relative CD19 CAR mRNA levels were calculated using standard dilution controls containing target sequences in plasmid DNA according to GLP standards. Relative CD19 CAR mRNA levels were calculated by the $\Delta(\Delta C_0T)$ method and normalized to the number of proviral copies per 100ng of genomic DNA.

V.2.10. Fluorescent microscopy analysis

For confocal microscopy, BB.z and 28.z CD19 CAR-Emerald T cells were incubated on silane coated glass slides for 10 minutes at 37°C. Cells were then permeabilized and fixed with BD Perm/Fix (BD Biosciences) solution for fifteen minutes at room temperature and stained for anti-Fas (mouse) and anti-FasL (rabbit) primary antibodies. Anti-mouse Alexa Fluor 568 and Anti-rabbit Alex Fluor 647 secondary antibodies (Life Technologies) were subsequently used to detect Fas and FasL. Cells were imaged as Z stacks of 0.2 µm thickness to cover their entire volume on a Leica TCS SP8 laser scanning microscope using a 100X objective. Images were acquired with the LASAF software (Leica) and analyzed with Volocity software (PerkinElmer) and Fiji. A region of interest (ROI) was drawn on cell surface or around the whole cell for measurement of amount of Fas and FasL.

V.2.11. Study approval

All animal experiments were conducted in compliance with the Baylor College of Medicine IACUC (Protocol# AN-4758). Primary human PBMC were isolated from healthy donors after informed consent using the protocol approved by the Baylor College of Medicine Institutional Review Board and in accordance with the Declaration of Helsinki.

V.3.Results

V.3.1. High Expression of 4-1BB.zeta CARs Impairs T Cell Expansion

High CAR expression on the cell surface, driven by strong promoters in viral vectors, can result in spontaneous ligand-independent clustering of CAR molecules and produce tonic signaling [226], [408]. To assess whether the 4-1BB costimulatory endodomain produces this effect in CAR T cells, we used 2nd generation CD19 CAR containing a CD8a stalk and 4-1BB (BB.z), a construct that has proven successful in clinical studies [202], [204] (Figure V-1A). To modulate the expression level of CARs in T cells—and hence the magnitude of tonic CAR signaling—we inserted the CAR cassettes in gammaretroviral vectors in which CAR expression was driven either directly by the retroviral long terminal repeat (LTR) promoter (BB.z) or attenuated by an upstream IRES element (IRES BB.z) (Figure V-1B). As control, we used a clinically validated CAR with CD28 endodomain (28.z CD19 CAR [190]).

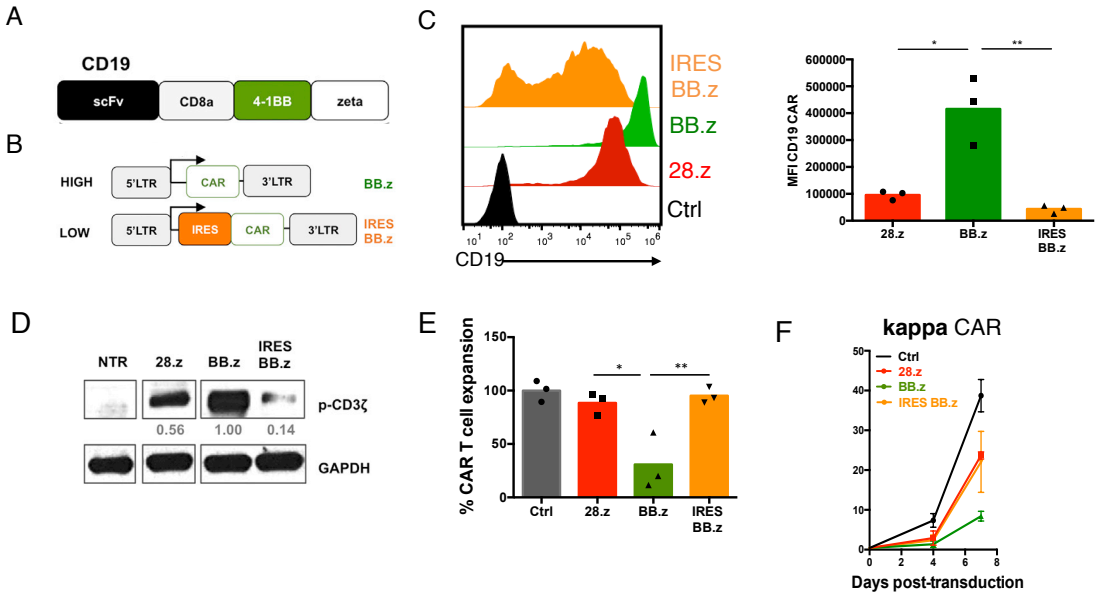

Figure V-1- High Expression of BB.z CARs in T Cells prevents normal expansion. (A) Schematic representation of the expression systems utilized to express BB.z CARs in T cells. **(B)** Expression of CD19-specific CARs on the cell surface of T cells 4 days post-transduction; mean fluorescence intensities are plotted on the bar graph. **(C)** Relative magnitude of tonic signaling in BB.z and IRES BB.z CAR T cells 7 days post-transduction measured by western blot analysis. Numbers indicate relative signal intensity normalized to GAPDH. **(D)** Overall expansion of T cells retrovirally transduced with CD19- and GD2-specific CARs relative to that of control mock-transduced T cells. **(F)** Expansion of T cells transduced with BB.z and IRES BB.z kappa-specific CAR.

We found that the expression of BB.z CD19-specific CAR was higher than control CD28-containing CARs, while incorporating an IRES reduced CAR expression (Figure V-

1C). As expected, decreasing CAR expression reduced tonic signaling and spontaneous phosphorylation of the CAR-derived CD3 zeta chain in transduced T cells (Figure V-1D). Notably, T cells expressing high levels of BB.z CARs expanded significantly less following transduction than control CAR T cells (Figure V-1E). Impaired expansion of BB.z CAR T cells over time was associated with dramatically increased apoptosis, as reflected by higher frequency of Annexin V+ cells 7 days post-transduction (Figure V- 2).

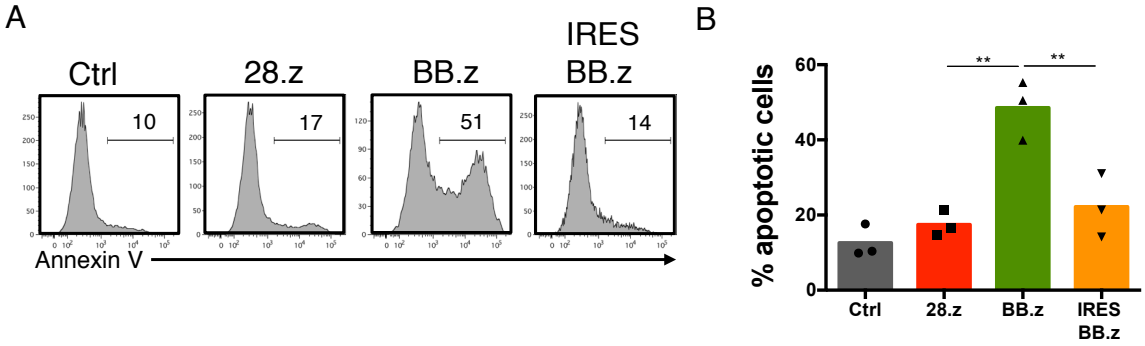

Figure V-2- High Expression of BB.z CARs in T Cells Enhances Apoptosis (A) Representative histograms showing Annexin V staining of CD19 CAR T cells 7 days post-transduction. **(B)** Bar graphs show summarized data for both CARs from 3 donors. Data represent two to five independent experiments. **P < 0.01; by one-way ANOVA.

We observed a similar effect in T cells transduced with BB.z kappa light chain-specific CAR (Figure V-1F), suggesting that high expression of BB.z CARs is toxic for T cells and that reduced CAR expression attenuates this ligand-independent signaling. As demonstrated in chapter IV, a CD7-specific CAR encoding 4-1BB also prevented normal activity of T cells, with lower viability when compared to CD28. Indeed, CD7 CAR T cells had higher expression (Figure V-3) of the CAR on the cell surface when the CAR included a 4-1BB endodomain, demonstrating that this phenomenon may not be specific to any particular CAR, since a different scFv had a similar effect.

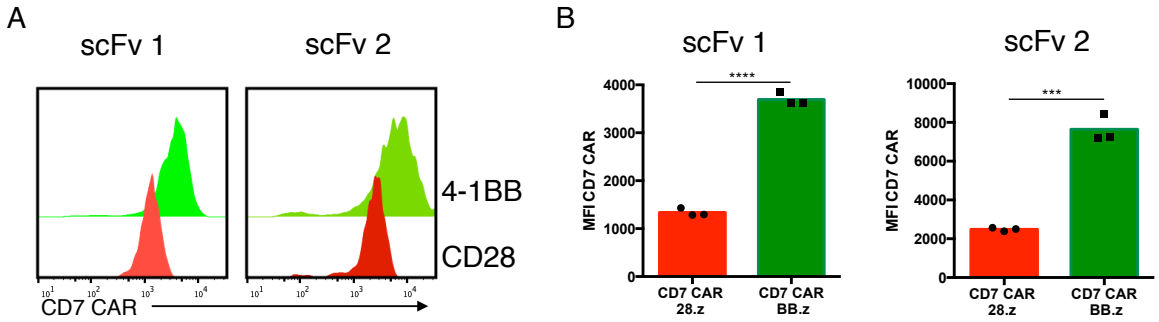

Figure V-3- High expression of CD7 CAR with 4-1BB in two different scFvs. (A) Representative histograms showing CD7 CAR expression in two different scFvs with CD28 or 4-1BB (B) Bar graphs with mean of fluorescence intensity of 3 donors detected by flow cytometry with 2 CD7 CAR T cells with CD28 or 4-1BB.

V.3.2. High Expression of BB.z CAR Alters the Phenotype and Reduces the Anti-tumor Activity of T Cells

Next, we sought to determine how CAR-derived tonic 4-1BB costimulation affected the anti-tumor function of BB.z CAR T cells. We observed increased frequency of central memory ($CCR7^+CD45RA^-$) cells among BB.z CD19 CAR T cells (Figure V-4A), consistent with the role of 4-1BB in promoting central memory differentiation [409], and an increased CD4:CD8 T cell ratio (Figure V-4B). Attenuated expression in IRES BB.z CAR T cells produced a T cell phenotype similar to that of non-transduced T cells and reverted the CD4:CD8 ratio (Figures V-4A and B).

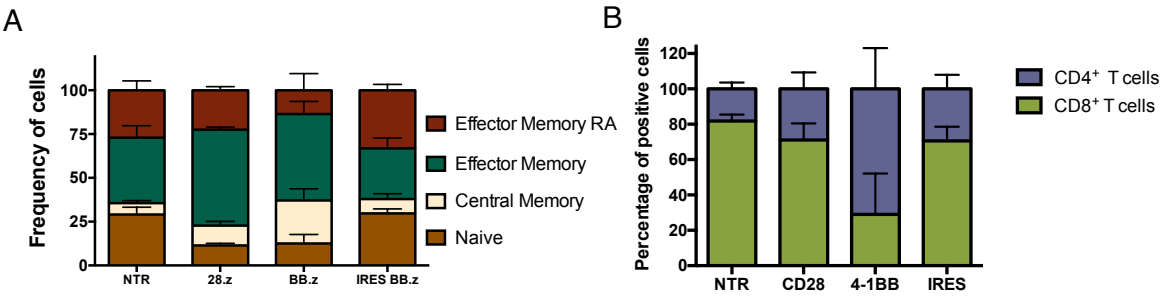

Figure V-4- High Expression of BB.z CD19 CAR Alters the Phenotype (A) Relative proportion of naive-phenotype (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector cells (T_{EMRA}) CD19 CAR T cells 14 days post-transduction defined by CCR7 and CD45RA expression. **(B)** Stacked bar graph showing relative proportion of $CD4^+$and $CD8^+$ cells among CD19 CAR T cells 14 days post-transduction.

We have shown that toxicity from high BB.z CAR expression can limit T cell expansion and alter their phenotype, but whether it affects CAR T cell anti-tumor function is unclear. We therefore measured the cytotoxicity of BB.z CD19 CAR T cells upon coculture with fluorescently labeled $CD19^+$ cell line Raji. We observed a significant increase in residual live tumor cells after coculture with BB.z CD19 CAR T cells (Figures V-5A and B) and reduced expansion of CAR T cells at the end of coculture compared to T cells transduced with IRES BB.z CD19 CAR (Figure V-5B). These results indicate that high expression of BB.z CD19 CAR from a gammaretroviral vector inhibits the anti-tumor function of T cells, and lower CAR expression in IRES BB.z cells still provides sufficient avidity to produce cytotoxicity.

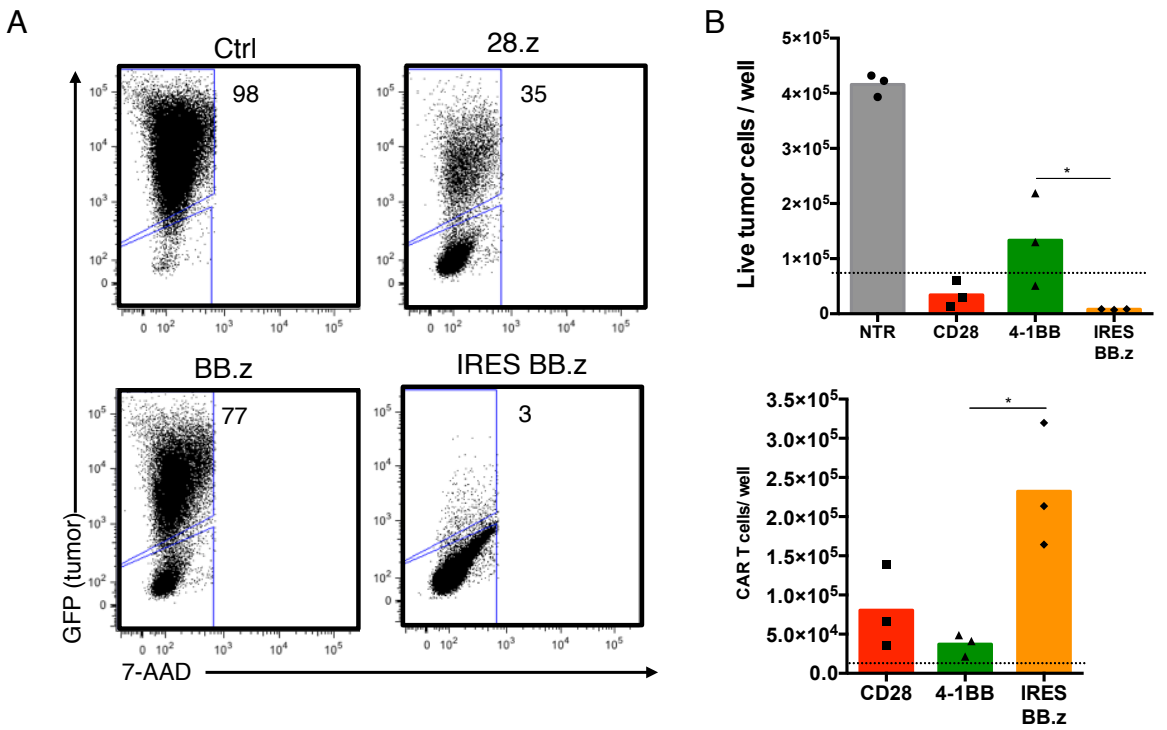

Figure V-5- High Expression of BB.z CD19 CAR Undermines the Anti-tumor Activity of CAR T Cells. (A) CD19 CAR T cells were cocultured with GFP$^+$ Raji cells (CD19$^+$) at a 1:4 ratio for 72 hr. Representative dot plots show the frequency of residual live tumor cells at the end of coculture. Numbers indicate percent of tumor cells among total live cells. **(B)** Absolute cell counts of Raji and CAR T cells at the end of coculture determined by flow cytometry using counting beads. Data from individual donors are shown on each bar graph.

To verify the impact of CAR expression on anti-tumor activity in vivo, we tested the ability of BB.z CD19 CAR T cells to suppress progression of leukemia in a xenograft mouse model of pre-B ALL (NALM-6). We chose an aggressive systemic model in which most mice succumb to the disease within 3 weeks if left untreated. A single low dose of CAR T cells was administered intravenously 3 days after tumor delivery (Figure V-5A). We observed significantly increased expansion of IRES BB.z CAR T cells in peripheral blood (Figure V-6B) compared to BB.z CAR T cells. Overall, the IRES BB.z CAR T cells demonstrated more potent suppression of leukemia progression (Figure V-6C,D), leading to a significant extension of survival compared to BB.z cells (Figure V-6E). Therefore, toxicity associated with high expression of BB.z CAR in T cells impedes their expansion and anti-tumor function and can be reversed by attenuating CAR expression.

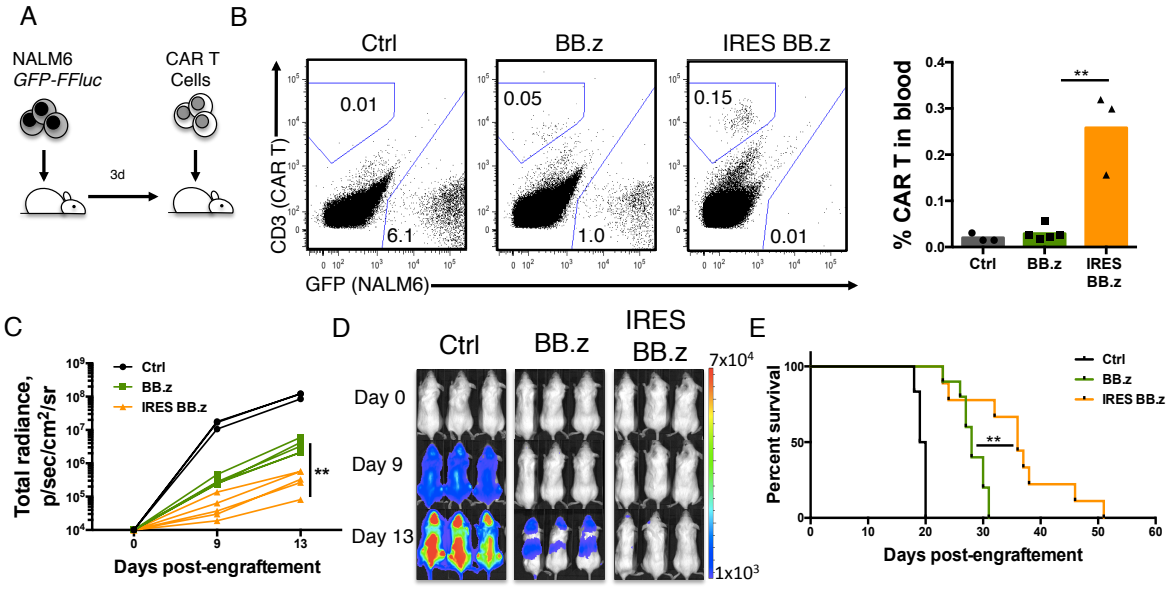

Figure V-6- High Expression of BB.z CD19 CAR impairs antitimur effect in vivo. (A) Schematic diagram of the experiment. NSG mice were intravenously engrafted with $1x10^6$ GFP^+ $FFluc^+$ NALM-6 cells followed by a single intravenous injection of $1x10^6$ CD19 CAR T cells 3 days later. **(B)** Representative staining of peripheral blood of tumor-bearing mice 17 days after tumor engraftment with relative frequency of CD19 CAR T cells and GFP^+ NALM-6 cells. Relative frequencies of CD19 CAR T cells in peripheral blood 17 days post-engraftment are shown on the bar graph. **(C)** Kinetics of NALM-6 luminescence in mice measured by IVIS. (D) Representative images showing leukemia progression in individual mice at day 9 and 13 after CAR T cell injection. **(D)** Kaplan-Meier curves showing advantage in overall survival of mice receiving IRES BB.z CD19 CAR T cells compared to mice receiving BB.z T cells (p = 0.0036 by Mantel-Cox log-rank test). Data represent 2–3 independent experiments. *p < 0.05; **p < 0.01 by Student's t test.

V.3.3. Disrupting TRAF2 Signaling in the 4-1BB Endodomain Reverses Toxicity and Normalizes CAR Expression

Because high expression of BB.z CARs led to cell toxicity regardless of CAR specificity, we hypothesized the enhanced apoptosis of BB.z CAR T cells was a generalizable phenomenon attributable to tonic signaling from the 4-1BB endodomain. We therefore substituted key amino acid residues in both TRAF-binding motifs of the 4-1BB endodomain; disruption of the N-terminal motif (*mut1*$^{QEED/\ QAAA}$) specifically abrogates TRAF2 binding, while mutation in the C-terminal motif (*mut2*$^{PEEEE/\ PEAAA}$) prevents binding with TRAF1, TRAF2, and TRAF3 [410] (Figure V-7A). Altering either of these motifs normalized expression of the BB.z CAR on the cell surface to levels comparable to the 28.z CAR (Figure V-7A). Importantly, disruption of a single TRAF2 binding site was sufficient to minimize BB.z CAR T cell apoptosis (Figures V-7B) and restore normal expansion (Figures V-7C and D), indicating that tonic TRAF2 signaling indeed produces toxicity in BB.z CAR T cells.

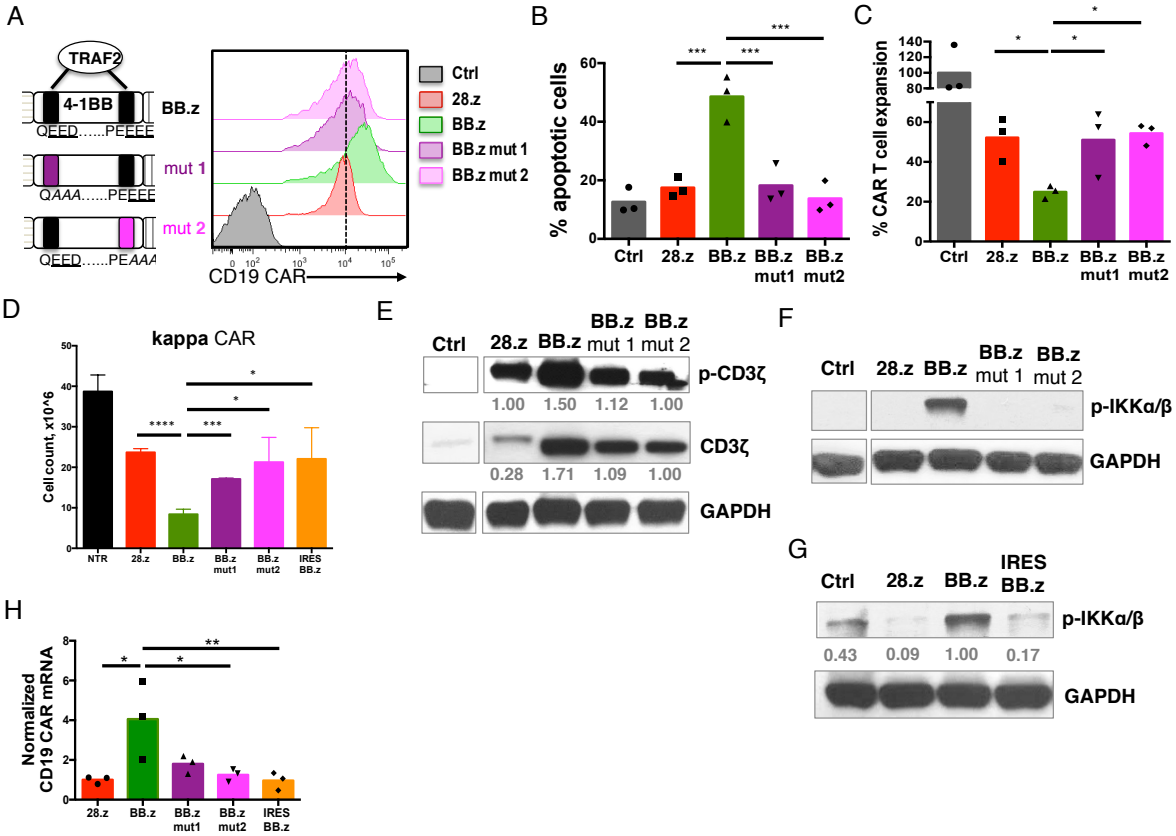

Figure V-7- Enhanced Apoptosis and Increased Expression of BB.z CAR in T Cells Requires 4-1BB-Derived TRAF2 Signaling. (A) A schematic drawing of TRAF2 binding motifs in the 4-1BB domain and corresponding alterations in the protein sequence to disrupt either the N-terminal (mut 1) or the C-terminal (mut 2) motif. Histogram on the right shows relative expression of CD19 CARs with mutated TRAF2 motifs. **(B)** Frequency of apoptotic cells among CD19 CAR T cells with intact or mutated costimulatory domains 7 days post-transduction. **(C)** Overall expansion of CD19 CAR T cells on day 7 post-transduction. **(D)** Expansion of kappa CAR T cells after disrupting CAR-derived TRAF signaling. **(E)** Tonic CD19 CAR signaling and total CAR expression in T cells 7 days post-transduction was measured with anti-pTyr (CD3z) and total anti-CD3z antibodies by western blot. Numbers indicate relative signal intensity normalized to GAPDH. **(F and G)** Relative phosphorylation of IKKa/b in normal and TRAF-mutant **(F)** and IRES-reduced **(G)** BB.z CD19 CAR T cells 7 days post-transduction by a western blot analysis. **(H)** Relative abundance of CD19 CAR transcripts was measured by calculating relative expression of retroviral mRNA normalized to the copy number of CD19 CAR-containing proviruses per 100 ng of genomic DNA by qPCR in T cells 7 days post-transduction. Data represent 2–3 independent experiments with n = 3 donors in each. *p < 0.05; **p < 0.01; ***p < 0.001 by one-way ANOVA.

To investigate whether 4-1BB affects tonic CAR signaling in T cells, we measured spontaneous phosphorylation of ITAM motifs of the CAR-embedded zeta chain by western blot. We observed increased ITAM phosphorylation (per cell) in BB.z CD19 CAR compared to the 28.z CAR control, while disrupting TRAF binding sites normalized spontaneous signaling (Figure V-7E). The elevated tonic signaling of BB.z CD19 CAR was consistently associated with increased total CAR protein levels in T cells (Figure V-7E). Of note, 28.z CAR produced more signaling per CAR molecule, corroborating the enhanced propensity for spontaneous signaling of 28.z CARs as previously demonstrated [226]. We therefore sought to determine the mechanism of enhanced CAR production in BB.z CAR-transduced T cells.

In non-self-inactivating gammaretroviral vectors, transgene expression is driven by the LTR promoter, which is positively regulated by the host transcription factor nuclear factor kB (NF-kB) [411], [412]. In HIV-infected T cells, signaling from 4-1BB and other tumor necrosis factor receptor (TNFR) genes can activate the LTR promoter and promote viral replication [412], [413]. We therefore checked whether tonic 4-1BB-derived TRAF2 signaling similarly increased CAR expression from gammaretroviral vectors by enhancing LTR promoter activity. TRAF2 signaling from 4-1BB activates NF-kB by interacting with and activating the IkB kinase complex (IKKa/b), a positive regulator of NF-kB [410], [414]. Thus, we could block phosphorylation of IKKa/b in BB.z CAR T cells by disrupting TRAF2 signaling or replacing 4-1BB with CD28 (Figure V-7F). Similarly, reducing the level of CAR expression in IRES BB.z CAR T cells attenuated tonic CAR signaling and decreased NF-kB pathway activation (Figure V-7G). To investigate whether this signaling pathway modulated LTR promoter activity, we measured the levels of CD19 CAR mRNA in T cells and normalized to the number of provirus integrations into the genomic DNA. We found significant upregulation of CD19 CAR mRNA per genomic copy of the provirus (Figure V-7H) in BB.z CAR T cells, suggesting that tonic 4-1BB signaling forms a positive feedback loop by enhancing activity of the gammaretroviral LTR promoter and amplifying CAR production in T cells.

V.3.4. Tonic 4-1BB Signaling Increases FAS-Dependent Apoptosis of CAR T Cells

Overstimulation of T cells can provoke apoptosis via the activation-induced cell death (AICD) mechanism, which is predominantly mediated by the FAS/FASL interaction and subsequent activation of caspase 8 [415]–[418]. Because both *FAS* and *FASL* genes can be directly activated by the NF-kB pathway [419]–[421], we investigated whether this mechanism causes apoptosis of BB.z CAR T cells. We observed an increase in surface expression of the proapoptotic genes *FAS*, *FASL*, and *TRAIL* in BB.z CAR T cells and reducing CAR expression or disrupting TRAF2 signaling reversed it (Figures V-8A,B). Furthermore, levels of FAS and FASL on the cell surface positively correlated with CD19 CAR expression and negatively correlated with T cell expansion (Figure V-8C), suggesting that the upregulation of pro-apoptotic molecules is directly proportional to the magnitude of tonic signaling in T cells. Analysis of CAR T cells by confocal microscopy revealed that while FASL was mainly localized intra-cellularly in 28.z CAR T cells, FAS and FASL became co-localized on the cell surface in BB.z CD19 CAR T cells (Figures V-8D).

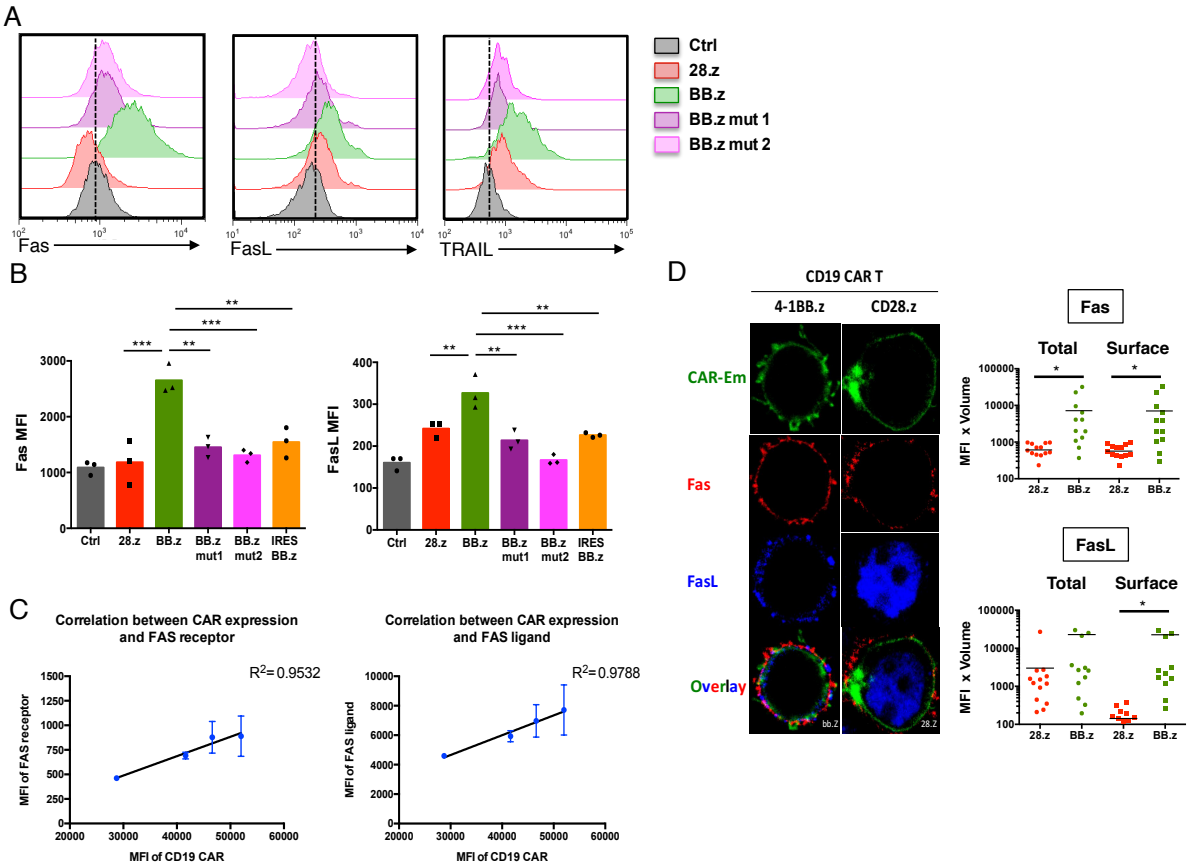

Figure V-8- Tonic 4-1BB Signaling up-regulates FAS in CAR T Cells. (A) Representative staining for surface FAS, FASL, and TRAIL by flow cytometry in CD19 CAR T cells 8 days post-transduction. **(B)** MFI of FAS and FASL on the surface of CD19 CAR on day 8 post-transduction. **p < 0.01; ***p < 0.001 by one-way ANOVA. **(C)** Linear correlation between the level of CD19 CAR and expression of Fas (left) and FasL (right). **(D)** Representative images showing cellular localization of CD19 CAR fused with Emerald fluorescent protein, FAS and FASL. Bottom panel shows overlay of all three signals. Scale bar, 5 um. Bar graphs show quantification of FAS (top) and FASL (bottom) total and surface signal by confocal microscopy. *p < 0.05; by Student's t test.

This co-localization may trigger FAS signaling and sub-sequent AICD [417]. In addition, we observed an increased aggregation of BB.z CD19 CAR T cells that correlated with an upregulation of intercellular adhesion molecule 1 (ICAM-1) (Figure V-9), which is directly activated by NF-kB in various cell types [422], [423]. ICAM-1 expression in T cells facilitates their clustering [424] and thus may enable *trans*-engagement of FAS in CAR T cells by neighboring FASL-expressing cells.

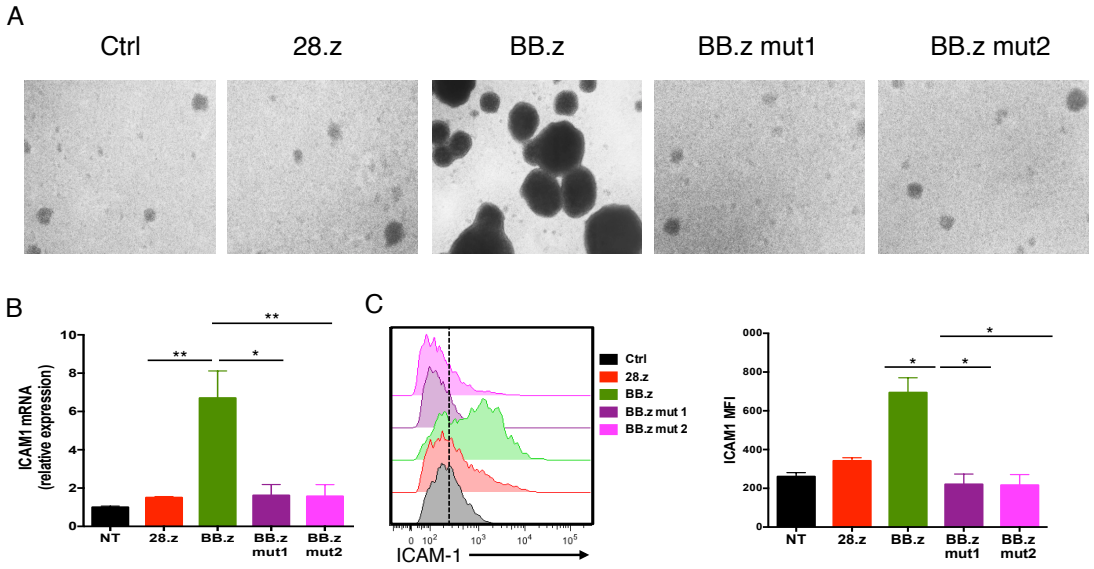

Figure V-9- Enhanced clustering of BB.z CD19 CAR T cells and upregulation of ICAM-1. (A) Morphology of CD19 CAR T cells 7 days post-transduction. **(B)** Relative expression of ICAM-1 mRNA in CD19 CAR T cells on day 7 post-transduction. **(C)** Surface expression of ICAM-1 in CD19 CAR T cells 7 days after transduction. MFI of ICAM-1 on T cells from 3 different donors shown on the bar graph. Data are shown as mean ± SD (n=3).

Indeed, we detected enhanced activation of caspase 8 in BB.z CD19 CAR T cells (Figure V-10A). In order to verify the role of FAS upregulation in BB.z CAR T cell death, we disrupted the *FAS* gene in BB.z CAR T cells using the CRISPR/Cas9 complex with a *FAS*-specific single guide RNA (sgRNA). We observed a moderate decrease in the frequency of Annexin V^+ cells among FAS-negative CAR T cells (Figure V-10B and C), indicating that FAS-mediated AICD contributes to the cell death of BB.z CAR T cells. Thus, tonic 4-1BB signaling activates pro-apoptotic pathways, and this toxicity is augmented in CARs expressed from a gammaretroviral vector via the formation of a positive feedback loop.

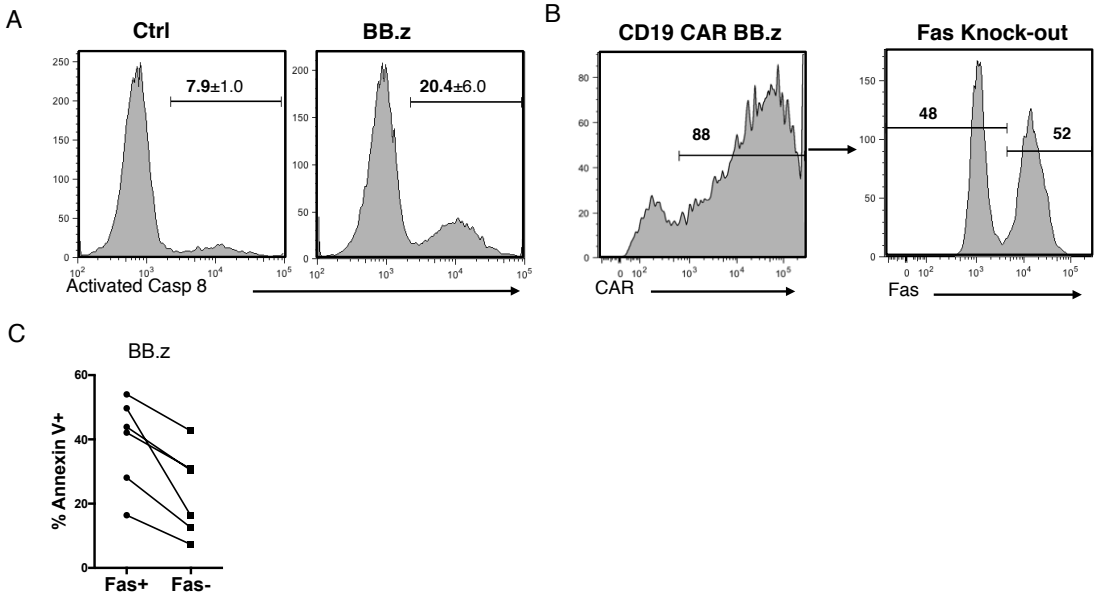

Figure V-10- Tonic 4-1BB Signaling Promotes FAS-Mediated Apoptosis in CAR T Cells. (A) Staining for activated caspase 8 in CD19 CAR T cells using a specific fluorescent inhibitor FAM-LETD-FMK.

Numbers indicate mean ± SD percentage of cells with activated caspase 8. **(B)** BB.z CD19 CAR T cells were electroporated with Cas9 protein and a *FAS*-specific sgRNA 24 hr post-transduction and allowed to expand for 7 days. Histograms show expression of CD19 CAR (left) and surface expression of FAS (right) on electroporated T cells. **(C)** Corresponding changes in the frequency of Annexin V cells gated on FAS^{+} and *FAS* populations are shown with lines indicating cells from the same donor. **p < 0.01 by paired two-tailed Student's t test. Experiments were replicated twice with n = 3 donors in each.

V.3.5. Expressing BB.z CARs from a Self-Inactivating Lentiviral Vector Reduces Toxicity and Improves the Anti-tumor Function of CAR T Cells

As our results indicate that expression of BB.z CARs from gammaretroviral LTR promoters amplify toxic 4-1BB signaling, we attenuated the NF-kB feedback loop by replacing the LTR promoter in a viral vector with an alternative promoter and measured effects on toxicity. We assessed the level of CD19 CAR expression from a clinically validated self-inactivating lentiviral vector [193], [204] in which transgene expression is driven by a non-LTR promoter, EF-1α (LV BB.z, Figure V-11A), that produces lower initial CAR expression level on the T cell surface (Figure V-11B). Indeed, this reduction normalized the expression of FAS and FASL on the cell surface (Figure V-11C), resembling attenuation of CAR expression via IRES. The expression of BB.z CD19 CAR from the lentiviral vector also significantly reduced cell death and restored overall expansion of CAR T cells in vitro (Figures V-11D,E). Lentiviral CAR expression promoted more efficient clearance of tumor cells during in vitro coculture (Figure V-11F).

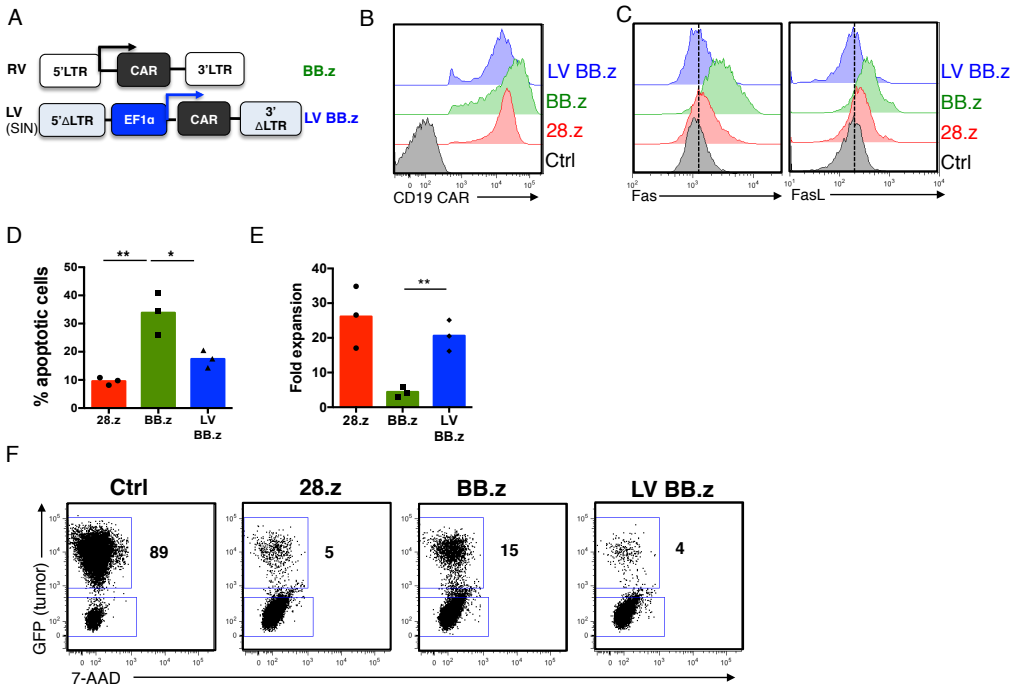

Figure V-11- Expression of BB.z CD19 CAR from a Lentiviral Vector Reduces Toxicity. (A) Schematic representation of the gammaretroviral (BB.z) and self-inactivating lentiviral (LV BB.z) vectors.

Arrows indicate promoter activity in each expression system. **(B)** Representative histograms showing CD19 CAR expression in T cells 7 days after transduction BB.z or LV BB.z vectors. **(C)** Representative histograms showing surface expression of FAS and FASL in CD19 CAR T cells. (D) Frequency of apoptotic cells in CD19 CAR T cells 7 days post-transduction measured by Annexin V staining. **(E)** Expansion of CD19 CAR T cells over 7 days. **(F)** CD19 CAR T cells were cocultured with GFP^+ $CD19^+$ Raji cells at a 1:4 ratio for 72 hr. Representative dot plots show the frequency of residual live tumor cells at the end of coculture. Numbers indicate percent of tumor cells among total live cells. Experiments were replicated three times

Similar to IRES BB.z CD19 CAR T cells, lentivirally transduced BB.z CD19 CAR T cells were more protective in the systemic mouse xenograft model of B-ALL, resulting in increased expansion of CAR T cells in peripheral blood (Figure V-12A) and lower frequencies of tumor cells (Figure V-12B). Overall, LV BB.z CD19 CAR T cells achieved more potent suppression of leukemia progression (Figure V-12C), leading to a significant extension of survival compared to BB.z control (Figure V-12D).

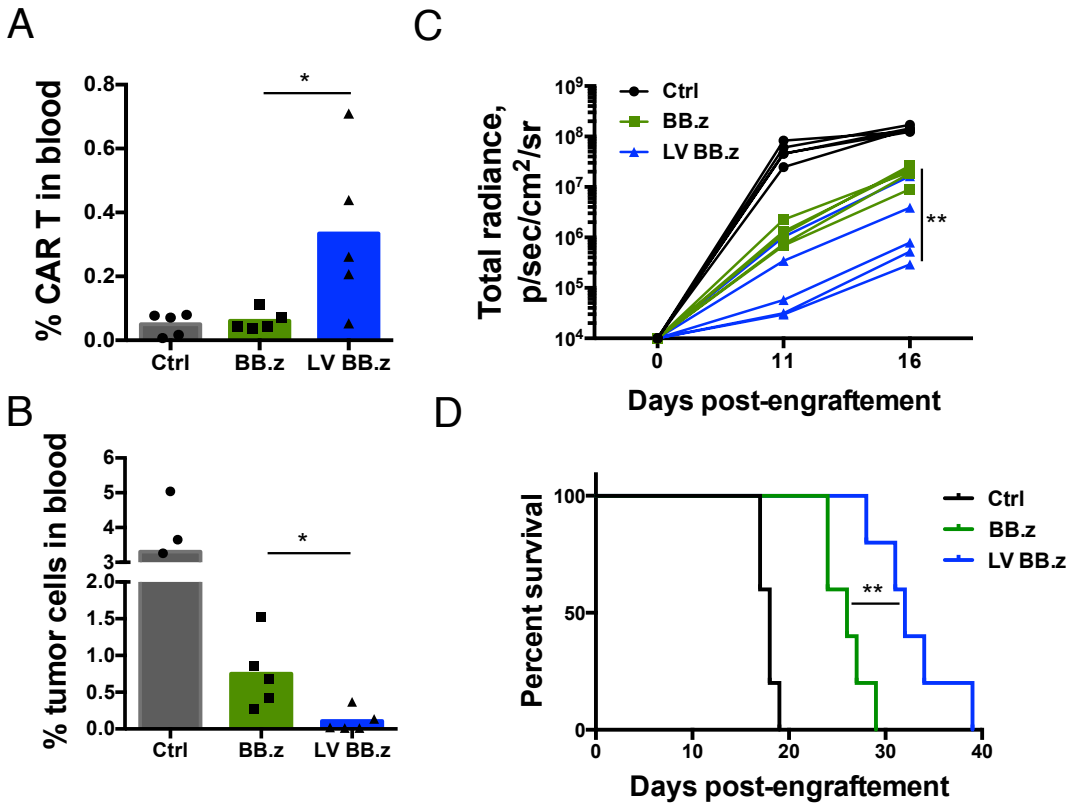

Figure V-12- Expression of BB.z CD19 CAR from a Lentiviral Vector Improves the Anti-tumor Function of CAR T Cells in vivo. **(A)** Expansion of LV BB.z CD19 CAR T cells in peripheral blood of NSG mice engrafted with $1x10^6$ GFP^+ $FFluc^+$ NALM-6 cells 15 days prior followed by a single intravenous injection of $8x10^5$ CD19 CAR T cells 3 days later. **(B)** Frequency of GFP^+ tumor cells in peripheral blood of mice 15 days post-engraftment. **(C)** Tumor burden in mice measured by IVIS imaging at indicated time points. **(D)** Survival of tumor-bearing mice receiving either BB.z or LV BB.z CD19 CAR T cells ($p = 0.0062$ by Mantel-Cox log-rank test). Experiments were replicated twice for. *$p < 0.05$; **$p < 0.01$ by Student's t test or one-way ANOVA.

V.3.6. Change of spacer decreases CD19 CAR expression and restores normal T cell function

Retroviruses are among the most commonly used vectors in clinical trials. Their efficiency and ease of production compared with lentiviral vectors are among the advantages former. We also demonstrated that it was possible to restore normal CAR T cell functionality by making changes in the vector (adding an IRES) or replacing it by a lentiviral vector, both approaches leading to a decrease of CAR expression and toxicity. The hinge connecting the scFv and the transmembrane domain is most often considered important to allow appropriate binding between the scFv and its epitope. For that reason, its size needs to be optimized for the molecule being targeted. However, when we replaced the CD8α hinge by the C_H3 IgG domain or the IgG1/IgG4-derived short hinge (Figure V-13A), lower CAR T cell expression was restored (Figure V-13B) and CAR T-cells expanded significantly better (Figure V-13C). This decrease in CD19 CAR expression was accompanied by beneficial effects, such as a small increase in naïve and central memory subsets (Figure V-13D) and a decrease in apoptotic cells (Figure V-13D), similar to those observed with the inclusion of the IRES or the use of a lentiviral vector.

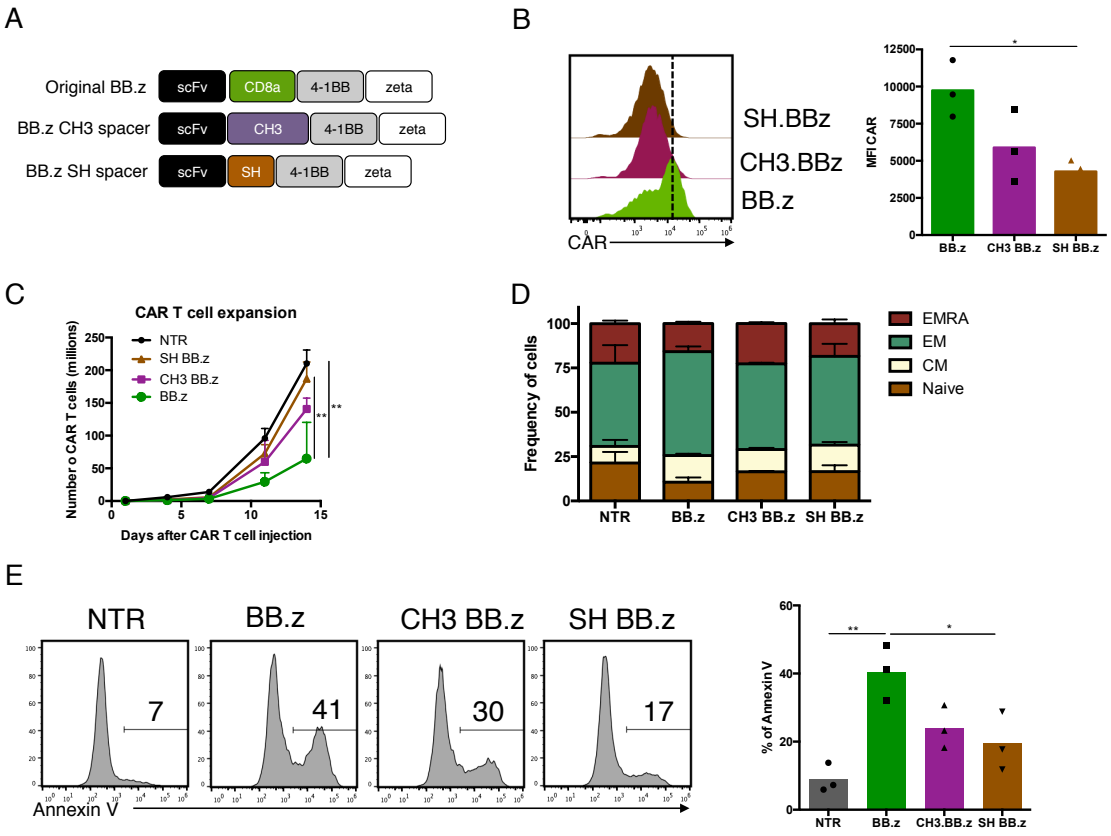

Figure V-13- IgG derived spacer decreases toxicity in 4-1BB CD19 CAR T cells. (A) Schematic representation of the expression systems utilized to express BB.z CARs in T cells. The CH3 and short hinge spacers have the same CD28 transmembrane domain while the original 4-1BB has CD8α hinge and transmembrane domain. **(B)** CD19 CAR expression in T cells with different spacers at day 7 after transduction. Bar graph denotes the median of florescence intensity of CAR expression for three donors. **(D)** Relative proportion of naive-phenotype (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated

effector cells (T_{EMRA}) CD19 CAR T cells 14 days post-transduction defined by CCR7 and CD45RA expression. **(E)** Expansion of T cells retrovirally transduced with 4-1BB CARs with different spacers for 14 days. **(E)** Representative histograms showing Annexin V staining of CD19 CAR T cells 7 days post-transduction. Bar graph shows summarized data for all three CARs from 3 donors. Data represent two to five independent experiments.

To demonstrate improved antitumor effect with this change of hinge domain, we tested 4-1BB CD19 CARs with CD8α hinge versus short hinge (which demonstrated a slight advantage over the C_H3 intermediate spacer in vitro) in the same xenograft mouse model used in previous *in vivo* experiments (Figure V-14A). Indeed, T cells whose CD19 CAR expression was lower due to the presence of the short IgG hinge performed better, with slower leukemic progression (Figure V-14B,C) and prolonged survival (Figure V-14D).

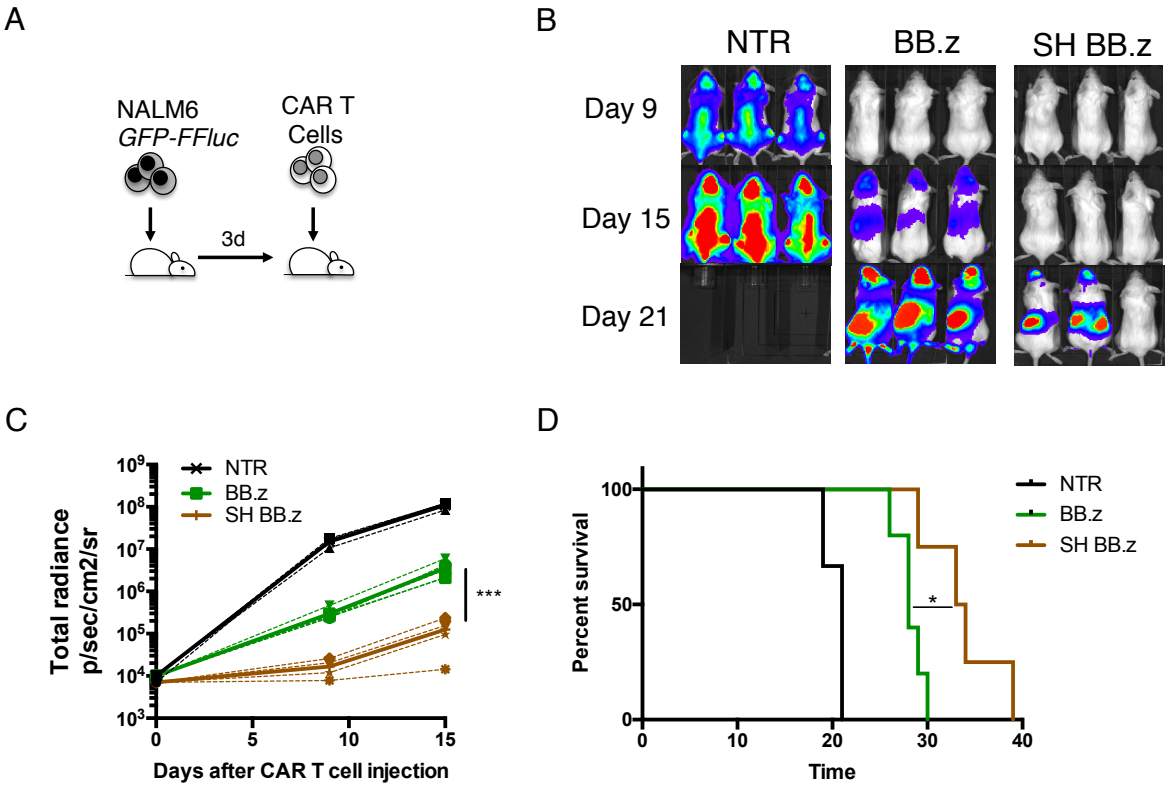

Figure V-14- CD19 CAR T cells withspacer derived from IgG4 sequence has stronger antitumor effect in vivo (A) Schematic diagram of the experiment. NSG mice were intravenously engrafted with $1x10^6$ GFP^+ $FFluc^+$ NALM-6 cells followed by a single intravenous injection of $1x10^6$ CD19 CAR T cells 3 days later. **(B)** Representative images showing leukemia progression in individual mice at day 9 and 15 and 21 after CAR T cell injection **(C)** Kinetics of NALM-6 luminescence in mice measured by IVIS. **(D)** Kaplan-Meier curves showing advantage in overall survival of mice receiving BB.z CD19 CAR T cells with CD8 and short IgG spacer. Data represent 2 independent experiments. *$p < 0.05$; ***$p < 0.001$ by Student's t test.

This demonstrates that CAR expression can be modulated solely by modifying the backbone of the CAR, an aspect often neglected, which led clearly to improved performance.

V.4.DISCUSSION

Clinical studies of BB.z CD19 CAR T cells demonstrated complete remissions in patients with B cell malignancies associated with increased persistence of transgenic T cells [202], [204], [294]. BB.z CAR T cells additionally have been shown to resist the functional exhaustion associated with constant CAR signaling, leading to the suggestion that 4-1BB costimulation may be universally beneficial for CAR T cells [226]. 4-1BB signaling in T cells is primarily mediated by TRAF adaptor proteins that can activate c- Jun N-terminal kinase (JNK) and p38 as well as NF-kB via both canonical and non-canonical pathways [248]. Acute stimulation of 4-1BB in human T cells protected CD28 tumor-specific cells against AICD upon restimulation [425], promoted mitochondrial biogenesis, and enhanced the development of central memory cells [409]. These studies indicate 4-1BB can have a protective role in T cell persistence. To study the effects of continuous 4-1BB costimulation in CAR T cells, we overexpressed BB.z CARs to induce their tonic antigen-independent signaling. In our studies, we did see an in- crease in the central memory population among cells expressing tonically signaling BB.z CARs. However, constant BB.z CAR signaling resulted in augmented T cell apoptosis, illustrating that in different conditions 4-1BB stimulation can have vastly different effects on T cells. In fact, NF-kB can play a pro-apoptotic role in T cells by directly activating expression of *TRAIL* [426]. We found that upregulation in BB.z CAR T cells of *FAS* and *FASL*—another pair of NF-kB target genes—contributed to the enhanced apoptosis. Our results suggest that by stable upregulation of pro-apoptotic target genes of NF-kB, tonic 4-1BB signaling may undermine the beneficial effects of BB.z sequences in the CARs expressed by T cells. Therefore, 4-1BB stimulation of T cells may not be universally beneficial; rather, the overall outcome of 4-1BB signaling may depend on its intensity and duration.

While T cells expressing CD19 CARs with either the CD28 or 4- 1BB costimulatory endodomain produced complete responses in patients with advanced $CD19^+$ tumors [203], [204], [294], [318], these studies used different expression systems. CD28.z CARs expression was driven by gammaretrovirus, whereas BB.z CARs were expressed from self-inactivating lentiviral vectors. Our results show that when BB.z CD19 CAR is expressed from a gammaretroviral system it can produce sufficient spontaneous activation to precipitate apoptosis in T cells, impairing their expansion and thereby potentially diminishing their anti-tumor activity in patients. This mechanism of toxicity from tonic signaling is not restricted to the CD19 CAR, but rather represents a more general mechanism, since we show the same effects in K CAR, a clinically implemented CAR, and in CD7 CAR.

Our data demonstrate the value of two alternative approaches to overcome the problem of excessive tonic signaling associated with a given CAR and its expression system. The first is to reduce CAR expression, because high CAR expression is not always necessary for T cell activation or efficient recognition of target cells. While insufficient avidity of the CAR-antigen interaction may impact formation of a productive immunologic synapse, for some antigens the optimal range of CAR expression may be much lower than that produced by a given expression system. Our results corroborated previous studies where lowering expression of 28.z c-Met and CD19 CARs by modulating the promoter reduced spontaneous activation of CAR T cells and augmented their anti-tumor function [274], [408]. Second, the level of activity of a given costimulatory molecule, and thus its capacity to induce tonic signaling, is additionally determined by the non-translated portions of the expression system.

Thus, while inclusion of the costimulatory 4-1BB endodomain in CAR constructs has improved their clinical success, we show that it can cause toxic tonic signaling even in CD19 CAR T cells. This signaling is not only a function of extracellular CAR domains, but can also be amplified by the interaction between costimulatory signaling and the expression system. Disrupting the positive feedback loop between tonic costimulation and a CAR expression system by utilizing alternative promoters may help reduce tonic signaling in T cells and thus mitigate its consequences. Taken together, these studies underscore the potential toxicity of tonic 4-1BB signaling CARs and thus will contribute to the rational design of CAR platforms for optimal clinical performance.

Conclusions and future directions

Adoptive T cell therapy using chimeric antigen receptors has demonstrated impressive objective clinical results in patients with refractory or relapsed hematologic malignancies. Despite this success, the technology is still in its infancy and its design is still empirical, with several aspects of CAR-T cell behavior not being fully understood yet. More than just the incorporation of an extra gene, transduction with a CAR can almost be considered as the introduction of new “source code” for biological systems to instruct T cells (or other lymphocytes) to eliminate diseased cells based on the recognition of a molecule that, owing to immunological rules, is most often considered a self-antigen by the organism. Due to the intrinsically unrestricted nature of this new command, normal cells can be equally affected, leading to potential destruction of any tissue that shares expression of the targeted molecule. The severity of such toxicity depends intimately on how many and which tissue(s), and how important they are for survival. Therefore, the first step in CAR development – identification of a target molecule – requires extensive consideration of the benefits and risks of tumor elimination versus normal tissue destruction. The success of any CAR in a clinic context depends on this perfect balance between avoiding extensive tissue destruction and achieving efficient elimination of all tumor cells. Even if certain aspects of CAR design are still not fully understood, selection of the best molecule to be used as a target is undoubtedly the most important. The targeted molecule defines which tumor and normal cells will be affected, even though the backbone, type and number of endodomains, and method of gene delivery can influence the potency of a particular CAR. However, the latter properties can and have been perfected at later time points, and several of these aspects were addressed through this work.

CAR-T cells designed against hematologic malignancies (especially B-cell leukemias) have led to the most successful results in human trials. Several reasons can explain this. First, hematologic tumors and normal hematopoietic cells are commonly defined by their characteristic cell surface proteins. A deeper knowledge of cell surface markers normally present in immune cells and aberrantly expressed in hematologic tumors makes CAR-T cell technology easier to design for blood cancers and can somewhat explain this biased success. Indeed, although only a reduced number of CARs are expected to reach commercial use, almost every type of hematologic malignancy has targets being tested for CAR-T cell therapy. CARs that target CD33, CD123, LeY, CCL-1, and NKG2D for myeloid leukemia; CD19, CD20, CD22, CD30, and CD37 for B-cell leukemias/lymphomas; CD138

and BCMA for multiple myeloma, are good examples, with several others being under investigation and likely to be tested in preclinical or clinical trials soon.

T-cell malignancies have been, on the other hand, less amenable to adoptive cell therapy. The difficulty in designing CARs for T cell leukemias/lymphomas lies on the extra challenge of avoiding the elimination of the therapeutic product (i.e., the modified T cells) during its generation. CD5, and to a lesser extent CD30, are expressed in T cells but transduction with CARs targeting these antigens demonstrated no or minor cellular fratricide [218], [219]. This success demonstrated that, at least for some CARs, targeting of molecules commonly expressed on T cells does not impose a challenge.

CD7 is a pan-T and NK cell marker with an activating role in T cell physiology [342]. Among several pan-T cell markers, CD7 is expressed earliest in T cell development, even before CD3 or the TCR [427]. Considering that its expression is usually not lost in T-cell neoplasms and that CD7 is not found in any other tissues, the therapeutic risk/benefit balance is fulfilled, and thus we selected this marker as a good candidate target for T-cell malignancies. However, due to the constant expression of CD7 on the surface of all T cells, co-expression of a CD7 CAR leads to detrimental toxicity that completely abrogates the efficient expansion of T cells bearing this CAR. Other types of cytotoxic lymphocytes are being tested as potential alternatives to CAR-T cell technology, which might even be advantageous for some applications [428]. However, CD7 is expressed in lymphocytes other than T cells, limiting the use of CD7 CAR in other cell platforms, such as NK cells. Although 10% of native T cells appear to be CD7-negative T cells and sorting this cell population has been proposed as a solution to the problem of fratricide in culture [429], so far this approach has never been tested and the low number of T cells available as starting material may be a significant downside, especially considering the high numbers of T cells required for adoptive therapy. To achieve those numbers of cells starting with just 10% of all T cells would require prolonged expansion, likely leading to T-cell exhaustion and reduced persistence, two crucial factors for the success of any adoptive T cell therapy.

To solve this problem, we decided instead to turn off CD7 expression by disrupting its gene. Among several gene editing tools available, CRISPR/cas9 has recently gotten more attention due to its usability and practicability. CRISPR/cas9, like other gene editing tools, creates a double break in a particular gene locus [430]. However, what makes this system attractive is that it relies on nucleic acid interaction (a RNA guide that interacts with the DNA of the cell host) to select the gene of interest to be disrupted, whereas systems like zinc fingers and TALEN are based on DNA-protein interaction, which is more difficult and time-consuming to design. Knockout of a gene does not remove the molecules already translated in the cell and, thus, after studying the kinetics of CD7 downregulation after disruption of its gene, a protocol to introduce the CD7 CAR into CD7-ablated cells with minor interference in

the overall standard operating procedures for CAR-T cell generation was developed. Creation of this protocol had in consideration its conversion to good manufacturing practices, maintaining reduced T-cell expansion time and T-cell exhaustion. Clinical trials using CRISPR/cas9 system in T cells have been launched (NCT03399448) but reports using these genetically modified cells in humans have not been published yet. Therefore, their use and safety profile in the clinical context are not yet known.

Interestingly, an alternative approach to this was recently published. Instead of knocking out CD7, Campana and collaborators introduced together with the CAR a second gene that sequesters CD7 in the endoplasmic reticulum [343]. Both approaches have advantages and disadvantages. Blockade of CD7 migration to the cellular membrane is a simpler process since it requires a single genetic modification instead of the two-step process used in our work. However, one can argue that although CAR activation may be prevented in a synapse between two T cells, it may still occur if CAR and CD7 molecules are both present in the endoplasmic reticulum. This undesired activation was never clearly proven for CD7, but a similar effect was seen with a CD5 CAR [431]: since expression of CD5 is not fully abrogated, only precluded from occurring on the cell surface, cells that express CD5 internally differentiate (and senesce) faster due to constant CAR activation. Indeed, disruption of the CD5 gene (as opposed to downregulation of its expression) in CD5 CAR-T cells reduced self-activation when compared to CD5 CAR-T cells without disruption [432]. If a similar effect will be observed for CD7 CAR is, for now, unknown.

In this work, we also tested the possibility of preventing CAR expression during expansion using a regulatory system based on a tetracycline responding promoter. The Tet-Off (tetracycline-controlled transcriptional activation) system can be used to prevent gene expression after genetic modification by providing doxycycline in culture medium [431]. However, this system never worked perfectly since small amounts of CAR molecules where still detectable on the cell surface, which was enough to produce fratricide. Minimal expression is commonly detectable in this type of system in the presence of doxycycline because of incomplete inhibition of gene expression [433]. Nonetheless, even if this strategy avoids fratricide during CAR T-cell expansion in vitro, as soon as they are injected into patients (in the absence of doxycycline) both the CAR and the CD7 molecules would be present on the cell surface. While tumor elimination might prevail over CAR-T cell elimination because of high concentration of tumor cells at sites of disease, reduced persistence of CAR-T cells would still be expected due to in vivo fratricide. Moreover, since the Tet-Off system introduces bacterial sequences into the proteome, there is potential immunogenicity of transduced cells, which may also reduce their persistence.

Regardless of the method used to avoid T-cell fratricide, a second hurdle to the use of adoptive T cell therapy in T-cell malignancies relates to the potential contamination of

normal T cells with malignant T cells. After collection of T cells from peripheral blood of tumor patients, a CD3 antibody is used as a stimulus to specifically expand T cells from a population of PBMCs. CD3 expression is absent in most patients with T-cell leukemia and, thus, activation of T-cell blasts should be a rare event. Nonetheless, even though only a minority of T-cell lymphomas are $CD7^+$, CD3 is expressed in the majority of them. While it is expected that lymphocytic blasts will rarely survive long periods of culture in vitro and repeated freeze/thaw cycles (as observed in the assays we performed with patient-derived samples), inadvertent introduction of a CD19 CAR into B-cell blasts has already been reported, proving that, although rare, such events do occur [434]. Those B-cell blasts gave rise to a CD19-negative population that lead to relapse.

In our particular application, owing to the presence of T-cell endodomains that promote expansion, expression of a CAR in malignant T cells could facilitate emergence of a more aggressive clone. Although our attempts at introducing a CAR in leukemic T-cell lines led to cell death, the effect in primary tumor T cells is unknown. More problematic, however, is the potential knockout of CD7 in malignant T cells, immediately rendering such clones invisible to our therapeutic approach. This risk could be averted by using allogeneic T cells collected from healthy donors. However, administration of native allogeneic T cells can lead to serious GvHD, which can be fatal.

Further genetic modification of T cells to allow their safe use in an allogeneic setting is, therefore, being intensely researched, since it would permit transforming CAR-T cells into an off-the-shelf product, a less cumbersome manufacturing system compared to the personalized nature of autologous products, which prevents fabrication in advance. Since CAR-T cells do not require their TCR for activation, removal of either the α or the β TCR chains allow target recognition while preventing GvHD, as already demonstrated in a report of two infant patients [317]. Since double knockout can be performed in one step, TCR disruption can be achieved at the same time as CD7 knockout, as was recently demonstrated [389]. Yet, although promising, this approach still has drawbacks.

As mentioned before, any CAR must be designed taking into account potential elimination of normal cells expressing the target antigen. For the CD7 CAR, T- and NK-cell aplasia is expected, in a similar fashion to the B-cell aplasia observed in CD19 CAR trials. While lack of humoral response can be replaced by immunoglobulin infusion, lack of T and NK cells is more difficult to substitute and can lead to fatal opportunistic infections. In chapter II, we demonstrated that knockout of CD7 does not compromise antiviral responses by T cells. Thus, after infusion into patients, a CAR T-cell product with intact TCRs can not only fight tumor cells through the CAR, but also repopulate the host and provide protection via the TCRs against pathogens (particularly, viruses) that commonly affect immunosuppressed individuals. TCR removal precludes CAR-T cells from eliminating

infected cells. Whereas the approach used in chapter II, which spares native TCRs, may reduce the risk of infections, it does not allow the use of allogeneic cells as a means to avoid the risk of genetically modifying malignant T cells during CD7 CAR-T cell product generation.

In chapter IV, nonetheless, we demonstrated that CD7 can also be efficiently knocked out in virus-specific T cells (VSTs). $CD7^-$ VSTs allow the administration of a more specialized T cell product that is active against viral infections, but that can also be used to generate CD7 CAR VSTs. The interesting point of this approach combining CAR T cells with VSTs is that VSTs can be used in the allogeneic setting provided partial HLA-match with the patient is ensured, as demonstrated in clinical trials conducted at Baylor College of Medicine [399], [400]. Moreover, generation of CAR VSTs has already been established for hematologic [435] and solid tumors [177], proving the feasibility of this approach. Thus, $CD7^-$ VSTs can, in theory, be used as a platform for CD7 CAR therapy. This approach, combining the antiviral activity of third-party T cells that still express a TCR specifically recognizing pathogens commonly found in immunocompromised patients with a CD7 CAR that can eliminate AML or T-ALL cells, will be tested in future studies. Although third-party VSTs cannot be considered a universal platform for T-cell therapy, they can still be viewed as an off-the-shelf product that is produced in advance and independently of the recipient. Its most important advantage is that it eliminates the risks associated with transduction of patient-derived PBMCs, which can be contaminated with tumor cells.

Use of TCR^+ CD7 CAR-T cells is of increased importance when considering their potential use in AML, since several therapies (including CAR-T cells) targeting myeloid antigens have an overall myeloablative effect. Because CD7 is not expressed in normal myeloid cells, these immune cells are not eliminated and thus can continue to present microbial peptides to T cells, reducing the risk of infection. Elimination of monocytes and monocyte-derived cells would not only affect innate immune responses dependent on phagocytosis but also prevent antigen presentation to adaptive immune cells, making the generation of TCR^+ CD7 CAR^+ T cells useless since TCR activation through APC presentation would not occur properly. CD33 and CD123 CARs are examples of constructs being tested now in clinical trials of AML. However, their toxicity profile may restrict their use to the period immediately before hematopoietic stem cell transplantation, since normal stem and progenitor cells share these antigens and are also eliminated, especially with a CD33 CAR [220].

AML is a very heterogeneous malignancy and a unique, low-toxicity CAR for this type of cancer may not be attainable. CD7 is expressed in 30% of AML patients, and thus roughly a third of the patients would be suitable for CD7 CAR-T cell treatment. It is conceivable that

several other CARs can be developed for this purpose and used according to the expression of their targets in leukemic cells.

Since the same CD7 CAR can be employed in T-ALL (Chapter II) and in AML (Chapter IV), T- and NK-cell aplasia is expected in both settings. A potential solution to avoid T-cell aplasia in AML would be to use an inhibitory CAR (iCAR) to target a second pan-T cell marker. Instead of activating T cells, iCARs trigger signaling pathways that prevent their activation. For example, a modified CD5 CAR with a PD-1 or CTLA-4 endodomain (CD5 iCAR) could prevent CD7 CAR-T cells from killing $CD5^+$ cells despite CD7 expression, and thus normal T cells (which express both CD5 and CD7) would be spared. Since CD5 is not expressed by AML cells, these CD5 iCAR CD7 CAR-T cells would be able to exclusively kill tumor cells. This approach would not work for T-cell leukemias since T-cell blasts can express both CD5 and CD7. Experience with iCARs is, however, limited [314] and their feasibility and robustness have not been confirmed yet.

Relapses caused by the loss of the target antigen in tumor cells have already been reported in the literature. For example, loss of CD19 expression has been detected in CD19-directed therapies, despite its rare occurrence with older chemotherapeutic approaches. Considering that loss of at least some of the T-cell markers is commonly observed in patients with T-cell malignancies [316], combined targeting becomes even more relevant for T-cell malignances, as it is for B-cell cancers. Combined targeting of CD5 and CD7 can be tested in the future as a prophylactic measure to avoid tumor relapses related to antigen escape.

However, even simultaneous targeting of two T-cell markers may not be guaranteed to prevent tumor escape. Therefore, we also tested in this work the feasibility of targeting CD3, an exclusive pan-T-cell marker. Since this antigen is constitutively expressed by T cells, we used the same CRISPR/cas9 protocol to first ablate CD3 and then transduce the CD3 CAR, so as to avoid T-cell fratricide. This work showed that the same the protocol developed to generate CD7 CAR can also be adapted to other target molecules that can produce fratricide, including CD3. In theory, any other surface marker also expressed by normal T cells and that could be a good target for adoptive cell therapy can be removed using CRISPR/cas9 with minor or no changes to the strategy before the introduction of a CAR.

Nonetheless, in the case of CD3, the unexpected elimination of CAR-bearing T cells by normal T cells would prevent CAR-T cell persistence and preclude an efficient antitumor effect, despite the observed elimination of tumor cell lines in vitro. Although several aspects of CAR-T cell therapy are not fully understood, compelling data suggest that higher T-cell persistence and expansion in vivo are key characteristics associated with better outcomes. Because CD3 CAR-T cells are quickly eliminated by normal T cells, we do not expect sufficient persistence of these adoptively transferred cells in order to produce a strong

antitumor effect. Thus, we decided to stop the development of CD3 CAR as a therapy for T-cell malignancies. In any case, since all CAR-expressing T cells are rapidly and specifically eliminated, controlled expression of this CD3 CAR could potentially be used as a suicide switch for CAR-T cell elimination. Conversion of this CD3 CAR to an inducible system (using a Tet-On system) is underway. If a modified CD3 CAR can be expressed in vivo only after injection of doxycycline, any CAR-T cell could be specifically eliminated when desired, for example when life-threatening side effects develop or when CAR-T cells survive longer than needed, as it is possibly the case in patients treated with a CD19 CAR containing a 4-1BB endodomain. Despite the positive effect of longer persistence of CD19 CAR T cells, these patients also experience extensive periods of B-cell aplasia, which may have negative consequences. Transduction of an inducible CD3 CAR together with CD19 CAR (or any other CAR) can be a way to control how long this product persists in the patient.

CD19 CAR is by far the most successful CAR in use today. Some aspects underlying this success are easy to identify: CD19 is expressed in all B-cell malignancies; loss of its expression is a rare event; less aggressive tumor microenvironment (especially compared with solid tumors); consequences of loss of B cells are manageable; and easy access by CAR-T cells to tumor cells. Others, however, are more difficult to grasp. CAR expression, albeit capable of redirecting a T cell to any target molecule, happens at the expense of increased differentiation in parallel with stronger CAR activation. Due to the nature of the transduced expression cassettes, CARs are constitutively expressed, with little or no regulation. This intrinsic lack of control exerts a potentially deleterious pressure on T cells. T cells engineered with a CAR are more susceptible to constitutive expansion and differentiation. Premature exhaustion of T cells is a phenomenon observed in all CAR-T cells, although some CARs, such as a GD2 CAR, seem to be more prone to this undesirable effect than others, such as CD19 CAR [226]. Tonic signaling (constitutive activation of CAR endodomains independent of its binding to the target antigen) is one example of an uncontrolled and not fully understood mechanism. Such effect seems to be more deleterious to some CARs than others and can explain differences observed in in vitro assays, which do not require T cell migration, are unable to replicate clinical side effects such as cytokine release syndrome, and use homogenous tumor cell lines. Although far from ideal, such assays control for several variables, allowing a cleaner characterization of the CAR. These assays allowed us to conclude than a CAR can behave differently if a minor change in its design is made, revealing the complexity of building a fully functional synthetic gene. All portions of the CAR molecule are important and any minor change can profoundly affect the overall CAR T-cell behavior. Increasing the number or changing the type of endodomains influences the number or type of signaling pathways that such CAR can activate and, in doing so, alters the nature of T-cell responses.

Because of the immunocompromised state expected in patients after receiving CD7 CAR-T cells, most of the work was done using the CD28 endodomain, which in several clinical trials seems to be associated with shorter CAR T-cell persistence than 4-1BB. Thus, use of CD28 instead of 4-1BB can modulate the expected side effects caused by on-target/off-tumor activity. Use of an on-demand suicide gene can also be a protective measure in case T-cell aplasia leads to life-threatening complications. Specific elimination of CD7 CAR-T cells should lead to fast restoration of normal T cells since CD7 is only partially expressed by lymphoid progenitors.

Unexpectedly, inclusion of 4-1BB was toxic not only for CD7 CAR-T cells but more importantly for clinically validated CD19, GD2, and kappa-light-chain CAR-T cells. Such phenomenon had never previously been reported, despite the frequent use of CD19 CARs with 4-1BB endodomain, which have been approved by the FDA as a therapy for patients with relapsed B-cell leukemias. Higher expression of CAR molecules on cell surface is observed in several CARs containing a 4-1BB endodomain and can be associated with stronger activation of T cells, but its dependency on the type of endodomain had never been demonstrated. This points to an ignored aspect of CAR constructs: gene regulation. Any endogenous gene is under strict regulation and is expressed according to specific stimuli that can originate from the cell cycle, stress, extracellular molecules, and other factors. CARs, especially those introduced via gamma-retroviral vectors, are highly and constitutively expressed, a characteristic that may not be desirable and, as we demonstrated in chapter V, exacerbated by a 4-1BB co-stimulatory domain.

Although in a different context, it has been demonstrated that a CD28-containing CD19 CAR performs better when introduced in the TCR locus, so that its expression is as tightly regulated as the TCR gene in T cells [274]. This system allowed lower and stable expression due regulation by a human promoter instead of the viral LTR promoter, but also due to the ability to control the number of transgene copies per genome, something that most engineering tools cannot do. In our work, use of a different promoter within self-inactivating lentiviral vectors restored the normal function of 4-1BB-containing CD19 CAR-T cells, similar to that observed in clinical trials. Interestingly, use of an IRES sequence, which forces downregulation of CAR expression, produced a similar effect, demonstrating that the toxicity of a CAR can be modulated not only by changing the vector/promoter but also the non-coding regions of its cassette.

Modification of the spacer and transmembrane domains also demonstrated to be effective in preventing constant T-cell activation. This establishes even further the importance of the full backbone in CAR design and function. Different backbones may not only change how the signal is sent from extracellular to intracellular domains but also change how the molecule folds. Different folding structures may be particularly important to

prevent the interaction in cis of CAR molecules on the surface of the same cell and provide optimal physiology to the CAR T-cell. CD19 CARs with an IgG1-derived short-hinge sequence will be used in future clinical trials at Baylor College of Medicine.

As demonstrated in Chapter V, improvements that lead to reduced tonic signaling are extremely important to guarantee the selection of an optimized CAR for clinical use, with beneficial outcomes to the patient. Nonetheless, from a purely theoretical point of view, tonic signaling may be a desirable effect at low level. The success of any adoptive CAR-T cell therapy is correlated with the ability of T cells to expand and persist in vivo. Such ability is undeniably correlated with the use of a co-stimulatory endodomain. However, it appears that some CAR-T cells can expand even in the absence of target tumor cells and cytokines, and such expansion has been correlated with the presence of 4-1BB in the CAR [193]. Expansion independent of cytokines seems to be of extreme importance in the tumor microenvironment, since tumor and stromal cells do not secrete T_H1 cytokines, required for proper T-cell activation. The ability of CAR-T cells to persist for years in patients in remission can potentially be considered a desired effect since relapses can occur months or years after cancer cells become undetectable. For example, leukemias with central nervous system involvement are associated with poor prognosis and later relapses. Thus, one can hypothesize that, in situations where CAR-T cell persistence does not pose a clear risk to the patient, prolonged presence of CAR-T cells after tumor cells become undetectable by common diagnostic tools can be important to reduce or delay relapses. Such persistence may depend on a minimal but continuous activation of T cells due to tonic signaling.

T cells engineered with a CAR were the first genetically modified product approved by a regulatory agency. This approval pushed adoptive cell therapy into a new era in which genetic transfer of these tumor-specific receptors into T cells can convert them into effective cancer killers. CARs allow antigen recognition without MHC presentation and thus have removed one of the obstacles to more widespread application of T-cell therapy. CAR-T cells allow precise recognition of specific target molecules, like monoclonal antibodies, but possess the additional benefits of active trafficking to cancer sites, in vivo expansion and potential long-term persistence. While antibodies targeting CD7 failed to demonstrate antitumor effect in preclinical models, conversion of such targeting to CAR-T cell technology demonstrated promising results in preclinical models developed in this work. However, generation of new, better CARs is not confined to changing binding domains. Optimized CAR design, better understanding of the processes leading to T-cell activation and memory, and improved gene transfer methods have allowed this strategy to be successfully brought to the clinic. Emerging data illustrate the complexity of CAR design and, in this work, we demonstrate how several different aspects, directly and indirectly related to the CAR structure, can influence its potency. Nevertheless, the striking results obtained with CD19-

specific CAR-T cells support the idea that this therapy will soon join the mainstream of oncologic treatment not only for B-cell malignancies but also other cancers.

Supplemental data

Table 3-Results from GUIDE-seq in T cells for off-targets using gRNA 72

Target Site	GUIDE-Seq # Reads	Mock-1	Mock-2	Mock-3	sgRNA_72-1	sgRNA_72-2	sgRNA_72-3	p-value		Sequence	hg38	Closest Gene	Distance	Feature
CD7_sgRNA_72	225	0.03	0.04	0.02	44.37	54.51	18.66	0.03		GAGGTCAATGTCTACGGCTCCGG	Chr17:82316687-82316709	CD7	n/a	Exon
sgRNA_72_OT1		0.00	0.01	0.01	0.01	0.01	0.00	0.29		TGGGACAGTTCTACGGCTCTGG	Chr2:21048127-21048149	APOB	4kb	Intergenic
sgRNA_72_OT2		0.02	0.02	0.03	0.02	0.02	0.01	0.26		AGGGAGAATGTCTAAGGCTCTGG	Chr12:9264891-9264913	LINC00987	2kb	Intergenic
sgRNA_72_OT3		0.03	0.02	0.00	0.01	0.02	0.02	0.47		GGGGCCATTGTCTAAGGCTCTGG	Chr3:180235335-180235357	PEX5L	198kb	Intergenic
sgRNA_72_OT4		0.01	0.00	0.01	0.01	0.00	0.01	0.25		TGGGGGCAGTGTCTAGGGCTCTGG	Chr3:58167470-58167492	FLNB	na	Intron
sgRNA_72_OT5		0.00	0.00	0.00	0.01	0.01	0.00	0.12		GGGCTTCAATGTATACCGCTCAGG	Chr11:95528043-95528065	FAM76B	240kb	Intergenic
sgRNA_72_OT6		0.01	0.00	0.00	0.00	0.01	0.00	0.46		GGGCTTCAATGTATACTGCTCAGG	Chr13:64137847-64137869	LINC00355	61kb	Intergenic
sgRNA_72_OT7		0.00	0.00	0.00	0.00	0.00	0.00	0.21		GGGCTTCAATGTATACTGCTCAGG	Chr3:4955113-4955135	BHLHE40-AS1	na	intron
sgRNA_72_OT8		0.00	0.00	0.01	0.01	0.02	0.00	0.22		GGGTTCAATGTATACTGGTCAGG	Chr11:101291874-101291896	PGR	162kb	Intergenic
sgRNA_72_OT9		0.01	0.01	0.00	0.00	0.00	0.01	0.48		GGGCTTCAATGTATACTGCTCCGG	Chr21:22331778-22331800	LINC00308	215kb	Intergenic
sgRNA_72_OT10		0.00	0.00	0.00	0.03	0.02	0.00	0.10		AGGTTCAATGTAATACTGCTCGGG	Chr1:193361779-193361801	LINC01031	na	Intron
sgRNA_72_OT11		0.00	0.00	0.00	0.00	0.00	0.00	0.00		GGGTTCAATGTATACTGCTCGGG	Chr2:29029841-29029863	TOGARAM2	na	Intron
sgRNA_72_OT12		0.01	0.02	0.02	0.01	0.01	0.01	0.14		GGGTTCAATGTATACTGCTCGGG	ChrX:122102686-122102708	MIR3672	731kb	Intergenic
sgRNA_72_OT13		0.00	0.00	0.00	0.00	0.01	0.00	0.21		GGGTTCAATGTATACTGGTCGGG	Chr2:240658268-240658290	AQP12B	18kb	Intergenic
sgRNA_72_OT14		0.01	0.00	0.01	0.00	0.00	0.00	0.12		GGGTTCAATGTATACTGCTCGGG	Chr16:77152201-77152223	MON1B	38kb	Intergenic
sgRNA_72_OT15		0.02	0.01	0.02	0.01	0.01	0.01	0.36		GGGGTGAATGTTTACAGCTCTGG	Chr3:167247629-167247651	ZBBX	na	Intron
sgRNA_72_OT16		0.04	0.02	0.01	0.02	0.00	0.01	0.16		GGGGTCAGTGTATACTGCTCAGG	Chr4:183891648-183891670	STOX2	na	Intron
sgRNA_72_OT17		0.01	0.00	0.00	0.00	0.01	0.01	0.45		GGGGTCAGTGTATACTGCTCGGG	Chr18:61411891-61411913	CDH20	na	Intron
sgRNA_72_OT18	9	0.00	0.00	0.00	0.03	0.05	0.03	0.02		AAGGTCAAAGTATACGGCCCCGG	ChrX:154362732-154362754	FLNA	na	Exon

Table 3- Results from GUIDE-seq in T cells for off-targets using gRNA 85

Target Site	GUIDE-Seq # Reads	Mock-1	Mock-2	Mock-3	sgRNA_72-1	sgRNA_72-2	sgRNA_72-3	p-value		Sequence	hg38	Closest Gene	Distance	Feature
CD7_sgRNA_85	560	0.07	0.07	0.05	49.20	53.00	54.01	0.00		GGAGCAGGTGATGTTGACGGAGG	Chr17:82318917-82316939	CD7	n/a	Exon
sgRNA_85_OT1		0.01	0.00	0.01	0.01	0.01	0.01	0.09		GAAGGAGGTGGTGTTGACGGAGG	Chr2:241000074-241000096	SNED1	n/a	Intron
sgRNA_85_OT2		0.00	0.00	0.00	0.00	0.00	0.00			GGAGGTGGTGATGTTGACGGTGG	Chr5:173810749-173810771	LINC01485	19kb	Intergenic
sgRNA_85_OT3		0.00	0.00	0.01	0.02	0.01	0.01	0.12		TGAGCAGGAAATGCTGACGGAGG	Chr11:119344520-119344542	MFRP	n/a	Intron
sgRNA_85_OT4		0.14	0.13	0.12	0.19	0.11	0.14	0.30		TGATCAGGTGCTGGTGACGGGGG	Chr19_359681_359703	THEG	2.4kb	Intergenic
sgRNA_85_OT5		0.00	0.01	0.01	0.01	0.01	0.00	0.40		CGAGAAGGTGAAGCTGACGGAGG	Chr7:157410395-157410417	DNAJB6	n/a	Intron
sgRNA_85_OT6		0.02	0.03	0.01	0.01	0.02	0.01	0.11		TTTGCAGGTGATGTTCACGGAGG	Chr1:26553384-26553406	RPS6KA1	n/a	Exon
sgRNA_85_OT7		0.02	0.01	0.02	0.00	0.02	0.00	0.24		AGAGAAGGTGCTGTTGGCGCCGG	Chr11:68705473-68705495	TESMIN	2kb	Intergenic
sgRNA_85_OT8		0.05	0.07	0.06	0.06	0.06	0.09	0.18		GGAGCAGGTGTTGGGGACGGAGG	Chr22:49935662-49935584	CRELD2	8kb	Intergenic
sgRNA_85_OT9		0.01	0.02	0.02	0.04	0.01	0.01	0.40		TTAGCAGGAGGATGTTGAAGAGG	Chr22:37540545-37540567	CARD10	50kb	Intergenic
sgRNA_85_OT10		0.21	0.22	0.22	0.14	0.18	0.24	0.19		GGAGAAGGTGATGATGGCGGTGG	Chr1:208543307-208543329	PLXNA2	521kb	Intergenic
sgRNA_85_OT11		0.00	0.00	0.02	0.00	0.00	0.01	0.21		AGAGGAGGTGATGCTGGCGGAGG	Chr7:31027070-31027092	ADCYAP1R1	25kb	Intergenic
sgRNA_85_OT12		0.02	0.01	0.01	0.01	0.02	0.01	0.28		GGATCAGGCGATGTTGATGGTGG	Chr8:55076022-55076044	XKR4	26kb	Intergenic
sgRNA_85_OT13		0.00	0.00	0.00	0.01	0.00	0.00	0.21		GGAGCAGGTGATGACTTGCCGGTGG	Chr10:16520568-16520590	C1QL3	n/a	Exon
sgRNA_85_OT14		0.04	0.07	0.01	0.02	0.03	0.02	0.22		GGAGCCGGTGATGGGTTGAGGTGG	Chr5:35136695-35136717	PRLR	n/a	Intron
sgRNA_85_OT15		0.00	0.01	0.01	0.00	0.01	0.00	0.47		GGAGCAGGTGATAGCTTCGGAGG	Chr1:94908297-94908319	CNN3	n/a	Intron
sgRNA_85_OT16		0.05	0.10	0.03	0.02	0.00	0.05	0.22		AGAGGAGGTGATGGTTGATGGTGG	Chr5:82315738-82315760	ATP6AP1L	n/a	Intron
sgRNA_85_OT17		0.04	0.03	0.04	0.03	0.02	0.01	0.03		GGAGGAGGTGATGGTGATGGTGG	Chr8:138854953-138854975	COL22A1	n/a	Intron
sgRNA_85_OT18		0.04	0.04	0.04	0.02	0.05	0.04	0.38		GGAGCTGGTGATGGTGATGGTGG	Chr7:155598659-155598681	LOC100506302	43kb	Intergenic
sgRNA_85_OT19		0.00	0.01	0.01	0.02	0.01	0.01	0.08		AGAGCAGGAGCCTGTTGACTGCCGG	Chr11:45821426-45821448	SLC35C1	8.4kb	Intergenic
sgRNA_85_OT20		0.01	0.03	0.02	0.03	0.01	0.02	0.44		AGAGCAGGTGAAGTTGCGGAAGG	Chr3:969270 9-969731	MTMR14	n/a	Intron
sgRNA_85_OT21	1388	5.50	2.76	6.74	3.94	12.11	9.89	0.18		GGACAAGGAGAAGTGTTGACGGNGG	Chr8:80911125-80911147	ZNF704	36kb	Intergenic
sgRNA_85_OT22	844	0.12	0.12	0.14	0.19	0.13	0.12	0.25		GGACGGAGGAGATGTTGACGGNGG	Chr17:82248920-82248942	CSNK1D	n/a	Exon
sgRNA_85_OT23	337	0.00	0.01	0.00	0.01	0.01	0.03	0.15		TATAGAGGTGATGTTGACAGNGG	Chr22:21004106-21004128	THAP7-AS1	n/a	Intron
sgRNA_85_OT24	137	0.03	0.01	0.04	0.07	0.05	0.02	0.19		GCAGCAGGAGATGCTGACGGNGG	Chr15:100975310-100975332	LRRK1	n/a	Intron
sgRNA_85_OT25	22	0.01	0.00	0.02	0.01	0.01	0.03	0.12		GGTGCAGGAGATGTAGACGTNGG	Chr17:78420049-78420071	PGS1	n/a	Intron
sgRNA_85_OT26	18	0.03	0.03	0.03	0.04	0.02	0.03	0.37		GGGGAAGATGATGTTGACAANGG	Chr11:61327658-61327680	DDB1	n/a	Intron
sgRNA_85_OT27	10	0.01	0.00	0.00	0.01	0.00	0.00	0.21		AAGGCAGGAGAGAGTTGACGGNAG	Chr3:49165918-49165940	CCDC71	n/a	Intron
sgRNA_85_OT28	9	0.15	0.11	0.12	0.16	0.10	0.09	0.22		AGGGCAGGAGGGGTGTTGACGGNGG	Chr4:650786-650808	PDE6B	n/a	Intron
sgRNA_85_OT29	7	0.01	0.01	0.00	0.01	0.00	0.00	0.41		GGAACAAGTCATGTTGACAGNGG	Chr4:151952981-151953003	LINC02273	147kb	Intergenic
sgRNA_85_OT30	6	0.02	0.01	0.00	0.01	0.01	0.00	0.18		ACAGCAGGTGGTGTTGACGGNCG	Chr20:25196986-25197008	ENTPD6	n/a	Intron
sgRNA_85_OT31	3	0.00	0.00	0.01	0.02	0.01	0.01	0.14		AGAGCAGGTGATGCTGACGGNAG	Chr15:78324172-78324194	CRABP1	16kb	Intergenic

References

[1] B. Beutler, “Innate immunity: An overview,” *Molecular Immunology*, vol. 40, no. 12. pp. 845–859, 2004.

[2] A. M. Silverstein, “Cellular versus humoral immunity: Determinants and consequences of an epic 19th century battle,” *Cell. Immunol.*, vol. 48, no. 1, pp. 208–221, Nov. 1979.

[3] E. Metschnikoff and M. B. Freund, “Ueber eine Sprosspilzkrankheit der Daphnien. Beitrag zur Lehre über den Kampf der Phagocyten gegen Krankheitserreger,” *Dtsch. Medizinische Wochenschrift*, vol. 10, no. 43, pp. 699–700, 1884.

[4] C. A. Janeway, “How the immune system protects the host from infection,” *Microbes and Infection*, vol. 3, no. 13. pp. 1167–1171, 2001.

[5] B. Geering, C. Stoeckle, S. Conus, and H. U. Simon, “Living and dying for inflammation: Neutrophils, eosinophils, basophils,” *Trends in Immunology*, vol. 34, no. 8. pp. 398–409, 2013.

[6] P. J. Murray and T. A. Wynn, “Protective and pathogenic functions of macrophage subsets,” *Nature Reviews Immunology*, vol. 11, no. 11. pp. 723–737, 2011.

[7] C. Reis E Sousa, “Toll-like receptors and dendritic cells: For whom the bug tolls,” *Seminars in Immunology*, vol. 16, no. 1. pp. 27–34, 2004.

[8] J. Magarian Blander and R. Medzhitov, “Toll-dependent selection of microbial antigens for presentation by dendritic cells,” *Nature*, vol. 440, no. 7085, pp. 808–812, 2006.

[9] L. Engell-Noerregaard, T. H. Hansen, M. H. Andersen, P. Thor Straten, and I. M. Svane, “Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: Assessment of correlation between clinical response and vaccine parameters,” *Cancer Immunology, Immunotherapy*, vol. 58, no. 1. pp. 1–14, 2009.

[10] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka, “Immunobiology of dendritic cells.,” *Annu. Rev. Immunol.*, vol. 18, pp. 767–811, 2000.

[11] E. Vivier, D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini, “Innate or adaptive immunity? The example of natural killer cells.,” *Science*, vol. 331, no. 6013, pp. 44–9, 2011.

[12] M. A. Cooper, M. Colonna, and W. M. Yokoyama, “Hidden talents of natural killers: NK cells in innate and adaptive immunity,” *EMBO Reports*, vol. 10, no. 10. pp. 1103–1110, 2009.

[13] J. M. Milush, B. R. Long, J. E. Snyder-Cappione, A. J. Cappione, V. A. York, L. C. Ndhlovu, L. L. Lanier, J. Michaëlsson, and D. F. Nixon, “Functionally distinct subsets of human NK cells and monocyte/DC-like cells identified by coexpression of CD56, CD7, and CD4,” *Blood*, vol. 114, no. 23, pp. 4823–4831, 2009.

[14] L. Van Kaer, “NKT cells: T lymphocytes with innate effector functions,” *Current Opinion in Immunology*, vol. 19, no. 3. pp. 354–364, 2007.

[15] T. Kawano, J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi, “CD1d-restricted and TCR-mediated activation of V(α)14 NKT cells by glycosylceramides,” *Science (80-.).*, vol. 278, no. 5343, pp. 1626–1629, 1997.

[16] P. J. Brennan, M. Brigl, and M. B. Brenner, “Invariant natural killer T cells: An innate activation scheme linked to diverse effector functions,” *Nature Reviews Immunology*, vol. 13, no. 2. pp. 101–117, 2013.

[17] H.-J. Gober, M. Kistowska, L. Angman, P. Jenö, L. Mori, and G. De Libero, “Human T

cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells.," *J. Exp. Med.*, vol. 197, no. 2, pp. 163–8, 2003.

[18] Y. Gao and A. P. Williams, "Role of innate T cells in anti-bacterial immunity," *Frontiers in Immunology*, vol. 6, no. JUN. 2015.

[19] P. Vantourout and A. Hayday, "Six-of-the-best: Unique contributions of γδ T cells to immunology," *Nature Reviews Immunology*, vol. 13, no. 2. pp. 88–100, 2013.

[20] J. Gertner-Dardenne, C. Bonnafous, C. Bezombes, A. H. Capietto, V. Scaglione, S. Ingoure, D. Cendron, E. Gross, J. F. Lepage, A. Quillet-Mary, L. Ysebaert, G. Laurent, H. Sicard, and J. J. Fournié, "Bromohydrin pyrophosphate enhances antibody-dependent cell-mediated cytotoxicity induced by therapeutic antibodies," *Blood*, vol. 113, no. 20, pp. 4875–4884, 2009.

[21] M. Brandes, K. Willimann, and B. Moser, "Professional antigen-presentation function by human gammadelta T Cells.," *Science*, vol. 309, no. 5732, pp. 264–8, 2005.

[22] J. N. Blattman and P. D. Greenberg, "Cancer immunotherapy: a treatment for the masses.," *Science*, vol. 305, no. 5681, pp. 200–205, 2004.

[23] F. Sallusto, J. Geginat, and A. Lanzavecchia, "Central memory and effector memory T cell subsets: function, generation, and maintenance," *Annu Rev Immunol*, vol. 22, pp. 745–763, 2004.

[24] T. Yamashita, "Toward rational antibody design: recent advancements in molecular dynamics simulations," *Int. Immunol.*, vol. 30, no. 4, pp. 133–140, Apr. 2018.

[25] D. N. Forthal, "Functions of Antibodies," *Microbiol. Spectr.*, vol. 2, no. 4, 2014.

[26] K. E. Tiller and P. M. Tessier, "Advances in Antibody Design," *Annu. Rev. Biomed. Eng.*, vol. 17, no. 1, pp. 191–216, 2015.

[27] I. S. Afonina, S. P. Cullen, and S. J. Martin, "Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B," *Immunological Reviews*, vol. 235, no. 1. pp. 105–116, 2010.

[28] X. Wang, C. Berger, C. W. Wong, S. J. Forman, S. R. Riddell, and M. C. Jensen, "Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice," *Blood*, vol. 117, no. 6, pp. 1888–1898, 2011.

[29] L. Gattinoni, E. Lugli, Y. Ji, Z. Pos, C. M. Paulos, M. F. Quigley, J. R. Almeida, E. Gostick, Z. Yu, C. Carpenito, E. Wang, D. C. Douek, D. A. Price, C. H. June, F. M. Marincola, M. Roederer, and N. P. Restifo, "A human memory T-cell subset with stem cell-like properties," *Nat. Med.*, vol. 17, no. 10, pp. 1290–1297, 2011.

[30] C. Berger, M. C. Jensen, P. M. Lansdorp, M. Gough, C. Elliott, S. R. Riddell, and others, "Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates," *J. Clin. Invest.*, vol. 118, no. 1, pp. 294–305, 2008.

[31] E. J. Wherry, V. Teichgräber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed, "Lineage relationship and protective immunity of memory CD8 T cell subsets.," *Nat. Immunol.*, vol. 4, no. 3, pp. 225–34, 2003.

[32] M. Schmueck-Henneresse, R. Sharaf, K. Vogt, B. J. D. Weist, S. Landwehr-Kenzel, H. Fuehrer, A. Jurisch, N. Babel, C. M. Rooney, P. Reinke, and H.-D. Volk, "Peripheral Blood–Derived Virus-Specific Memory Stem T Cells Mature to Functional Effector Memory Subsets with Self-Renewal Potency," *J. Immunol.*, vol. 194, no. 11, pp. 5559–5567, 2015.

[33] M. Schmueck, A. M. Fischer, B. Hammoud, G. Brestrich, H. Fuehrer, S.-H. Luu, K. Mueller, N. Babel, H.-D. Volk, and P. Reinke, "Preferential expansion of human virus-specific multifunctional central memory T cells by partial targeting of the IL-2 receptor signaling pathway: the key role of CD4+ T cells.," *J. Immunol.*, vol. 188, no. 10, pp. 5189–98, 2012.

[34] E. S. Trombetta and I. Mellman, "Cell biology of antigen processing in vitro and in

vivo," *Annu Rev Immunol*, vol. 23, pp. 975–1028, 2005.

[35] T. Boehm and J. B. Swann, "Origin and Evolution of Adaptive Immunity," *Annu. Rev. Anim. Biosci.*, vol. 2, no. 1, pp. 259–283, 2014.

[36] M. S. Krangel, "Mechanics of T cell receptor gene rearrangement," *Current Opinion in Immunology*, vol. 21, no. 2. pp. 133–139, 2009.

[37] L. Klein, B. Kyewski, P. M. Allen, and K. Hogquist, "Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see).," *Nat. Rev. Immunol.*, vol. 14, no. 6, pp. 377–91, 2014.

[38] J. Sprent and S. R. Webb, "Intrathymic and extrathymic clonal deletion of T cells," *Curr. Opin. Immunol.*, vol. 7, no. 2, pp. 196–205, 1995.

[39] M. E. Call and K. W. Wucherpfennig, "Molecular mechanisms for the assembly of the T cell receptor-CD3 complex," *Molecular Immunology*, vol. 40, no. 18. pp. 1295–1305, 2004.

[40] J. M. Smith-Garvin JE, Koretzky GA, "T cell activation," *Annu Rev Immunol*, vol. 27, pp. 591–619, 2009.

[41] G. P. Morris and P. M. Allen, "How the TCR balances sensitivity and specificity for the recognition of self and pathogens," *Nature Immunology*, vol. 13, no. 2. pp. 121–128, 2012.

[42] E. M. Hiltbold and P. A. Roche, "Trafficking of MHC class II molecules in the late secretory pathway," *Current Opinion in Immunology*, vol. 14, no. 1. pp. 30–35, 2002.

[43] A. L. Goldberg, P. Cascio, T. Saric, and K. L. Rock, "The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides," *Molecular Immunology*, vol. 39, no. 3–4. pp. 147–164, 2002.

[44] K. L. Rock, I. A. York, and A. L. Goldberg, "Post-proteasomal antigen processing for major histocompatibility complex class I presentation," *Nature Immunology*, vol. 5, no. 7. pp. 670–677, 2004.

[45] A. Lanzavecchia and F. Sallusto, "Antigen decoding by T lymphocytes: From synapses to fate determination," *Nature Immunology*, vol. 2, no. 6. pp. 487–492, 2001.

[46] D. J. Lenschow, T. L. Walunas, and J. A. Bluestone, "CD28/B7 system of T cells costimulation," *Annu. Rev. Immunol.*, vol. 14, no. 1, pp. 233–258, 1996.

[47] C. Wang, G. H. Y. Lin, A. J. McPherson, and T. H. Watts, "Immune regulation by 4-1BB and 4-1BBL: Complexities and challenges," *Immunological Reviews*, vol. 229, no. 1. pp. 192–215, 2009.

[48] M. K. Jenkins and R. H. Schwartz, "Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo," *J. Exp. Med.*, vol. 165, no. 2, pp. 302–319, 1987.

[49] E. R. Westra, D. C. Swarts, R. H. J. Staals, M. M. Jore, S. J. J. Brouns, and J. van der Oost, "The CRISPRs, They Are A-Changin': How Prokaryotes Generate Adaptive Immunity," *Annu. Rev. Genet.*, vol. 46, no. 1, pp. 311–339, 2012.

[50] T. E. O'Sullivan, J. C. Sun, and L. L. Lanier, "Natural Killer Cell Memory," *Immunity*, vol. 43, no. 4. pp. 634–645, 2015.

[51] S. Meraviglia, S. El Daker, F. Dieli, F. Martini, and A. Martino, "γδ T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection.," *Clin. Dev. Immunol.*, vol. 2011, p. 587315, 2011.

[52] T. A. Wynn and K. M. Vannella, "Macrophages in Tissue Repair, Regeneration, and Fibrosis," *Immunity*, vol. 44, no. 3. pp. 450–462, 2016.

[53] P. K. Coyle, "Dissecting the immune component of neurologic disorders: A grand challenge for the 21st century," *Front. Neurol.*, vol. JUN, 2011.

[54] N. Zmora, S. Bashiardes, M. Levy, and E. Elinav, "The Role of the Immune System in

Metabolic Health and Disease," *Cell Metabolism*, vol. 25, no. 3. pp. 506–521, 2017.

[55] B. F. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, "GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012 v1.0," *IARC CancerBase No. 11*, vol. 978, no. 92, 2012.

[56] L. A. Liotta and E. C. Kohn, "The microenvironment of the tumour-host interface.," *Nature*, vol. 411, no. 6835, pp. 375–379, 2001.

[57] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1. pp. 27–30, 1995.

[58] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer.," *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[59] F. Wang, W. Meng, B. Wang, and L. Qiao, "Helicobacter pylori-induced gastric inflammation and gastric cancer," *Cancer Letters*, vol. 345, no. 2. pp. 196–202, 2014.

[60] F. Colotta, P. Allavena, A. Sica, C. Garlanda, and A. Mantovani, "Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability," *Carcinogenesis*, vol. 30, no. 7. pp. 1073–1081, 2009.

[61] M. Karin, "Nuclear factor-kappaB in cancer development and progression.," *Nature*, vol. 441, no. 7092, pp. 431–436, 2006.

[62] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The Three Es of Cancer Immunoediting," *Annu. Rev. Immunol.*, vol. 22, no. 1, pp. 329–360, 2004.

[63] D. Shou, L. Wen, Z. Song, J. Yin, Q. Sun, and W. Gong, "Suppressive role of myeloid-derived suppressor cells (MDSCs) in the microenvironment of breast cancer and targeted immunotherapies," *Oncotarget*, vol. 7, no. 39, 2016.

[64] K. A. Ward-Hartstonge and R. A. Kemp, "Regulatory T-cell heterogeneity and the cancer immune response," *Clin. Transl. Immunol.*, vol. 6, no. 9, p. e154, 2017.

[65] L. M. Coussens, C. L. Tinkle, D. Hanahan, and Z. Werb, "MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis.," *Cell*, vol. 103, no. 3, pp. 481–490, 2000.

[66] R. D. Schreiber, L. J. Old, and M. J. Smyth, "Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion," *Science*, vol. 331, no. 6024. pp. 1565–1570, 2011.

[67] F. M. Burnet, "The concept of immunological surveillance.," *Prog. Exp. Tumor Res.*, vol. 13, pp. 1–27, 1970.

[68] R. A. Gatti and R. A. Good, "Occurrence of malignancy in immunodeficiency diseases: A literature review," *Cancer*, vol. 28, no. 1, pp. 89–98, 1971.

[69] B. Mueller and P. Pizzo, "Cancer in children with primary or secondary immunodeficiencies," *J. Pediatr.*, vol. 126, no. January, pp. 1–10, 1995.

[70] K. Salavoura, A. Kolialexi, G. Tsangaris, and A. Mavrou, "Development of cancer in patients with primary immunodeficiencies," *Anticancer Research*, vol. 28, no. 2 B. pp. 1263–1269, 2008.

[71] L. J. Kinlen, A. D. Webster, A. G. Bird, R. Haile, J. Peto, J. F. Soothill, and R. A. Thompson, "Prospective study of cancer in patients with hypogammaglobulinaemia.," *Lancet*, vol. 1, no. 8423, pp. 263–6, 1985.

[72] W. F. Davidson, T. Giese, and T. N. Fredrickson, "Spontaneous development of plasmacytoid tumors in mice with defective Fas-Fas ligand interactions.," *J. Exp. Med.*, vol. 187, no. 11, pp. 1825–38, 1998.

[73] R. M. Siegel, F. K. Chan, H. J. Chun, and M. J. Lenardo, "The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity.," *Nat. Immunol.*, vol. 1, no. 6, pp. 469–474, 2000.

[74] E. A. Engels, R. M. Pfeiffer, J. F. Fraumeni, B. L. Kasiske, A. K. Israni, J. J. Snyder,

R. A. Wolfe, N. P. Goodrich, A. R. Bayakly, C. A. Clarke, G. Copeland, J. L. Finch, M. Lou Fleissner, M. T. Goodman, A. Kahn, L. Koch, C. F. Lynch, M. M. Madeleine, K. Pawlish, C. Rao, M. A. Williams, D. Castenson, M. Curry, R. Parsons, G. Fant, and M. Lin, “Spectrum of Cancer Risk Among US Solid Organ Transplant Recipients,” *JAMA*, vol. 306, no. 17, p. 1891, 2011.

[75] S. a Birkeland, H. H. Storm, L. U. Lamm, L. Barlow, I. Blohmé, B. Forsberg, B. Eklund, O. Fjeldborg, M. Friedberg, and L. Frödin, “Cancer risk after renal transplantation in the Nordic countries, 1964-1986.,” *Int. J. Cancer*, vol. 60, no. 2, pp. 183–9, 1995.

[76] R. Hoover and J. F. Fraumeni, “Risk of cancer in renal-transplant recipients,” *Lancet*, vol. 302, no. 7820, pp. 55–57, 1973.

[77] P. L. Ward, H. K. Koeppen, T. Hurteau, D. A. Rowley, and H. Schreiber, “Major Histocompatibility Complex Class I and Unique Antigen Expression by Murine Tumors That Escaped from CD8+ T-Cell-dependent Surveillance,” *Cancer Res.*, vol. 50, no. 13, pp. 3851–3858, 1990.

[78] N. Yusuf, T. H. Nasti, S. K. Katiyar, M. K. Jacobs, M. D. Seibert, A. C. Ginsburg, L. Timares, H. Xu, and C. A. Elmets, “Antagonistic roles of CD4+and CD8+T-cells in 7,12-dimethylbenz(a)anthracene cutaneous carcinogenesis,” *Cancer Res.*, vol. 68, no. 10, pp. 3924–3930, 2008.

[79] L. Zhang, J. R. Conejo-Garcia, D. Katsaros, P. A. Gimotty, M. Massobrio, G. Regnani, A. Makrigiannakis, H. Gray, K. Schlienger, M. N. Liebman, S. C. Rubin, and G. Coukos, “Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.,” *N. Engl. J. Med.*, vol. 348, no. 3, pp. 203–13, 2003.

[80] Y. Naito, K. Saito, K. Shiiba, A. Ohuchi, K. Saigenji, H. Nagura, and H. Ohtani, “CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer,” *Cancer Res.*, vol. 58, no. 16, pp. 3491–3494, 1998.

[81] K. I. Al-Shibli, T. Donnem, S. Al-Saad, M. Persson, R. M. Bremnes, and L. T. Busund, “Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer,” *Clin. Cancer Res.*, vol. 14, no. 16, pp. 5220–5227, 2008.

[82] G. Alexe, G. S. Dalgin, D. Scanfeld, P. Tamayo, J. P. Mesirov, C. DeLisi, L. Harris, N. Barnard, M. Martel, A. J. Levine, S. Ganesan, and G. Bhanote, “High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates,” *Cancer Res.*, vol. 67, no. 22, pp. 10669–10676, 2007.

[83] Y. Cho, M. Miyamoto, K. Kato, A. Fukunaga, T. Shichinohe, Y. Kawarada, Y. Hida, T. Oshikiri, T. Kurokawa, M. Suzuoki, Y. Nakakubo, K. Hiraoka, S. Murakami, T. Shinohara, T. Itoh, S. Okushiba, S. Kondo, and H. Katoh, “CD4+ and CD8+ T cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma.,” *Cancer Res.*, vol. 63, no. 7, pp. 1555–9, 2003.

[84] S. V Sharma and J. Settleman, “Oncogene addiction: setting the stage for molecularly targeted cancer therapy.,” *Genes Dev.*, vol. 21, no. 24, pp. 3214–3231, 2007.

[85] D. Pardoll, “Does the immune system see tumors as foreign or self?,” *Annu Rev Immunol*, vol. 21, no. Table 1, p. 807–39., 2003.

[86] C. J. M. Melief, T. Van Hall, R. Arens, F. Ossendorp, and S. H. Van Der Burg, “Therapeutic cancer vaccines,” *J. Clin. Invest.*, vol. 125, no. 9, pp. 3401–3412, 2015.

[87] E. Jäger, D. Jäger, J. Karbach, Y. T. Chen, G. Ritter, Y. Nagata, S. Gnjatic, E. Stockert, M. Arand, L. J. Old, and a Knuth, “Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma.,” *J. Exp. Med.*, vol. 191, no. 4, pp. 625–630, 2000.

[88] Y. Kawakami, S. Eliyahu, K. Sakaguchi, P. F. Robbins, L. Rivoltini, J. R. Yannelli, E. Appella, and S. A. Rosenberg, “Identification of the immunodominant peptides of the

MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes.," *J. Exp. Med.*, vol. 180, no. 1, pp. 347–52, 1994.

[89] Y. Kawakami, S. Eliyahu, C. Jennings, K. Sakaguchi, X. Kang, S. Southwood, P. F. Robbins, A. Sette, E. Appella, and S. A. Rosenberg, "Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression.," *J. Immunol.*, vol. 154, no. 8, pp. 3961–8, 1995.

[90] L. A. Johnson, R. A. Morgan, M. E. Dudley, L. Cassard, J. C. Yang, M. S. Hughes, U. S. Kammula, R. E. Royal, R. M. Sherry, J. R. Wunderlich, C. C. R. Lee, N. P. Restifo, S. L. Schwarz, A. P. Cogdill, R. J. Bishop, H. Kim, C. C. Brewer, S. F. Rudy, C. VanWaes, J. L. Davis, A. Mathur, R. T. Ripley, D. A. Nathan, C. M. Laurencot, and S. A. Rosenberg, "Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen," *Blood*, vol. 114, no. 3, pp. 535–546, 2009.

[91] T. Wölfel, M. Hauer, J. Schneider, M. Serrano, C. Wölfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K. H. Meyer Zum Büschenfelde, and D. Beach, "A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma," *Science (80-.).*, vol. 269, no. 5228, pp. 1281–1284, 1995.

[92] I. Saeterdal, J. Bjorheim, K. Lislerud, M. K. Gjertsen, I. K. Bukholm, O. C. Olsen, J. M. Nesland, J. A. Eriksen, M. Moller, A. Lindblom, and G. Gaudernack, "Frameshift-mutation-derived peptides as tumor-specific antigens in inherited and spontaneous colorectal cancer," *Proc. Natl. Acad. Sci.*, vol. 98, no. 23, pp. 13255–13260, 2001.

[93] R. E. Clark, I. Anthony Dodi, S. C. Hill, J. R. Lill, G. Aubert, A. R. Macintyre, J. Rojas, A. Bourdon, P. L. R. Bonner, L. Wang, S. E. Christmas, P. J. Travers, C. S. Greaser, R. C. Rees, and J. Alejandro Madrigal, "Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein," *Blood*, vol. 98, no. 10, pp. 2887–2893, 2001.

[94] O. Preynat-Seauve, P. Schuler, E. Contassot, F. Beermann, B. Huard, and L. E. French, "Tumor-Infiltrating Dendritic Cells Are Potent Antigen-Presenting Cells Able to Activate T Cells and Mediate Tumor Rejection," *J. Immunol.*, vol. 176, no. 1, pp. 61–67, 2006.

[95] F. Garrido, F. Ruiz-Cabello, T. Cabrera, J. J. Pérez-Villar, M. López-Botet, M. Duggan-Keen, and P. L. Stern, "Implications for immunosurveillance of altered HLA class I phenotypes in human tumours," *Immunology Today*, vol. 18, no. 2. pp. 89–95, 1997.

[96] B. Pérez, R. Benitez, M. a Fernández, M. R. Oliva, J. L. Soto, S. Serrano, M. a López Nevot, and F. Garrido, "A new β2 microglobulin mutation found in a melanoma tumor cell line.," *Tissue Antigens*, vol. 53, no. 6, pp. 569–572, 1999.

[97] C. M. D'Urso, Z. Wang, Y. Cao, R. Tatake, R. A. Zeff, and S. Ferrone, "Lack of HLA class I antigen expression by cultured melanoma cells FO-1 due to a defect in B2m gene expression," *J. Clin. Invest.*, vol. 87, no. 1, pp. 284–292, 1991.

[98] M. S. Lawrence, P. Stojanov, C. H. Mermel, J. T. Robinson, L. A. Garraway, T. R. Golub, M. Meyerson, S. B. Gabriel, E. S. Lander, and G. Getz, "Discovery and saturation analysis of cancer genes across 21 tumour types," *Nature*, vol. 505, no. 7484, pp. 495–501, 2014.

[99] P. Jiménez, J. Cantón, A. Collado, T. Cabrera, A. Serrano, L. M. Real, A. García, F. Ruiz-Cabello, and F. Garrido, "Chromosome loss is the most frequent mechanism contributing to HLA haplotype loss in human tumors," *Int. J. Cancer*, vol. 83, no. 1, pp. 91–97, 1999.

[100] S. Charni, G. de Bettignies, M. G. Rathore, J. I. Aguilo, P. J. van den Elsen, D. Haouzi, R. A. Hipskind, J. A. Enriquez, M. Sanchez-Beato, J. Pardo, A. Anel, and M. Villalba, "Oxidative Phosphorylation Induces De Novo Expression of the MHC Class I in Tumor Cells through the ERK5 Pathway," *J. Immunol.*, vol. 185, no. 6, pp. 3498–

3503, 2010.

[101] F. Concha-Benavente, R. M. Srivastava, S. Ferrone, and R. L. Ferris, “EGFR-mediated tumor immunoescape The imbalance between phosphorylated STAT1 and phosphorylated STAT3,” *Oncoimmunology*, vol. 2, no. 12, pp. 1–3, 2013.

[102] S. Vertuani, C. Triulzi, A. K. Roos, J. Charo, H. Norell, F. Lemonnier, P. Pisa, B. Seliger, and R. Kiessling, “HER-2/neu mediated down-regulation of MHC class i antigen processing prevents CTL-mediated tumor recognition upon DNA vaccination in HLA-A2 transgenic mice,” *Cancer Immunol. Immunother.*, vol. 58, no. 5, pp. 653–664, 2009.

[103] D. Hanahan and R. A. Weinberg, “Hallmarks of cancer: The next generation,” *Cell*, vol. 144, no. 5. pp. 646–674, 2011.

[104] P. Dalerba, R. W. Cho, and M. F. Clarke, “Cancer stem cells: models and concepts,” *Annu Rev Med*, vol. 58, pp. 267–284, 2007.

[105] D. Bonnet and J. E. Dick, “Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell,” *Nat. Med.*, vol. 3, no. 7, pp. 730–737, 1997.

[106] S. K. Singh, C. Hawkins, I. D. Clarke, J. A. Squire, J. Bayani, T. Hide, R. M. Henkelman, M. D. Cusimano, and P. B. Dirks, “Identification of human brain tumour initiating cells,” *Nature*, vol. 432, no. 7015, pp. 396–401, 2004.

[107] M. Al-Hajj, M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, and M. F. Clarke, “Prospective identification of tumorigenic breast cancer cells,” *Proc. Natl. Acad. Sci.*, vol. 100, no. 7, pp. 3983–3988, 2003.

[108] M. V. Blagosklonny, “Why therapeutic response may not prolong the life of a cancer patient: Selection for oncogenic resistance,” *Cell Cycle*, vol. 4, no. 12. pp. 1693–1698, 2005.

[109] B. Cooper, “The origins of bone marrow as the seedbed of our blood: from antiquity to the time of Osler.,” *Proc. (Bayl. Univ. Med. Cent).*, vol. 24, no. 2, pp. 115–8, Apr. 2011.

[110] I. L. Seita, Jun; Weissman, “Hematopoietic Stem Cell: Self-renewal versus Differentiation,” *Wiley Interdiscip Rev Syst Biol Med*, vol. 2, no. 6, pp. 640–653, 2010.

[111] S. P. Hunger, X. Lu, M. Devidas, B. M. Camitta, P. S. Gaynon, N. J. Winick, G. H. Reaman, and W. L. Carroll, “Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: A report from the children’s oncology group,” *J. Clin. Oncol.*, vol. 30, no. 14, pp. 1663–1669, 2012.

[112] R. Bassan and D. Hoelzer, “Modern therapy of acute lymphoblastic leukemia,” *J Clin Oncol*, vol. 29, no. 5, pp. 532–543, 2011.

[113] C. Cobaleda and I. Sánchez-García, “B-cell acute lymphoblastic leukaemia: Towards understanding its cellular origin,” *BioEssays*, vol. 31, no. 6. pp. 600–609, 2009.

[114] D. M. Oliansky, B. Camitta, P. Gaynon, M. L. Nieder, S. K. Parsons, M. A. Pulsipher, H. Dillon, T. A. Ratko, D. Wall, P. L. McCarthy Jr., and T. Hahn, “Role of cytotoxic therapy with hematopoietic stem cell transplantation in the treatment of pediatric acute lymphoblastic leukemia: Update of the 2005 evidence-based review,” *Biol. Blood Marrow Transplant.*, vol. 18, no. 4, 2012.

[115] M. A. Pulsipher, C. Peters, and C. H. Pui, “High-Risk Pediatric Acute Lymphoblastic Leukemia: To Transplant or Not to Transplant?,” *Biol. Blood Marrow Transplant.*, vol. 17, no. 1 SUPPL, 2011.

[116] S. J. Forman and J. M. Rowe, “The myth of the second remission of acute leukemia in the adult,” *Blood*, vol. 121, no. 7. pp. 1077–1082, 2013.

[117] M. J. Borowitz, M. Devidas, S. P. Hunger, W. P. Bowman, A. J. Carroll, W. L. Carroll, S. Linda, P. L. Martin, D. J. Pullen, D. Viswanatha, C. L. Willman, N. Winick, and B. M. Camitta, “Clinical significance of minimal residual disease in childhood acute

lymphoblastic leukemia and its relationship to other prognostic factors: A Children's Oncology Group study," *Blood*, vol. 111, no. 12, pp. 5477–5485, 2008.

[118] A. Verma and W. Stock, "Management of adult acute lymphoblastic leukemia: moving toward a risk-adapted approach.," *Curr. Opin. Oncol.*, vol. 13, no. 1, pp. 14–20, 2001.

[119] C. R. Bartram, A. Schrauder, R. Köhler, and M. Schrappe, "Acute lymphoblastic leukemia in children: treatment planning via minimal residual disease assessment.," *Dtsch. Ärzteblatt Int.*, vol. 109, no. 40, pp. 652–8, 2012.

[120] J. Pullen, J. J. Shuster, M. Link, M. Borowitz, M. Amylon, A. J. Carroll, V. Land, A. T. Look, B. McIntyre, and B. Camitta, "Significance of commonly used prognostic factors differs for children with T cell acute lymphocytic leukemia (ALL), as compared to those with B-precursor ALL, a Pediatric Oncology Group (POG) study," *Leukemia*, vol. 13, no. 11, pp. 1696–1707, 1999.

[121] N. Chiorazzi, K. R. Rai, and M. Ferrarini, "Chronic lymphocytic leukemia.," *N. Engl. J. Med.*, vol. 352, no. 8, pp. 804–815, 2005.

[122] M. Hallek, B. D. Cheson, D. Catovsky, F. Caligaris-Cappio, G. Dighiero, H. Döhner, P. Hillmen, M. J. Keating, E. Montserrat, K. R. Rai, and T. J. Kipps, "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. pp. 5446–5456, 2008.

[123] E. A. McCulloch, "Stem cells in normal and leukemic hemopoiesis (Henry Stratton Lecture, 1982).," *Blood*, vol. 62, no. 1, pp. 1–13, 1983.

[124] I. De Kouchkovsky and M. Abdul-Hay, "'Acute myeloid leukemia: A comprehensive review and 2016 update,'" *Blood Cancer Journal*, vol. 6, no. 7. 2016.

[125] F. Buccisano, L. Maurillo, V. Gattei, G. Del Poeta, M. I. Del Principe, M. C. Cox, P. Panetta, M. I. Consalvo, C. Mazzone, B. Neri, L. Ottaviani, D. Fraboni, a Tamburini, F. Lo-Coco, S. Amadori, and a Venditti, "The kinetics of reduction of minimal residual disease impacts on duration of response and survival of patients with acute myeloid leukemia.," *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K*, vol. 20, no. 10, pp. 1783–1789, 2006.

[126] A. Shah, T. M. L. Andersson, B. Rachet, M. Björkholm, and P. C. Lambert, "Survival and cure of acute myeloid leukaemia in England, 1971-2006: a population-based study," *Br. J. Haematol.*, vol. 162, no. 4, pp. 509–516, 2013.

[127] J. Meyers, Y. Yu, J. A. Kaye, and K. L. Davis, "Medicare fee-for-service enrollees with primary acute myeloid leukemia: An analysis of treatment patterns, survival, and healthcare resource utilization and costs," *Appl. Health Econ. Health Policy*, vol. 11, no. 3, pp. 275–286, 2013.

[128] P. C. Nowell, "A minute chromosome in human chronic granulocytic leukemia," *Science (80-.).*, vol. 8, p. 19, 1985.

[129] I. Sloma, X. Jiang, a C. Eaves, and C. J. Eaves, "Insights into the stem cells of chronic myeloid leukemia.," *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K*, vol. 24, no. 11, pp. 1823–1833, 2010.

[130] S. H. Swerdlow, E. Campo, N. L. Harris, E. S. Jaffe, S. A. Pileri, H. Stein, J. Thiele, and J. . Vardiman, *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, vol. 4th. 2008.

[131] D. W. Scott and R. D. Gascoyne, "The tumour microenvironment in B cell lymphomas," *Nature Reviews Cancer*, vol. 14, no. 8. pp. 517–534, 2014.

[132] S. V. Rajkumar, "Evolving diagnostic criteria for multiple myeloma," *Hematology*, vol. 2015, no. 1. pp. 272–278, 2015.

[133] P. Moreau, M. Attal, and T. Facon, "Frontline therapy of multiple myeloma," *Blood*, vol. 125, no. 20. pp. 3076–3084, 2015.

[134] G. Bianchi, P. G. Richardson, and K. C. Anderson, “Promising therapies in multiple myeloma,” *Blood*, vol. 126, no. 3. pp. 300–310, 2015.

[135] W. I. Bensinger, C. H. Weaver, F. R. Appelbaum, S. Rowley, T. Demirer, J. Sanders, R. Storb, and C. D. Buckner, “Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor.,” *Blood*, vol. 85, no. 6, pp. 1655–1658, 1995.

[136] P. Ljungman, M. Bregni, M. Brune, J. Cornelissen, T. De Witte, G. Dini, H. Einsele, H. B. Gaspar, A. Gratwohl, J. Passweg, C. Peters, V. Rocha, R. Saccardi, H. Schouten, A. Sureda, A. Tichelli, A. Velardi, and D. Niederwieser, “Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: Current practice in Europe 2009,” *Bone Marrow Transplantation*, vol. 45, no. 2. pp. 219–234, 2010.

[137] L. M. Poon, A. Hamdi, R. Saliba, G. Rondon, C. Ledesma, M. Kendrick, M. Qazilbash, C. Hosing, R. B. Jones, U. R. Popat, Y. Nieto, A. Alousi, S. Ciurea, E. J. Shpall, R. E. Champlin, and P. Kebriaei, “Outcomes of Adults with Acute Lymphoblastic Leukemia Relapsing after Allogeneic Hematopoietic Stem Cell Transplantation,” *Biol. Blood Marrow Transplant.*, vol. 19, no. 7, pp. 1059–1064, 2013.

[138] E. F. McCarthy, “The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas.,” *Iowa Orthop. J.*, vol. 26, pp. 154–8, 2006.

[139] K. R. Loughlin, “Understanding intravesical BCG: A window to the future treatment of noninvasive bladder cancer,” *Urologic Oncology: Seminars and Original Investigations*, vol. 26, no. 4, pp. 339–340, 2008.

[140] Leibowitz, “Head and Neck Cancer Immunotherapy: Clinical Evaluation,” *Curr Oncol Rep*, 2008.

[141] J. Christiansen and A. K. Rajasekaran, “Biological impediments to monoclonal antibody-based cancer immunotherapy.,” *Mol. Cancer Ther.*, vol. 3, no. 11, pp. 1493–501, 2004.

[142] D. M. Goldenberg, “Targeting of cancer with radiolabeled antibodies. Prospects for imaging and therapy,” *Archives of Pathology and Laboratory Medicine*, vol. 112, no. 6. pp. 580–587, 1988.

[143] Z. Shulman, S. J. Cohen, B. Roediger, V. Kalchenko, R. Jain, V. Grabovsky, E. Klein, V. Shinder, L. Stoler-Barak, S. W. Feigelson, T. Meshel, S. M. Nurmi, I. Goldstein, O. Hartley, C. G. Gahmberg, A. Etzioni, W. Weninger, A. Ben-Baruch, and R. Alon, “Transendothelial migration of lymphocytes mediated by intraendothelial vesicle stores rather than by extracellular chemokine depots,” *Nat. Immunol.*, vol. 13, no. 1, pp. 67–76, 2012.

[144] D. Masopust and J. M. Schenkel, “The integration of T cell migration, differentiation and function,” *Nat Rev Immunol*, vol. 13, no. 5, pp. 309–320, 2013.

[145] H. E. Heslop, K. S. Slobod, M. A. Pule, G. A. Hale, A. Rousseau, C. A. Smith, C. M. Bollard, H. Liu, M. F. Wu, R. J. Rochester, P. J. Amrolia, J. L. Hurwitz, M. K. Brenner, and C. M. Rooney, “Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients,” *Blood*, vol. 115, no. 5, pp. 925–935, 2010.

[146] G. Mathe, J. L. Amiel, L. Schwarzenberg, A. Cattan, M. Schneider, M. J. Devrires, M. Tubiana, C. Lalanne, J. L. Binet, M. Papiernik, G. Seman, M. Matsukura, A. M. Mery, V. Schwazmann, and A. Flaiser, “Succesful allogenic bone marrow transplantation in man: chimerism, induced specific tolerance and possible anti-leukemic effects,” *Blood*, vol. 25, no. 2, pp. 179–96, 1965.

[147] A. Saad and L. S. Lamb, “Ex vivo T-cell depletion in allogeneic hematopoietic stem cell transplant: Past, present and future,” *Bone Marrow Transplantation*, vol. 52, no. 9. pp. 1241–1248, 2017.

[148] D. L. Porter, W.-T. Hwang, N. V. Frey, S. F. Lacey, P. A. Shaw, A. W. Loren, A. Bagg, K. T. Marcucci, A. Shen, V. Gonzalez, D. Ambrose, S. A. Grupp, A. Chew, Z. Zheng, M. C. Milone, B. L. Levine, J. J. Melenhorst, and C. H. June, "Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia," *Sci. Transl. Med.*, vol. 7, no. 303, p. 303, 2015.

[149] E. Sato, S. H. Olson, J. Ahn, B. Bundy, H. Nishikawa, F. Qian, A. A. Jungbluth, D. Frosina, S. Gnjatic, C. Ambrosone, J. Kepner, T. Odunsi, G. Ritter, S. Lele, Y.-T. Chen, H. Ohtani, L. J. Old, and K. Odunsi, "Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer," *Proc. Natl. Acad. Sci.*, vol. 102, no. 51, pp. 18538–18543, 2005.

[150] G. Parmiani and A. Anichini, "T cell infiltration and prognosis in HCC patients," *Journal of Hepatology*, vol. 45, no. 2, pp. 178–181, 2006.

[151] 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. U. S. A.*, vol. 86, no. 24, pp. 10024–8, Dec. 1989.

[152] S. A. Rosenberg, P. Spiess, and R. Lafreniere, "A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes.," *Science*, vol. 233, no. 4770, pp. 1318–21, 1986.

[153] S. A. Rosenberg, B. S. Packard, P. M. Aebersold, D. Solomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, C. A. Seipp, C. Simpson, C. Carter, S. Bock, D. Schwartzentruber, J. P. Wei, and D. E. White, "Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma," *N. Engl. J. Med.*, vol. 319, no. 25, pp. 1676–1680, 1988.

[154] R. L. Kradin, D. S. Lazarus, S. M. Dubinett, J. Gifford, B. Grove, J. T. Kurnick, F. I. Preffer, C. E. Pinto, E. Davidson, R. J. Callahan, and H. W. Strauss, "Tumour-inflitrating lymphocytes and interleukin-2 in treatment of advanced cancer," *Lancet*, vol. 333, no. 8638, pp. 577–580, 1989.

[155] S. L. Topalian, D. Solomon, F. P. Avis, A. E. Chang, D. L. Freerksen, W. M. Linehan, M. T. Lotze, C. N. Robertson, C. A. Seipp, and P. Simon, "Immunotherapy of patients with advanced cancer using tumor-infiltrating lymphocytes and recombinant interleukin-2: a pilot study.," *J. Clin. Oncol.*, vol. 6, no. 5, pp. 839–53, 1988.

[156] R. A. Figlin, W. C. Pierce, R. Kaboo, C. L. Tso, N. Moldawer, B. Gitlitz, J. Dekernion, and A. Belldegrun, "Treatment of metastatic renal cell carcinoma with nephrectomy, interleukin-2 and cytokine-primed or CD8(+) selected tumor infiltrating lymphocytes from primary tumor," *J. Urol.*, vol. 158, no. 3, pp. 740–745, 1997.

[157] K. B. Quattrocchi, C. H. Miller, S. Cush, S. a Bernard, S. T. Dull, M. Smith, S. Gudeman, and M. a Varia, "Pilot study of local autologous tumor infiltrating lymphocytes for the treatment of recurrent malignant gliomas.," *J. Neurooncol.*, vol. 45, no. 2, pp. 141–57, 1999.

[158] M. E. Dudley, J. R. Wunderlich, T. E. Shelton, J. Even, and S. A. Rosenberg, "Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients.," *J. Immunother.*, vol. 26, no. 4, pp. 332–42, 2008.

[159] S. A. Rosenberg, J. R. Yannelli, J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White, "Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2.," *J. Natl. Cancer Inst.*, vol. 86, no. 15, pp. 1159–66, 1994.

[160] M. E. Dudley, C. A. Gross, R. P. T. Somerville, Y. Hong, N. P. Schaub, S. F. Rosati, D. E. White, D. Nathan, N. P. Restifo, S. M. Steinberg, J. R. Wunderlich, U. S. Kammula, R. M. Sherry, J. C. Yang, G. Q. Phan, M. S. Hughes, C. M. Laurencot, and S. A. Rosenberg, "Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients

with melanoma," *J. Clin. Oncol.*, vol. 31, no. 17, pp. 2152–2159, 2013.

[161] S. a Rosenberg, J. C. Yang, R. M. Sherry, U. S. Kammula, S. Marybeth, G. Q. Phan, D. E. Citrin, N. P. Restifo, P. F. Robbins, R. John, K. E. Morton, C. M. Laurencot, S. M. Steinberg, E. Donald, and M. E. Dudley, "Durable Complete Responses in Heavily Pretreated Patients with Metastatic Melanoma Using T Cell Transfer Immunotherapy," *Clin. Cancer Res.*, vol. 17, no. 13, pp. 4550–4557, 2011.

[162] M. E. Dudley, J. R. Wunderlich, J. C. Yang, R. M. Sherry, S. L. Topalian, N. P. Restifo, R. E. Royal, U. Kammula, D. E. White, S. A. Mavroukakis, L. J. Rogers, G. J. Gracia, S. A. Jones, D. P. Mangiameli, M. M. Pelletier, J. Gea-Banacloche, M. R. Robinson, D. M. Berman, A. C. Filie, A. Abati, and S. A. Rosenberg, "Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma.," *J. Clin. Oncol.*, vol. 23, no. 10, pp. 2346–57, 2005.

[163] G. Q. Phan and S. A. Rosenberg, "Adoptive cell transfer for patients with metastatic melanoma: the potential and promise of cancer immunotherapy.," *Cancer Control*, vol. 20, no. 4, pp. 289–97, 2013.

[164] N. Zacharakis, H. Chinnasamy, M. Black, H. Xu, Y.-C. Lu, Z. Zheng, A. Pasetto, M. Langhan, T. Shelton, T. Prickett, J. Gartner, L. Jia, K. Trebska-McGowan, R. P. Somerville, P. F. Robbins, S. A. Rosenberg, S. L. Goff, and S. A. Feldman, "Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer," *Nat. Med.*, vol. 24, no. 6, pp. 724–730, 2018.

[165] T. Connerotte, A. Van Pel, D. Godelaine, E. Tartour, B. Schuler-Thurner, S. Lucas, K. Thielemans, G. Schuler, and P. G. Coulie, "Functions of anti-MAGE T-cells induced in melanoma patients under different vaccination modalities," *Cancer Res.*, vol. 68, no. 10, pp. 3931–3940, 2008.

[166] V. Karanikas, C. Lurquin, D. Colau, N. van Baren, C. De Smet, B. Lethe, T. Connerotte, V. Corbiere, M.-A. Demoitie, D. Lienard, B. Dreno, T. Velu, T. Boon, and P. G. Coulie, "Monoclonal Anti-MAGE-3 CTL Responses in Melanoma Patients Displaying Tumor Regression after Vaccination with a Recombinant Canarypox Virus," *J. Immunol.*, vol. 171, no. 9, pp. 4898–4904, 2003.

[167] E. Lanitis, J. B. Smith, D. Dangaj, S. Flingai, M. Poussin, S. Xu, B. J. Czerniecki, Y. F. Li, P. F. Robbins, and D. J. Powell, "A Human ErbB2-Specific T-Cell Receptor Confers Potent Antitumor Effector Functions in Genetically Engineered Primary Cytotoxic Lymphocytes," *Hum. Gene Ther.*, vol. 25, no. 8, pp. 730–739, 2014.

[168] R. A. Morgan, N. Chinnasamy, D. Abate-Daga, A. Gros, P. F. Robbins, Z. Zheng, M. E. Dudley, S. A. Feldman, J. C. Yang, R. M. Sherry, G. Q. Phan, M. S. Hughes, U. S. Kammula, A. D. Miller, C. J. Hessman, A. A. Stewart, N. P. Restifo, M. M. Quezado, M. Alimchandani, A. Z. Rosenberg, A. Nath, T. Wang, B. Bielekova, S. C. Wuest, N. Akula, F. J. McMahon, S. Wilde, B. Mosetter, D. J. Schendel, C. M. Laurencot, and S. A. Rosenberg, "Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy," *J. Immunother.*, vol. 36, no. 2, pp. 133–151, 2013.

[169] P. F. Robbins, R. A. Morgan, S. A. Feldman, J. C. Yang, R. M. Sherry, M. E. Dudley, J. R. Wunderlich, A. V. Nahvi, L. J. Helman, C. L. Mackall, U. S. Kammula, M. S. Hughes, N. P. Restifo, M. Raffeld, C. C. R. Lee, C. L. Levy, Y. F. Li, M. El-Gamil, S. L. Schwarz, C. Laurencot, and S. A. Rosenberg, "Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1," *J. Clin. Oncol.*, vol. 29, no. 7, pp. 917–924, 2011.

[170] P. F. Robbins, S. H. Kassim, T. L. N. Tran, J. S. Crystal, R. A. Morgan, S. A. Feldman, J. C. Yang, M. E. Dudley, J. R. Wunderlich, R. M. Sherry, U. S. Kammula, M. S. Hughes, N. P. Restifo, M. Raffeld, C. C. R. Lee, Y. F. Li, M. El-Gamil, and S. A. Rosenberg, "A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-

reactive T-cell receptor: Long-term follow-up and correlates with response," *Clin. Cancer Res.*, vol. 21, no. 5, pp. 1019–1027, 2015.

[171] G. M. Bendle, C. Linnemann, A. I. Hooijkaas, L. Bies, M. A. De Witte, A. Jorritsma, A. D. M. Kaiser, N. Pouw, R. Debets, E. Kieback, W. Uckert, J. Y. Song, J. B. A. G. Haanen, and T. N. M. Schumacher, "Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy," *Nat. Med.*, vol. 16, no. 5, pp. 565–570, 2010.

[172] S. L. Goff, L. A. Johnson, M. A. Black, H. Xu, Z. Zheng, C. J. Cohen, R. A. Morgan, S. A. Rosenberg, and S. A. Feldman, "Enhanced receptor expression and in vitro effector function of a murine-human hybrid MART-1-reactive T cell receptor following a rapid expansion," *Cancer Immunol. Immunother.*, vol. 59, no. 10, pp. 1551–1560, 2010.

[173] K. N. Pollizzi and J. D. Powell, "Integrating canonical and metabolic signalling programmes in the regulation of T cell responses," *Nat. Rev. Immunol.*, vol. 14, no. 7, pp. 435–446, Jun. 2014.

[174] I. Tirapu, E. Huarte, C. Guiducci, A. Arina, M. Zaratiegui, O. Murillo, A. Gonzalez, C. Berasain, P. Berraondo, P. Fortes, J. Prieto, M. P. Colombo, L. Chen, and I. Melero, "Low Surface Expression of B7-1 (CD80) Is an Immunoescape Mechanism of Colon Carcinoma," *Cancer Res.*, vol. 66, no. 4, pp. 2442–2450, Feb. 2006.

[175] D. J. Hicklin, F. M. Marincola, S. Ferrone, and S. Ferrone, "HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story.," *Mol. Med. Today*, vol. 5, no. 4, pp. 178–86, Apr. 1999.

[176] Y. Kuwana, Y. Asakura, N. Utsunomiya, M. Nakanishi, Y. Arata, S. Itoh, F. Nagase, and Y. Kurosawa, "Expression of chimeric receptor composed of immunoglobulin-derived V resions and T-cell receptor-derived C regions," *Biochem. Biophys. Res. Commun.*, vol. 149, no. 3, pp. 960–968, Dec. 1987.

[177] C. U. Louis, B. Savoldo, G. Dotti, M. Pule, E. Yvon, G. D. Myers, C. Rossig, H. V. Russell, O. Diouf, E. Liu, H. Liu, M.-F. Wu, A. P. Gee, Z. Mei, C. M. Rooney, H. E. Heslop, and M. K. Brenner, "Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma," *Blood*, vol. 118, no. 23, pp. 6050–6056, Dec. 2011.

[178] D. S. Ritchie, P. J. Neeson, A. Khot, S. Peinert, T. Tai, K. Tainton, K. Chen, M. Shin, D. M. Wall, D. Hönemann, P. Gambell, D. A. Westerman, J. Haurat, J. A. Westwood, A. M. Scott, L. Kravets, M. Dickinson, J. A. Trapani, M. J. Smyth, P. K. Darcy, M. H. Kershaw, and H. M. Prince, "Persistence and efficacy of second generation CAR T Cell against the LeY Antigen in acute myeloid leukemia," *Mol. Ther.*, vol. 21, no. 11, pp. 2122–2129, Nov. 2013.

[179] S. Rafiq, T. J. Purdon, A. F. Daniyan, M. Koneru, T. Dao, C. Liu, D. A. Scheinberg, and R. J. Brentjens, "Optimized T-cell receptor-mimic chimeric antigen receptor T cells directed toward the intracellular Wilms Tumor 1 antigen," *Leukemia*, vol. 31, no. 8, pp. 1788–1797, 2017.

[180] M. V. Maus, A. R. Haas, G. L. Beatty, S. M. Albelda, B. L. Levine, X. Liu, Y. Zhao, M. Kalos, and C. H. June, "T Cells Expressing Chimeric Antigen Receptors Can Cause Anaphylaxis in Humans," *Cancer Immunol. Res.*, vol. 1, no. 1, pp. 26–31, 2013.

[181] S. L. Maude, D. M. Barrett, D. E. Ambrose, S. R. Rheingold, R. Aplenc, D. T. Teachey, C. Callahan, C. S. Barker, M. Mudambi, P. A. Shaw, J. Brogdon, R. M. Young, J. Scholler, A. Loew, K. T. Marcucci, J. Finklestein, I. Kulikovskaya, F. Nazimuddin, Z. Zheng, B. L. Levine, D. L. Porter, S. F. Lacey, J. J. Melenhorst, C. H. June, and S. A. Grupp, "Efficacy and Safety of Humanized Chimeric Antigen Receptor (CAR)-Modified T Cells Targeting CD19 in Children with Relapsed/Refractory ALL," *Blood*, vol. 126, no. 23, p. 683, 2015.

[182] M. H. Kershaw, J. a Westwood, L. L. Parker, G. Wang, Z. Eshhar, S. a Mavroukakis, D. E. White, J. R. Wunderlich, S. Canevari, L. Rogers-Freezer, C. C. Chen, J. C.

Yang, S. a Rosenberg, and P. Hwu, “A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer.,” *Clin. Cancer Res.*, vol. 12, no. 20 Pt 1, pp. 6106–15, 2006.

[183] C. H. J. Lamers, S. Sleijfer, A. G. Vulto, W. H. J. Kruit, M. Kliffen, R. Debets, J. W. Gratama, G. Stoter, and E. Oosterwijk, “Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience.,” *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, vol. 24, no. 13. 2006.

[184] J. R. Park, D. L. DiGiusto, M. Slovak, C. Wright, A. Naranjo, J. Wagner, H. B. Meechoovet, C. Bautista, W. C. Chang, J. R. Ostberg, and M. C. Jensen, “Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma,” *Mol. Ther.*, vol. 15, no. 4, pp. 825–833, 2007.

[185] B. G. Till, M. C. Jensen, J. Wang, E. Y. Chen, B. L. Wood, H. A. Greisman, X. Qian, S. E. James, A. Raubitschek, S. J. Forman, A. K. Gopal, J. M. Pagel, C. G. Lindgren, P. D. Greenberg, S. R. Riddell, and O. W. Press, “Adoptive immunotherapy for indolent non-hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells,” *Blood*, vol. 112, no. 6, pp. 2261–2271, 2008.

[186] F. Letourneur and R. D. Klausner, “T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor zeta family proteins.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 88, no. 20, pp. 8905–9, 1991.

[187] C. Romeo and B. Seed, “Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides,” *Cell*, vol. 64, no. 5, pp. 1037–1046, 1991.

[188] B. A. 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, no. 5, pp. 891–901, 1991.

[189] T. Brocker, “Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes,” *J. Exp. Med.*, vol. 181, no. 5, pp. 1653–1659, 1995.

[190] B. Savoldo, C. A. Ramos, E. Liu, M. P. Mims, M. J. Keating, G. Carrum, R. T. Kamble, C. M. Bollard, A. P. Gee, Z. Mei, H. Liu, B. Grilley, C. M. Rooney, H. E. Heslop, M. K. Brenner, and G. Dotti, “CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients.,” *J. Clin. Invest.*, vol. 121, no. 5, pp. 1822–6, May 2011.

[191] C. Imai, K. Mihara, M. Andreansky, I. C. Nicholson, C. H. Pui, T. L. Geiger, and D. Campana, “Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia,” *Leukemia*, vol. 18, no. 4, pp. 676–684, 2004.

[192] X. S. Zhong, M. Matsushita, J. Plotkin, I. Riviere, and M. Sadelain, “Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI 3 kinase/AKT/Bcl-X L activation and CD8 T cell-mediated tumor eradication,” *Mol. Ther.*, vol. 18, no. 2, pp. 413–420, 2010.

[193] M. C. Milone, J. D. Fish, C. Carpenito, R. G. Carroll, G. K. Binder, D. Teachey, M. Samanta, M. Lakhal, B. Gloss, G. Danet-Desnoyers, D. Campana, J. L. Riley, S. A. Grupp, and C. H. June, “Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo,” *Mol. Ther.*, vol. 17, no. 8, pp. 1453–1464, 2009.

[194] M. Cartellieri, A. Feldmann, S. Koristka, C. Arndt, S. Loff, A. Ehninger, M. von Bonin, E. P. Bejestani, G. Ehninger, and M. P. Bachmann, “Switching CAR T cells on and off: a novel modular platform for retargeting of T cells to AML blasts,” *Blood Cancer J.*, vol. 6, no. 8, p. e458, 2016.

[195] C. Turtle, S. Riddell, and D. Maloney, “CD19-Targeted chimeric antigen receptor-modified T-cell immunotherapy for B-cell malignancies,” *Clin. Pharmacol. Ther.*, vol. 100, no. 3, pp. 252–258, Sep. 2016.

[196] J. H. Park, M. B. Geyer, and R. J. Brentjens, “CD19-targeted CAR T-cell therapeutics for hematologic malignancies: interpreting clinical outcomes to date.,” *Blood*, vol. 127, no. 26, pp. 3312–20, Jun. 2016.

[197] D. C. Otero, A. N. Anzelon, and R. C. Rickert, “CD19 function in early and late B cell development: I. Maintenance of follicular and marginal zone B cells requires CD19-dependent survival signals.,” *J. Immunol.*, vol. 170, no. 1, pp. 73–83, 2003.

[198] D. C. Otero and R. C. Rickert, “CD19 Function in Early and Late B Cell Development. II. CD19 Facilitates the Pro-B/Pre-B Transition,” *J. Immunol.*, vol. 171, no. 11, pp. 5921–5930, 2003.

[199] H. B. Gaspar and M. E. Conley, “Early B cell defects.,” *Clin. Exp. Immunol.*, vol. 119, no. 3, pp. 383–9, Mar. 2000.

[200] J. N. Kochenderfer, M. E. Dudley, S. A. Feldman, W. H. Wilson, D. E. Spaner, I. Maric, M. S. Stevenson, G. Q. Phan, M. S. Hughes, R. M. Sherry, J. C. Yang, U. S. Kammula, L. Devillier, R. Carpenter, D.-A. N. Nathan, R. A. Morgan, C. Laurencot, and S. A. Rosenberg, “B--cell depletion and remissions of malignancy along with cytokine- associated toxicity in a clinical trial of anti-CD19 chimeric-antigen- receptor–transduced T cells,” *Blood*, vol. 119, no. 12, pp. 2709–2720, 2012.

[201] J. N. Kochenderfer, W. H. Wilson, J. E. Janik, M. E. Dudley, M. Stetler-Stevenson, S. A. Feldman, I. Maric, M. Raffeld, D. A. N. Nathan, B. J. Lanier, R. A. Morgan, and S. A. Rosenberg, “Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19,” *Blood*, vol. 116, no. 20, pp. 4099–4102, 2010.

[202] D. L. Porter, B. L. Levine, M. Kalos, A. Bagg, and C. H. June, “Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia.,” *N. Engl. J. Med.*, vol. 365, no. 8, pp. 725–33, 2011.

[203] D. W. Lee, J. N. Kochenderfer, M. Stetler-Stevenson, Y. K. Cui, C. Delbrook, S. A. Feldman, T. J. Fry, R. Orentas, M. Sabatino, N. N. Shah, S. M. Steinberg, D. Stroncek, N. Tschernia, C. Yuan, H. Zhang, L. Zhang, S. A. Rosenberg, A. S. Wayne, and C. L. Mackall, “T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial,” *Lancet*, vol. 385, no. 9967, pp. 517–528, Feb. 2015.

[204] S. L. Maude, N. Frey, P. A. Shaw, R. Aplenc, D. M. Barrett, N. J. Bunin, A. Chew, V. E. Gonzalez, Z. Zheng, S. F. Lacey, Y. D. Mahnke, J. J. Melenhorst, S. R. Rheingold, A. Shen, D. T. Teachey, B. L. Levine, C. H. June, D. L. Porter, and S. A. Grupp, “Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia,” *N. Engl. J. Med.*, vol. 371, no. 16, pp. 1507–1517, Oct. 2014.

[205] S. L. Maude, M. A. Pulsipher, M. W. Boyer, S. A. Grupp, S. M. Davies, C. L. Phillips, M. R. Verneris, K. J. August, K. Schlis, T. A. Driscoll, R. Mody, C. M. Capitini, C. H. June, B. L. Levine, P. A. Wood, L. Yi, and J. E. Levine, “Efficacy and Safety of CTL019 in the First US Phase II Multicenter Trial in Pediatric Relapsed/Refractory Acute Lymphoblastic Leukemia: Results of an Interim Analysis,” *Blood*, vol. 128, no. 22, 2016.

[206] R. J. Brentjens, I. Rivière, J. H. Park, M. L. Davila, X. Wang, J. Stefanski, C. Taylor, R. Yeh, S. Bartido, O. Borquez-Ojeda, M. Olszewska, Y. Bernal, H. Pegram, M. Przybylowski, D. Hollyman, Y. Usachenko, D. Pirraglia, J. Hosey, E. Santos, E. Halton, P. Maslak, D. Scheinberg, J. Jurcic, M. Heaney, G. Heller, M. Frattini, and M. Sadelain, “Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias,” *Blood*, vol. 118, no. 18, pp. 4817–4828, 2011.

[207] S. Gill, M. V. Maus, and D. L. Porter, “Chimeric antigen receptor T cell therapy: 25years in the making,” *Blood Rev.*, vol. 30, no. 3, pp. 157–167, May 2016.

[208] S. A. Ali, V. Shi, I. Maric, M. Wang, D. F. Stroncek, J. J. Rose, J. N. Brudno, M.

Stetler-Stevenson, S. A. Feldman, B. G. Hansen, V. S. Fellowes, F. T. Hakim, R. E. Gress, and J. N. Kochenderfer, “T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma.,” *Blood*, vol. 128, no. 13, pp. 1688–700, Sep. 2016.

[209] B. G. Till, M. C. Jensen, J. Wang, X. Qian, A. K. Gopal, D. G. Maloney, C. G. Lindgren, Y. Lin, J. M. Pagel, L. E. Budde, A. Raubitschek, S. J. Forman, P. D. Greenberg, S. R. Riddell, and O. W. Press, “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, no. 17, pp. 3940–50, Apr. 2012.

[210] Q. Wang, Y. Wang, H. Lv, Q. Han, H. Fan, B. Guo, L. Wang, and W. Han, “Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia.,” *Mol. Ther.*, vol. 23, no. 1, pp. 184–91, Jan. 2015.

[211] Y. Luo, L.-J. Chang, Y. Hu, L. Dong, G. Wei, and H. Huang, “First-in-Man CD123-Specific Chimeric Antigen Receptor-Modified T Cells for the Treatment of Refractory Acute Myeloid Leukemia,” *Blood*, vol. 126, no. 23, p. 3778, 2015.

[212] D. M. O’Rourke, M. P. Nasrallah, A. Desai, J. J. Melenhorst, K. Mansfield, J. J. D. Morrissette, M. Martinez-Lage, S. Brem, E. Maloney, A. Shen, R. Isaacs, S. Mohan, G. Plesa, S. F. Lacey, J. M. Navenot, Z. Zheng, B. L. Levine, H. Okada, C. H. June, J. L. Brogdon, and M. V. Maus, “A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma,” *Sci. Transl. Med.*, vol. 9, no. 399, 2017.

[213] R. P. Junghans, Q. Ma, R. Rathore, E. M. Gomes, A. J. Bais, A. S. Y. Lo, M. Abedi, R. A. Davies, H. J. Cabral, A. S. Al-Homsi, and S. I. Cohen, “Phase I Trial of Anti-PSMA Designer CAR-T Cells in Prostate Cancer: Possible Role for Interacting Interleukin 2-T Cell Pharmacodynamics as a Determinant of Clinical Response,” *Prostate*, vol. 76, no. 14, pp. 1257–1270, 2016.

[214] N. Ahmed, V. S. Brawley, M. Hegde, C. Robertson, A. Ghazi, C. Gerken, E. Liu, O. Dakhova, A. Ashoori, A. Corder, T. Gray, M.-F. Wu, H. Liu, J. Hicks, N. Rainusso, G. Dotti, Z. Mei, B. Grilley, A. Gee, C. M. Rooney, M. K. Brenner, H. E. Heslop, W. S. Wels, L. L. Wang, P. Anderson, and S. Gottschalk, “Human Epidermal Growth Factor Receptor 2 (HER2) –Specific Chimeric Antigen Receptor–Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma,” *J. Clin. Oncol.*, vol. 33, no. 15, pp. 1688–1696, May 2015.

[215] A. L. Garfall, M. V. Maus, W.-T. Hwang, S. F. Lacey, Y. D. Mahnke, J. J. Melenhorst, Z. Zheng, D. T. Vogl, A. D. Cohen, B. M. Weiss, K. Dengel, N. D. S. Kerr, A. Bagg, B. L. Levine, C. H. June, and E. A. Stadtmauer, “Chimeric Antigen Receptor T Cells against CD19 for Multiple Myeloma,” *N. Engl. J. Med.*, vol. 373, no. 11, pp. 1040–1047, 2015.

[216] G. Bo, C. M. Xia, H. Q. Wang, F. Hui, D. H. Ren, Z. W. Ying, Z. Y. Jing, W. Yao, Z. H. Li, and H. Wei Dong, “CD138-directed adoptive immunotherapy of chimeric antigen receptor (CAR)-modified T cells for multiple myeloma,” *J. Cell. Immunother.*, pp. 4–11, 2015.

[217] J. G. Berdeja, Y. Lin, N. Raje, N. Munshi, D. Siegel, M. Liedtke, S. Jagannath, M. V. Maus, A. Turka, L. P. Lam, K. Hege, R. A. Morgan, M. T. Quigley, and J. N. Kochenderfer, “Durable Clinical Responses in Heavily Pretreated Patients with Relapsed/Refractory Multiple Myeloma: Updated Results from a Multicenter Study of bb2121 Anti-Bcma CAR T Cell Therapy,” *Blood*, vol. 130, no. Suppl 1, 2017.

[218] C. A. Ramos, B. Ballard, H. Zhang, O. Dakhova, A. P. Gee, Z. Mei, M. Bilgi, M. F. Wu, H. Liu, B. Grilley, C. M. Bollard, B. H. Chang, C. M. Rooney, M. K. Brenner, H. E. Heslop, G. Dotti, and B. Savoldo, “Clinical and immunological responses after CD30-specific chimeric antigen receptor-redirected lymphocytes,” *J. Clin. Invest.*, vol. 127,

no. 9, pp. 3462–3471, 2017.

[219] M. Mamonkin, R. H. Rouce, H. Tashiro, and M. K. Brenner, “A T-cell-directed chimeric antigen receptor for the selective treatment of T-cell malignancies,” *Blood*, vol. 126, no. 8, pp. 983–992, 2015.

[220] S. S. Kenderian, M. Ruella, O. Shestova, M. Klichinsky, V. Aikawa, J. J. D. Morrissette, J. Scholler, D. Song, D. L. Porter, M. Carroll, C. H. June, and S. Gill, “CD33-specific chimeric antigen receptor T cells exhibit potent preclinical activity against human acute myeloid leukemia,” *Leukemia*, vol. 29, no. 8, pp. 1637–1647, 2015.

[221] J. D. Hoyer, K. L. Grogg, C. A. Hanson, J. D. Gamez, and A. Dogan, “CD33 detection by immunohistochemistry in paraffin-embedded tissues: A new antibody shows excellent specificity and sensitivity for cells of myelomonocytic lineage,” *Am. J. Clin. Pathol.*, vol. 129, no. 2, pp. 316–323, 2008.

[222] L. Budde, J. Y. Song, Y. Kim, S. Blanchard, J. Wagner, A. S. Stein, L. Weng, M. Del Real, R. Hernandez, E. Marcucci, J. K. Shepphird, X. Wang, B. Wood, G. Marcucci, C. E. Brown, and S. J. Forman, “Remissions of Acute Myeloid Leukemia and Blastic Plasmacytoid Dendritic Cell Neoplasm Following Treatment with CD123-Specific CAR T Cells: A First-in-Human Clinical Trial,” *Blood*, vol. 130, no. Suppl 1, 2017.

[223] S. Z. He, S. Busfield, D. S. Ritchie, M. S. Hertzberg, S. Durrant, I. D. Lewis, P. Marlton, A. J. McLachlan, I. Kerridge, K. F. Bradstock, G. Kennedy, A. W. Boyd, T. M. Yeadon, A. F. Lopez, H. S. Ramshaw, H. Iland, S. Bamford, M. Barnden, M. DeWitte, R. Basser, and A. W. Roberts, “A Phase 1 study of the Safety, Pharmacokinetics, and Anti-leukemic Activity of the anti-CD123 monoclonal antibody, CSL360, in Relapsed, Refractory or High-Risk Acute Myeloid Leukemia (AML),” *Leuk Lymphoma*, pp. 1–10, 2014.

[224] E. Feldman, M. Kalaycio, G. Weiner, S. Frankel, P. Schulman, L. Schwartzberg, J. Jurcic, E. Velez-Garcia, K. Seiter, D. Scheinberg, D. Levitt, and N. Wedel, “Treatment of relapsed or refractory acute myeloid leukemia with humanized anti-CD33 monoclonal antibody HuM195.,” *Leukemia*, vol. 17, no. 2, pp. 314–8, 2003.

[225] S. Srivastava and S. R. Riddell, “Engineering CAR-T cells: Design concepts.,” *Trends Immunol.*, vol. 36, no. 8, pp. 494–502, Aug. 2015.

[226] A. H. Long, W. M. Haso, J. F. Shern, K. M. Wanhainen, M. Murgai, M. Ingaramo, J. P. Smith, A. J. Walker, M. E. Kohler, V. R. Venkateshwara, R. N. Kaplan, G. H. Patterson, T. J. Fry, R. J. Orentas, and C. L. Mackall, “4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors,” *Nat. Med.*, vol. 21, no. 6, pp. 581–590, May 2015.

[227] M. Hudecek, D. Sommermeyer, P. L. Kosasih, A. Silva-Benedict, L. Liu, C. Rader, M. C. Jensen, and S. R. Riddell, “The Nonsignaling Extracellular Spacer Domain of Chimeric Antigen Receptors Is Decisive for In Vivo Antitumor Activity,” *Cancer Immunol. Res.*, vol. 3, no. 2, pp. 125–135, Feb. 2015.

[228] M. L. Dustin and D. Depoil, “New insights into the T cell synapse from single molecule techniques,” *Nature Reviews Immunology*, vol. 11, no. 10. pp. 672–684, 2011.

[229] W. Haso, D. W. Lee, N. N. Shah, M. Stetler-Stevenson, C. M. Yuan, I. H. Pastan, D. S. Dimitrov, R. A. Morgan, D. J. FitzGerald, D. M. Barrett, A. S. Wayne, C. L. MacKall, and R. J. Orentas, “Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia,” *Blood*, vol. 121, no. 7, pp. 1165–1171, 2013.

[230] M. Hudecek, M. T. Lupo-Stanghellini, P. L. Kosasih, D. Sommermeyer, M. C. Jensen, C. Rader, and S. R. Riddell, “Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells,” *Clin. Cancer Res.*, vol. 19, no. 12, pp. 3153–3164, 2013.

[231] K. S. Kahlon, C. Brown, L. J. N. Cooper, A. Raubitschek, S. J. Forman, and M. C. Jensen, “Specific recognition and killing of glioblastoma multiforme by interleukin 13-

zetakine redirected cytolytic T cells," *Cancer Res.*, vol. 64, no. 24, pp. 9160–9166, 2004.

[232] A. Barber, K. R. Meehan, and C. L. Sentman, "Treatment of multiple myeloma with adoptively transferred chimeric NKG2D receptor-expressing T cells," *Gene Ther.*, vol. 18, no. 5, pp. 509–516, 2011.

[233] H. Almåsbak, E. Walseng, A. Kristian, M. R. Myhre, E. M. Suso, L. A. Munthe, J. T. Andersen, M. Y. Wang, G. Kvalheim, G. Gaudernack, and J. A. Kyte, "Inclusion of an IgG1-Fc spacer abrogates efficacy of CD19 CAR T cells in a xenograft mouse model," *Gene Ther.*, vol. 22, no. 5, pp. 391–403, 2015.

[234] S. E. James, P. D. Greenberg, M. C. Jensen, Y. Lin, J. Wang, B. G. Till, A. A. Raubitschek, S. J. Forman, and O. W. Press, "Antigen Sensitivity of CD22-Specific Chimeric TCR Is Modulated by Target Epitope Distance from the Cell Membrane," *J. Immunol.*, vol. 180, no. 10, pp. 7028–7038, 2008.

[235] M. Jonnalagadda, A. Mardiros, R. Urak, X. Wang, L. J. Hoffman, A. Bernanke, W. C. Chang, W. Bretzlaff, R. Starr, S. Priceman, J. R. Ostberg, S. J. Forman, and C. E. Brown, "Chimeric antigen receptors with mutated IgG4 Fc spacer avoid fc receptor binding and improve T cell persistence and antitumor efficacy," *Mol. Ther.*, vol. 23, no. 4, pp. 757–768, 2015.

[236] R. D. Guest, R. E. Hawkins, N. Kirillova, E. J. Cheadle, J. Arnold, A. O'Neill, J. Irlam, K. A. Chester, J. T. Kemshead, D. M. Shaw, M. J. Embleton, P. L. Stern, and D. E. Gilham, "The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens," *J Immunother*, vol. 28, no. 3, pp. 203–211, 2005.

[237] S. Guedan, A. D. Posey, C. Shaw, A. Wing, T. Da, P. R. Patel, S. E. McGettigan, V. Casado-Medrano, O. U. Kawalekar, M. Uribe-Herranz, D. Song, J. J. Melenhorst, S. F. Lacey, J. Scholler, B. Keith, R. M. Young, and C. H. June, "Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation," *JCI Insight*, vol. 3, no. 1, 2018.

[238] J. L. Riley, M. Mao, S. Kobayashi, M. Biery, J. Burchard, G. Cavet, B. P. Gregson, C. H. June, and P. S. Linsley, "Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors," *Proc. Natl. Acad. Sci.*, vol. 99, no. 18, pp. 11790–11795, 2002.

[239] M. Diehn, A. A. Alizadeh, O. J. Rando, C. L. Liu, K. Stankunas, D. Botstein, G. R. Crabtree, and P. O. Brown, "Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation," *Proc. Natl. Acad. Sci.*, vol. 99, no. 18, pp. 11796–11801, 2002.

[240] R. L. Smeets, W. W. M. Fleuren, X. He, P. M. Vink, F. Wijnands, M. Gorecka, H. Klop, S. Bauerschmidt, A. Garritsen, H. J. P. M. Koenen, I. Joosten, A. M. H. Boots, and W. Alkema, "Molecular pathway profiling of T lymphocyte signal transduction pathways; Th1 and Th2 genomic fingerprints are defined by TCR and CD28-mediated signaling.," *BMC Immunol.*, vol. 13, p. 12, 2012.

[241] G. J. Boonen, A. M. van Dijk, L. F. Verdonck, R. A. van Lier, G. Rijksen, and R. H. Medema, "CD28 induces cell cycle progression by IL-2-independent down-regulation of p27kip1 expression in human peripheral T lymphocytes," *Eur J Immunol*, vol. 29, no. 3, pp. 789–798, 1999.

[242] A. Viola and A. Lanzavecchia, "T cell activation determined by T cell receptor number and tunable thresholds.," *Science*, vol. 273, no. 5271, pp. 104–6, 1996.

[243] J. S. Boomer and J. M. Green, "An enigmatic tail of CD28 signaling.," *Cold Spring Harbor perspectives in biology*, vol. 2, no. 8. 2010.

[244] M. Croft, "Costimulation of T cells by OX40, 4-1BB, and CD27," *Cytokine and Growth Factor Reviews*, vol. 14, no. 3–4. pp. 265–273, 2003.

[245] D. S. Vinay and B. S. Kwon, "Role of 4-1BB in immune responses," *Seminars in*

Immunology, vol. 10, no. 6. pp. 481–489, 1998.

[246] K.-O. Nam, H. Kang, S.-M. Shin, K.-H. Cho, B. Kwon, B. S. Kwon, S.-J. Kim, and H.-W. Lee, "Cross-Linking of 4-1BB Activates TCR-Signaling Pathways in CD8+ T Lymphocytes," *J. Immunol.*, vol. 174, no. 4, pp. 1898–1905, 2005.

[247] I. Daniel-Meshulam, M. Horovitz-Fried, and C. J. Cohen, "Enhanced antitumor activity mediated by human 4-1BB-engineered T cells," *Int. J. Cancer*, vol. 133, no. 12, pp. 2903–13, 2013.

[248] A. J. McPherson, L. M. Snell, T. W. Mak, and T. H. Watts, "Opposing roles for TRAF1 in the alternative versus classical NF-κB pathway in T cells," *J. Biol. Chem.*, vol. 287, no. 27, pp. 23010–23019, 2012.

[249] B. Zhang, Y. Zhang, L. Niu, A. T. Vella, and R. S. Mittler, "Dendritic Cells and Stat3 Are Essential for CD137-Induced CD8 T Cell Activation-Induced Cell Death," *J. Immunol.*, vol. 184, no. 9, pp. 4770–4778, 2010.

[250] R. J. Brentjens, E. Santos, Y. Nikhamin, R. Yeh, M. Matsushita, K. La Perle, A. Quintás-Cardama, S. M. Larson, and M. Sadelain, "Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts," *Clin. Cancer Res.*, vol. 13, no. 18, pp. 5426–5435, 2007.

[251] J. Maher, R. J. Brentjens, G. Gunset, I. Rivière, and M. Sadelain, "Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRζ/CD28 receptor," *Nat. Biotechnol.*, vol. 20, no. 1, pp. 70–75, 2002.

[252] F. J. Plunkett, O. Franzese, H. M. Finney, J. M. Fletcher, L. L. Belaramani, M. Salmon, I. Dokal, D. Webster, A. D. G. Lawson, and A. N. Akbar, "The Loss of Telomerase Activity in Highly Differentiated CD8+CD28-CD27- T Cells Is Associated with Decreased Akt (Ser473) Phosphorylation," *J. Immunol.*, vol. 178, no. 12, pp. 7710–7719, 2007.

[253] J. Wang, M. Jensen, Y. Lin, X. Sui, E. Chen, C. G. Lindgren, B. Till, A. Raubitschek, S. J. Forman, X. Qian, S. James, P. Greenberg, S. Riddell, and O. W. Press, "Optimizing Adoptive Polyclonal T Cell Immunotherapy of Lymphomas, Using a Chimeric T Cell Receptor Possessing CD28 and CD137 Costimulatory Domains," *Hum. Gene Ther.*, vol. 18, no. 8, pp. 712–725, 2007.

[254] M. A. Pulè, K. C. Straathof, G. Dotti, H. E. Heslop, C. M. Rooney, and M. K. Brenner, "A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells.," *Mol. Ther.*, vol. 12, no. 5, pp. 933–41, 2005.

[255] C. Carpenito, M. C. Milone, R. Hassan, J. C. Simonet, M. Lakhal, M. M. Suhoski, A. Varela-Rohena, K. M. Haines, D. F. Heitjan, S. M. Albelda, R. G. Carroll, J. L. Riley, I. Pastan, and C. H. June, "Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains," *Proc. Natl. Acad. Sci.*, vol. 106, no. 9, pp. 3360–3365, 2009.

[256] D. Gomes da Silva, M. Mukherjee, S. Madhuwanti, O. Dakhova, H. Liu, B. Grilley, A. Gee, S. S. Neelapu, C. Rooney, H. Heslop, B. Savoldo, G. Dotti, M. Brenner, M. Mamonkin, and C. A. Ramos, "Direct comparison of in vivo fate of second and third-generation CD19-specific chimeric antigen receptor (CAR)-T cells in patients with b cell non-hodgkin lymphoma (B-NHL): Reversal of toxicity from tonic signaling," *Cytotherapy*, vol. 19, no. 5, p. S9, May 2017.

[257] M. C. Milone, J. D. Fish, C. Carpenito, R. G. Carroll, G. K. Binder, D. Teachey, M. Samanta, M. Lakhal, B. Gloss, G. Danet-Desnoyers, D. Campana, J. L. Riley, S. a Grupp, and C. H. June, "Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo.," *Mol. Ther.*, vol. 17, no. 8, pp. 1453–1464, 2009.

[258] U. Modlich, S. Navarro, D. Zychlinski, T. Maetzig, S. Knoess, M. H. Brugman, A. Schambach, S. Charrier, A. Galy, A. J. Thrasher, J. Bueren, and C. Baum, "Insertional

transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors," *Mol. Ther.*, vol. 17, no. 11, pp. 1919–1928, 2009.

[259] C. Baum, J. Düllmann, Z. Li, B. Fehse, J. Meyer, D. A. Williams, and C. Von Kalle, "Side effects of retroviral gene transfer into hematopoietic stem cells," *Blood*, vol. 101, no. 6. pp. 2099–2114, 2003.

[260] S. Hacein-Bey-Abina, A. Garrigue, G. P. Wang, J. Soulier, A. Lim, E. Morillon, E. Clappier, L. Caccavelli, E. Delabesse, K. Beldjord, V. Asnafi, E. MacIntyre, L. Dal Cortivo, I. Radford, N. Brousse, F. Sigaux, D. Moshous, J. Hauer, A. Borkhardt, B. H. Belohradsky, U. Wintergerst, M. C. Velez, L. Leiva, R. Sorensen, N. Wulffraat, S. Blanche, F. D. Bushman, A. Fischer, and M. Cavazzana-Calvo, "Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1," *J. Clin. Invest.*, vol. 118, no. 9, pp. 3132–3142, 2008.

[261] S. Stein, M. G. Ott, S. Schultze-Strasser, A. Jauch, B. Burwinkel, A. Kinner, M. Schmidt, A. Krämer, J. Schwäble, H. Glimm, U. Koehl, C. Preiss, C. Ball, H. Martin, G. Göhring, K. Schwarzwaelder, W. K. Hofmann, K. Karakaya, S. Tchatchou, R. Yang, P. Reinecke, K. Kühlcke, B. Schlegelberger, A. J. Thrasher, D. Hoelzer, R. Seger, C. Von Kalle, and M. Grez, "Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease," *Nat. Med.*, vol. 16, no. 2, pp. 198–204, 2010.

[262] I. Avedillo Díez, D. Zychlinski, E. G. Coci, M. Galla, U. Modlich, R. A. Dewey, A. Schwarzer, T. Maetzig, N. Mpofu, E. Jaeckel, K. Boztug, C. Baum, C. Klein, and A. Schambach, "Development of novel efficient sin vectors with improved safety features for Wiskott-Aldrich syndrome stem cell based gene therapy," *Mol. Pharm.*, vol. 8, no. 5, pp. 1525–1537, 2011.

[263] T. Iwakuma, Y. Cui, and L. J. Chang, "Self-Inactivating lentiviral vectors with U3 and U5 modifications," *Virology*, vol. 261, no. 1, pp. 120–132, 1999.

[264] G. J. McGarrity, G. Hoyah, A. Winemiller, K. Andre, D. Stein, G. Blick, R. N. Greenberg, C. Kinder, A. Zolopa, G. Binder-Scholl, P. Tebas, C. H. June, L. M. Humeau, and T. Rebello, "Patient monitoring and follow-up in lentiviral clinical trials," *J. Gene Med.*, vol. 15, no. 2, pp. 78–82, Feb. 2013.

[265] Z. Ivics, P. B. Hackett, R. H. Plasterk, and Z. Izsvák, "Molecular reconstruction of sleeping beauty, a Tc1-like transposon from fish, and its transposition in human cells," *Cell*, vol. 91, no. 4, pp. 501–510, 1997.

[266] R. Monjezi, C. Miskey, T. Gogishvili, M. Schleef, M. Schmeer, H. Einsele, Z. Ivics, and M. Hudecek, "Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors," *Leukemia*, vol. 31, no. 1, pp. 186–194, Jan. 2017.

[267] P. Kebriaei, H. Singh, M. H. Huls, M. J. Figliola, R. Bassett, S. Olivares, B. Jena, M. J. Dawson, P. R. Kumaresan, S. Su, S. Maiti, J. Dai, B. Moriarity, M.-A. Forget, V. Senyukov, A. Orozco, T. Liu, J. McCarty, R. N. Jackson, J. S. Moyes, G. Rondon, M. Qazilbash, S. Ciurea, A. Alousi, Y. Nieto, K. Rezvani, D. Marin, U. Popat, C. Hosing, E. J. Shpall, H. Kantarjian, M. Keating, W. Wierda, K. A. Do, D. A. Largaespada, D. A. Lee, P. B. Hackett, R. E. Champlin, and L. J. N. Cooper, "Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells," *J. Clin. Invest.*, vol. 126, no. 9, pp. 3363–3376, Aug. 2016.

[268] D. M. Barrett, Y. Zhao, X. Liu, S. Jiang, C. Carpenito, M. Kalos, R. G. Carroll, C. H. June, and S. A. Grupp, "Treatment of Advanced Leukemia in Mice with mRNA Engineered T Cells," *Hum. Gene Ther.*, vol. 22, no. 12, pp. 1575–1586, 2011.

[269] Y. Zhao, E. Moon, C. Carpenito, C. M. Paulos, X. Liu, A. L. Brennan, A. Chew, R. G. Carroll, J. Scholler, B. L. Levine, S. M. Albelda, and C. H. June, "Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor," *Cancer Res.*, vol. 70, no. 22, pp. 9053–

9061, 2010.

[270] S. H. Yoon, J. M. Lee, H. I. Cho, E. K. Kim, H. S. Kim, M. Y. Park, and T. G. Kim, “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, no. 6, pp. 489–497, Jun. 2009.

[271] G. L. Beatty, A. R. Haas, M. V Maus, D. a Torigian, M. C. Soulen, G. Plesa, A. Chew, Y. Zhao, B. L. Levine, S. M. Albelda, M. Kalos, and C. H. June, “Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies.,” *Cancer Immunol. Res.*, vol. 2, no. 2, pp. 112–20, 2014.

[272] G. L. Beatty, M. H. O’Hara, S. F. Lacey, D. A. Torigian, F. Nazimuddin, F. Chen, I. M. Kulikovskaya, M. C. Soulen, M. McGarvey, A. M. Nelson, W. L. Gladney, B. L. Levine, J. J. Melenhorst, G. Plesa, and C. H. June, “Activity of Mesothelin-specific Chimeric Antigen Receptor T cells Against Pancreatic Carcinoma Metastases in a Phase 1 Trial,” *Gastroenterology*, 2018.

[273] K. D. Cummins, N. Frey, A. M. Nelson, A. Schmidt, S. Luger, R. E. Isaacs, S. F. Lacey, E. Hexner, J. J. Melenhorst, C. H. June, D. L. Porter, and S. I. Gill, “Treating Relapsed / Refractory (RR) AML with Biodegradable Anti-CD123 CAR Modified T Cells,” *Blood*, vol. 130, no. Suppl 1, 2017.

[274] J. Eyquem, J. Mansilla-Soto, T. Giavridis, S. J. C. van der Stegen, M. Hamieh, K. M. Cunanan, A. Odak, M. Gönen, and M. Sadelain, “Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection,” *Nature*, vol. 543, no. 7643, pp. 113–117, Feb. 2017.

[275] S. Kaneko, S. Mastaglio, A. Bondanza, M. Ponzoni, F. Sanvito, L. Aldrighetti, M. Radrizzani, S. La Seta-Catamancio, E. Provasi, A. Mondino, T. Nagasawa, K. Fleischhauer, V. Russo, C. Traversari, F. Ciceri, C. Bordignon, and C. Bonini, “IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes,” *Blood*, vol. 113, no. 5, pp. 1006–1015, Sep. 2008.

[276] N. Pouw, E. Treffers-Westerlaken, J. Kraan, F. Wittink, T. ten Hagen, J. Verweij, and R. Debets, “Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells,” *Cancer Immunol. Immunother.*, vol. 59, no. 6, pp. 921–931, Jun. 2010.

[277] K. J. Maloy and F. Powrie, “Fueling regulation: IL-2 keeps CD4+ Treg cells fit,” *Nat. Immunol.*, vol. 6, no. 11, pp. 1071–1072, Nov. 2005.

[278] R. Perret and F. Ronchese, “Memory T cells in cancer immunotherapy: Which CD8+ T-cell population provides the best protection against tumours?,” *Tissue Antigens*, vol. 72, no. 3. pp. 187–194, 2008.

[279] T. Gebhardt, S. N. Mueller, W. R. Heath, and F. R. Carbone, “Peripheral tissue surveillance and residency by memory T cells,” *Trends in Immunology*, vol. 34, no. 1. pp. 27–32, 2013.

[280] S. Maude and D. M. Barrett, “Current status of chimeric antigen receptor therapy for haematological malignancies,” *British Journal of Haematology*, vol. 172, no. 1. pp. 11–22, 2016.

[281] S. Ghorashian, M. Pule, and P. Amrolia, “CD19 chimeric antigen receptor T cell therapy for haematological malignancies,” *British Journal of Haematology*, vol. 169, no. 4. pp. 463–478, 2015.

[282] D. W. Lee, R. Gardner, D. L. Porter, C. U. Louis, N. Ahmed, M. Jensen, S. A. Grupp, and C. L. Mackall, “Current concepts in the diagnosis and management of cytokine release syndrome,” *Blood*, vol. 124, no. 2, pp. 188–195, 2014.

[283] F. Chen, D. T. Teachey, E. Pequignot, N. Frey, D. Porter, S. L. Maude, S. A. Grupp, C. H. June, J. J. Melenhorst, and S. F. Lacey, “Measuring IL-6 and sIL-6R in serum

from patients treated with tocilizumab and/or siltuximab following CAR T cell therapy," *J. Immunol. Methods*, vol. 434, pp. 1–8, 2016.

[284] U. Winkler, M. Jensen, O. Manzke, H. Schulz, V. Diehl, and a Engert, "Cytokine-release syndrome in patients with B-cell chronic lymphocytic leukemia and high lymphocyte counts after treatment with an anti-CD20 monoclonal antibody (rituximab, IDEC-C2B8).," *Blood*, vol. 94, no. 7, pp. 2217–24, 1999.

[285] M. L. Davila, I. Riviere, X. Wang, S. Bartido, J. Park, K. Curran, S. S. Chung, J. Stefanski, O. Borquez-Ojeda, M. Olszewska, J. Qu, T. Wasielewska, Q. He, M. Fink, H. Shinglot, M. Youssif, M. Satter, Y. Wang, J. Hosey, H. Quintanilla, E. Halton, Y. Bernal, D. C. G. Bouhassira, M. E. Arcila, M. Gonen, G. J. Roboz, P. Maslak, D. Douer, M. G. Frattini, S. Giralt, M. Sadelain, and R. Brentjens, "Efficacy and Toxicity Management of 19-28z CAR T Cell Therapy in B Cell Acute Lymphoblastic Leukemia," *Sci. Transl. Med.*, vol. 6, no. 224, p. 224ra25-224ra25, 2014.

[286] Y. Hu, J. Sun, Z. Wu, J. Yu, Q. Cui, C. Pu, B. Liang, Y. Luo, J. Shi, A. Jin, L. Xiao, and H. Huang, "Predominant cerebral cytokine release syndrome in CD19-directed chimeric antigen receptor-modified T cell therapy," *J. Hematol. Oncol.*, vol. 9, no. 1, 2016.

[287] R. A. Morgan, J. C. Yang, M. Kitano, M. E. Dudley, C. M. Laurencot, and S. A. Rosenberg, "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, no. 4, pp. 843–51, Apr. 2010.

[288] H. G. Caruso, L. V. Hurton, A. Najjar, D. Rushworth, S. Ang, S. Olivares, T. Mi, K. Switzer, H. Singh, H. Huls, D. A. Lee, A. B. Heimberger, R. E. Champlin, and L. J. N. Cooper, "Tuning sensitivity of CAR to EGFR density limits recognition of normal tissue while maintaining potent antitumor activity," *Cancer Res.*, vol. 75, no. 17, pp. 3505–3518, 2015.

[289] X. Liu, S. Jiang, C. Fang, S. Yang, D. Olalere, E. C. Pequignot, A. P. Cogdill, N. Li, M. Ramones, B. Granda, L. Zhou, A. Loew, R. M. Young, C. H. June, and Y. Zhao, "Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice," *Cancer Res.*, vol. 75, no. 17, pp. 3596–3607, 2015.

[290] C. A. Ramos, B. Savoldo, V. Torrano, B. Ballard, H. Zhang, O. Dakhova, E. Liu, G. Carrum, R. T. Kamble, A. P. Gee, Z. Mei, M. F. Wu, H. Liu, B. Grilley, C. M. Rooney, M. K. Brenner, H. E. Heslop, and G. Dotti, "Clinical responses with T lymphocytes targeting malignancy-associated κ light chains," *J. Clin. Invest.*, vol. 126, no. 7, pp. 2588–2596, 2016.

[291] K. C. Straathof, M. A. Pulè, P. Yotnda, G. Dotti, E. F. Vanin, M. K. Brenner, H. E. Heslop, D. M. Spencer, and C. M. Rooney, "An inducible caspase 9 safety switch for T-cell therapy," *Blood*, vol. 105, no. 11, pp. 4247–4254, 2005.

[292] G. Akpek, S. M. Lee, V. Anders, and G. B. Vogelsang, "A high-dose pulse steroid regimen for controlling active chronic graft-versus-host disease," *Biol. Blood Marrow Transplant.*, vol. 7, no. 9, pp. 495–502, 2001.

[293] J. L. Ferrara, J. E. Levine, P. Reddy, and E. Holler, "Graft-versus-host disease," *The Lancet*, vol. 373, no. 9674. pp. 1550–1561, 2009.

[294] C. J. Turtle, L.-A. Hanafi, C. Berger, T. A. Gooley, S. Cherian, M. Hudecek, D. Sommermeyer, K. Melville, B. Pender, T. M. Budiarto, E. Robinson, N. N. Steevens, C. Chaney, L. Soma, X. Chen, C. Yeung, B. Wood, D. Li, J. Cao, S. Heimfeld, M. C. Jensen, S. R. Riddell, and D. G. Maloney, "CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients.," *J. Clin. Invest.*, vol. 126, no. 6, pp. 2123–38, Jun. 2016.

[295] M. S. Topp, N. Gökbuget, G. Zugmaier, P. Klappers, M. Stelljes, S. Neumann, A. Viardot, R. Marks, H. Diedrich, C. Faul, A. Reichle, H. A. Horst, M. Brüggemann, D.

Wessiepe, C. Holland, S. Alekar, N. Mergen, H. Einsele, D. Hoelzer, and R. C. Bargou, “Phase II trial of the anti-CD19 bispecific T cell-engager blinatumomab shows hematologic and molecular remissions in patients with relapsed or refractory B-precursor acute lymphoblastic leukemia,” *J. Clin. Oncol.*, vol. 32, no. 36, pp. 4134–4140, 2014.

[296] E. Sotillo, D. M. Barrett, K. L. Black, A. Bagashev, D. Oldridge, G. Wu, R. Sussman, C. Lanauze, M. Ruella, M. R. Gazzara, N. M. Martinez, C. T. Harrington, E. Y. Chung, J. Perazzelli, T. J. Hofmann, S. L. Maude, P. Raman, A. Barrera, S. Gill, S. F. Lacey, J. J. Melenhorst, D. Allman, E. Jacoby, T. Fry, C. Mackall, Y. Barash, K. W. Lynch, J. M. Maris, S. A. Grupp, and A. Thomas-Tikhonenko, “Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy,” *Cancer Discov.*, vol. 5, no. 12, pp. 1282–1295, 2015.

[297] R. Gardner, D. Wu, S. Cherian, M. Fang, L. A. Hanafi, O. Finney, H. Smithers, M. C. Jensen, S. R. Riddell, D. G. Maloney, and C. J. Turtle, “Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy,” *Blood*, vol. 127, no. 20, pp. 2406–2410, 2016.

[298] E. Jacoby, S. M. Nguyen, T. J. Fountaine, K. Welp, B. Gryder, H. Qin, Y. Yang, C. D. Chien, A. E. Seif, H. Lei, Y. K. Song, J. Khan, D. W. Lee, C. L. Mackall, R. A. Gardner, M. C. Jensen, J. F. Shern, and T. J. Fry, “CD19 CAR immune pressure induces B-precursor acute lymphoblastic leukaemia lineage switch exposing inherent leukaemic plasticity,” *Nat. Commun.*, vol. 7, 2016.

[299] A. Rayes, R. L. Mcmasters, and M. M. O’Brien, “Lineage Switch in MLL-Rearranged Infant Leukemia Following CD19-Directed Therapy,” *Pediatr. Blood Cancer*, vol. 63, no. 6, pp. 1113–1115, 2016.

[300] J. Hiraga, A. Tomita, T. Sugimoto, K. Shimada, M. Ito, S. Nakamura, H. Kiyoi, T. Kinoshita, and T. Naoe, “Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: Its prevalence and clinical significance,” *Blood*, vol. 113, no. 20, pp. 4885–4893, 2009.

[301] A. Mayer and P. Vaupel, “Hypoxia, lactate accumulation, and acidosis: Siblings or accomplices driving tumor progression and resistance to therapy?,” in *Advances in Experimental Medicine and Biology*, 2013, vol. 789, pp. 203–209.

[302] L. Baitsch, S. A. Fuertes-Marraco, A. Legat, C. Meyer, and D. E. Speiser, “The three main stumbling blocks for anticancer T cells,” *Trends in Immunology*, vol. 33, no. 7. pp. 364–372, 2012.

[303] T. F. Gajewski, S.-R. Woo, Y. Zha, R. Spaapen, Y. Zheng, L. Corrales, and S. Spranger, “Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment,” *Curr. Opin. Immunol.*, vol. 25, no. 2, pp. 268–276, 2013.

[304] E. K. Moon, L. C. Wang, D. V. Dolfi, C. B. Wilson, R. Ranganathan, J. Sun, V. Kapoor, J. Scholler, E. Puré, M. C. Milone, C. H. June, J. L. Riley, E. J. Wherry, and S. M. Albelda, “Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor-transduced human T cells in solid tumors,” *Clin. Cancer Res.*, vol. 20, no. 16, pp. 4262–4273, 2014.

[305] S. Radoja and a B. Frey, “Cancer-induced defective cytotoxic T lymphocyte effector function: another mechanism how antigenic tumors escape immune-mediated killing.,” *Mol. Med.*, vol. 6, no. 6, pp. 465–79, 2000.

[306] W. Zhang, Y. Wang, Y. Guo, H. Dai, Q. Yang, Y. Zhang, Y. Zhang, M. Chen, C. Wang, K. Feng, S. Li, Y. Liu, F. Shi, C. Luo, and W. Han, “Treatment of CD20-directed Chimeric Antigen Receptor-modified T cells in patients with relapsed or refractory B-cell non-Hodgkin lymphoma: an early phase IIa trial report,” *Signal Transduct. Target. Ther.*, vol. 1, p. 16002, 2016.

[307] M. Hegde, M. Mukherjee, Z. Grada, A. Pignata, D. Landi, S. A. Navai, A. Wakefield, K. Fousek, K. Bielamowicz, K. K. H. Chow, V. S. Brawley, T. T. Byrd, S. Krebs, S.

Gottschalk, W. S. Wels, M. L. Baker, G. Dotti, M. Mamonkin, M. K. Brenner, J. S. Orange, and N. Ahmed, “Tandem CAR T cells targeting HER2 and IL13Rα2 mitigate tumor antigen escape,” *J. Clin. Invest.*, vol. 126, no. 8, pp. 3036–3052, 2016.

[308] Z. Grada, M. Hegde, T. Byrd, D. R. Shaffer, A. Ghazi, V. S. Brawley, A. Corder, K. Schönfeld, J. Koch, G. Dotti, H. E. Heslop, S. Gottschalk, W. S. Wels, M. L. Baker, and N. Ahmed, “TanCAR: A novel bispecific chimeric antigen receptor for cancer immunotherapy,” *Mol. Ther. - Nucleic Acids*, vol. 2, 2013.

[309] U. Anurathapan, R. C. Chan, H. F. Hindi, R. Mucharla, P. Bajgain, B. C. Hayes, W. E. Fisher, H. E. Heslop, C. M. Rooney, M. K. Brenner, A. M. Leen, and J. F. Vera, “Kinetics of tumor destruction by chimeric antigen receptor-modified T cells,” *Mol. Ther.*, vol. 22, no. 3, pp. 623–633, 2014.

[310] M. Hegde, A. Corder, K. K. H. Chow, M. Mukherjee, A. Ashoori, Y. Kew, Y. J. Zhang, D. S. Baskin, F. A. Merchant, V. S. Brawley, T. T. Byrd, S. Krebs, M. F. Wu, H. Liu, H. E. Heslop, S. Gottschalk, S. Gottachalk, E. Yvon, and N. Ahmed, “Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma.,” *Mol. Ther.*, vol. 21, no. 11, pp. 2087–101, 2013.

[311] J. Galon, A. Costes, F. Sanchez-Cabo, A. Kirilovsky, B. Mlecnik, C. Lagorce-Pagès, M. Tosolini, M. Camus, A. Berger, P. Wind, F. Zinzindohoué, P. Bruneval, P.-H. Cugnenc, Z. Trajanoski, W.-H. Fridman, and F. Pagès, “Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.,” *Science*, vol. 313, no. 5795, pp. 1960–4, 2006.

[312] W. Wang, Y. Ma, J. Li, H. S. Shi, L. Q. Wang, F. C. Guo, J. Zhang, D. Li, B. H. Mo, F. Wen, T. Liu, Y. T. Liu, Y. S. Wang, and Y. Q. Wei, “Specificity redirection by CAR with human VEGFR-1 affinity endows T lymphocytes with tumor-killing ability and anti-angiogenic potency,” *Gene Ther.*, vol. 20, no. 10, pp. 970–978, 2013.

[313] C. C. Kloss, M. Condomines, M. Cartellieri, M. Bachmann, and M. Sadelain, “Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells,” *Nat. Biotechnol.*, vol. 31, no. 1, pp. 71–75, 2013.

[314] V. D. Fedorov, M. Themeli, and M. Sadelain, “PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses,” *Sci. Transl. Med.*, vol. 5, no. 215, 2013.

[315] A. Gouble, B. Philip, L. Poirot, C. Schiffer-Mannioui, R. Galetto, S. Derniame, G. Weng-Kit Cheung, S. Arnould, C. Desseaux, M. Pule, and J. Smith, “In Vivo Proof of Concept of Activity and Safety of UCART19, an Allogeneic ‘Off-the-Shelf’ Adoptive T-Cell Immunotherapy Against CD19+ B-Cell Leukemias,” *Blood*, vol. 124, no. 21, p. 4689, 2014.

[316] L. Poirot, B. Philip, C. Schiffer-Mannioui, D. Le Clerre, I. Chion-Sotinel, S. Derniame, P. Potrel, C. Bas, L. Lemaire, R. Galetto, C. Lebuhotel, J. Eyquem, G. W.-K. Cheung, A. Duclert, A. Gouble, S. Arnould, K. Peggs, M. Pule, A. M. Scharenberg, and J. Smith, “Multiplex Genome-Edited T-cell Manufacturing Platform for ‘Off-the-Shelf’ Adoptive T-cell Immunotherapies,” *Cancer Res.*, vol. 75, no. 18, p. 3853, 2015.

[317] W. Qasim, H. Zhan, S. Samarasinghe, S. Adams, P. Amrolia, S. Stafford, K. Butler, C. Rivat, G. Wright, K. Somana, S. Ghorashian, D. Pinner, G. Ahsan, K. Gilmour, G. Lucchini, S. Inglott, W. Mifsud, R. Chiesa, K. S. Peggs, L. Chan, F. Farzaneh, A. J. Thrasher, A. Vora, M. Pule, and P. Veys, “Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells,” *Sci. Transl. Med.*, vol. 9, no. 374, p. 2013, 2017.

[318] R. J. Brentjens, M. L. Davila, I. Riviere, J. Park, X. Wang, L. G. Cowell, S. Bartido, J. Stefanski, C. Taylor, M. Olszewska, O. Borquez-Ojeda, J. Qu, T. Wasielewska, Q. He, Y. Bernal, I. V Rijo, C. Hedvat, R. Kobos, K. Curran, P. Steinherz, J. Jurcic, T. Rosenblat, P. Maslak, M. Frattini, and M. Sadelain, “CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute

lymphoblastic leukemia.," *Sci. Transl. Med.*, vol. 5, no. 177, p. 177ra38, Mar. 2013.

[319] N. a Kernan, R. W. Knowles, M. J. Burns, H. E. Broxmeyer, L. Lu, H. M. Lee, R. T. Kawahata, P. J. Scannon, and B. Dupont, "Specific inhibition of in vitro lymphocyte transformation by an anti-pan T cell (gp67) ricin A chain immunotoxin.," *J. Immunol.*, vol. 133, no. 1, pp. 137–146, 1984.

[320] A. P. Rapoport, E. A. Stadtmauer, G. K. Binder-Scholl, O. Goloubeva, D. T. Vogl, S. F. Lacey, A. Z. Badros, A. Garfall, B. Weiss, J. Finklestein, I. Kulikovskaya, S. K. Sinha, S. Kronsberg, M. Gupta, S. Bond, L. Melchiori, J. E. Brewer, A. D. Bennett, A. B. Gerry, N. J. Pumphrey, D. Williams, H. K. Tayton-Martin, L. Ribeiro, T. Holdich, S. Yanovich, N. Hardy, J. Yared, N. Kerr, S. Philip, S. Westphal, D. L. Siegel, B. L. Levine, B. K. Jakobsen, M. Kalos, and C. H. June, "NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma," *Nat. Med.*, vol. 21, no. 8, pp. 914–921, 2015.

[321] B. F. Haynes, G. S. Eisenbarth, and A. S. Fauci, "Human lymphocyte antigens: production of a monoclonal antibody that defines functional thymus-derived lymphocyte subsets.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 76, no. 11, pp. 5829–33, Nov. 1979.

[322] H. Rabinowich, L. Pricop, R. B. Herberman, and T. L. Whiteside, "Expression and function of CD7 molecule on human natural killer cells.," *J. Immunol.*, vol. 152, no. 2, pp. 517–26, 1994.

[323] S. Juneja, *Atlas of Differential Diagnosis in Neoplastic Hematopathology*, vol. 37, no. 3. 2005.

[324] D. Campana, J. J. van Dongen, a Mehta, E. Coustan-Smith, I. L. Wolvers-Tettero, K. Ganeshaguru, and G. Janossy, "Stages of T-cell receptor protein expression in T-cell acute lymphoblastic leukemia.," *Blood*, vol. 77, no. 7, pp. 1546–54, 1991.

[325] S. D. Lyman, S. Escobar, A. M. Rousseau, A. Armstrong, and W. C. Fanslow, "Identification of CD7 as a cognate of the human K12 (SECTM1) protein," *J. Biol. Chem.*, vol. 275, no. 5, pp. 3431–3437, 2000.

[326] a S. Chan, J. L. Mobley, G. B. Fields, and Y. Shimizu, "CD7-mediated regulation of integrin adhesiveness on human T cells involves tyrosine phosphorylation-dependent activation of phosphatidylinositol 3-kinase.," *J. Immunol.*, vol. 159, no. 2, pp. 934–42, 1997.

[327] S. G. Ward, R. Parry, C. Lefeuvre, D. M. Sansom, J. Westwick, and A. I. Lazarovits, "Antibody ligation of CD7 leads to association with phosphoinositide 3-kinase and phosphatidylinositol 3,4,5-trisphosphate formation in T lymphocytes," *Eur. J. Immunol.*, vol. 25, no. 2, pp. 502–507, 1995.

[328] F. A. Bonilla, C. M. Kokron, P. Swinton, and R. S. Geha, "Targeted gene disruption of murine CD7," *Int. Immunol.*, vol. 9, no. 12, pp. 1875–1883, 1997.

[329] D. M. Lee, H. F. Staats, J. S. Sundy, D. D. Patel, G. D. Sempowski, R. M. Scearce, D. M. Jones, and B. F. Haynes, "Immunologic characterization of CD7-deficient mice.," *J. Immunol.*, vol. 160, no. 12, pp. 5749–56, 1998.

[330] F. W. M. B. Preijers, W. J. M. Tax, T. De Witte, A. Janssen, H. Van der Heijden, H. Vidal, J. M. C. Wessels, and P. J. A. Capel, "Relationship between internalization and cytotoxicity of ricin A-chain immunotoxins," *Br. J. Haematol.*, vol. 70, no. 3, pp. 289–294, 1988.

[331] A. E. Frankel, J. H. Laver, M. C. Willingham, L. J. Burns, J. H. Kersey, and D. A. Vallera, "Therapy of Patients with T-cell Lymphomas and Leukemias Using an Anti-CD7 Monoclonal Antibody-Rich a Chain Immunotoxin," *Leuk. Lymphoma*, vol. 26, no. 42067, pp. 287–298, 1997.

[332] M. E. Pauza, S. O. Doumbia, and C. A. Pennell, "Construction and characterization of human CD7-specific single-chain Fv immunotoxins.," *J. Immunol.*, vol. 158, no. 7, pp.

3259–69, 1997.

[333] W. Baum, H. Steininger, H. J. Bair, W. Becker, T. E. Hansen-Hagge, M. Kressel, E. Kremmer, J. R. Kalden, and M. Gramatzki, “Therapy with CD7 monoclonal antibody TH-69 is highly effective for xenografted human T-cell ALL,” *Br. J. Haematol.*, vol. 95, no. 2, pp. 327–338, 1996.

[334] M. A. Moreno-Mateos, C. E. Vejnar, J. D. Beaudoin, J. P. Fernandez, E. K. Mis, M. K. Khokha, and A. J. Giraldez, “CRISPRscan: Designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo,” *Nat. Methods*, vol. 12, no. 10, pp. 982–988, 2015.

[335] T. J. Cradick, P. Qiu, C. M. Lee, E. J. Fine, and G. Bao, “COSMID: A web-based tool for identifying and validating CRISPR/Cas off-target sites,” *Mol. Ther. - Nucleic Acids*, vol. 3, no. 12, 2014.

[336] M. C. Gundry, L. Brunetti, A. Lin, A. E. Mayle, A. Kitano, D. Wagner, J. I. Hsu, K. A. Hoegenauer, C. M. Rooney, M. A. Goodell, and D. Nakada, “Highly Efficient Genome Editing of Murine and Human Hematopoietic Progenitor Cells by CRISPR/Cas9,” *Cell Rep.*, vol. 17, no. 5, pp. 1453–1461, 2016.

[337] C. M. Lee, H. Zhu, T. H. Davis, H. Deshmukh, and G. Bao, “Design and validation of CRISPR/cas9 systems for targeted gene modification in induced pluripotent stem cells,” in *Methods in Molecular Biology*, 2017, vol. 1498, pp. 3–21.

[338] L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, and F. Zhang, “Multiplex genome engineering using CRISPR/Cas systems,” *Science (80-.).*, vol. 339, no. 6121, pp. 819–823, 2013.

[339] S. Q. Tsai, Z. Zheng, N. T. Nguyen, M. Liebers, V. V. Topkar, V. Thapar, N. Wyvekens, C. Khayter, A. J. Iafrate, L. P. Le, M. J. Aryee, and J. K. Joung, “GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases,” *Nat. Biotechnol.*, vol. 33, no. 2, pp. 187–198, 2015.

[340] C. M. Lee, T. J. Cradick, and G. Bao, “The neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells,” *Mol. Ther.*, vol. 24, no. 3, pp. 645–654, 2016.

[341] Y. Lin, T. J. Cradick, M. T. Brown, H. Deshmukh, P. Ranjan, N. Sarode, B. M. Wile, P. M. Vertino, F. J. Stewart, and G. Bao, “CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences,” *Nucleic Acids Res.*, vol. 42, no. 11, pp. 7473–7485, 2014.

[342] D. M. Lee, D. D. Patel, A. M. Pendergast, and B. F. Haynes, “Functional association of CD7 with phosphatidylinositol 3-kinase: Interaction via a YEDM motif,” *Int. Immunol.*, vol. 8, no. 8, pp. 1195–1203, 1996.

[343] Y. T. Png, N. Vinanica, T. Kamiya, N. Shimasaki, E. Coustan-Smith, and D. Campana, “Blockade of CD7 expression in T cells for effective chimeric antigen receptor targeting of T-cell malignancies,” *Blood Adv.*, vol. 1, no. 25, pp. 2348–2360, 2017.

[344] P. Tebas, D. Stein, W. W. Tang, I. Frank, S. Q. Wang, G. Lee, S. K. Spratt, R. T. Surosky, M. a Giedlin, G. Nichol, M. C. Holmes, P. D. Gregory, D. G. Ando, M. Kalos, R. G. Collman, G. Binder-Scholl, G. Plesa, W.-T. Hwang, B. L. Levine, and C. H. June, “Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV.,” *N. Engl. J. Med.*, vol. 370, no. 10, pp. 901–10, 2014.

[345] W. Qasim, P. J. Amrolia, S. Samarasinghe, S. Ghorashian, H. Zhan, S. Stafford, K. Butler, G. Ahsan, K. Gilmour, S. Adams, D. Pinner, R. Chiesa, S. Chatters, S. Swift, N. Goulden, K. Peggs, A. J. Thrasher, P. Veys, and M. Pule, “First Clinical Application of Talen Engineered Universal CAR19 T Cells in B-ALL,” *Blood*, vol. 126, no. 23, p. 2046, 2015.

[346] D. B. T. Cox, R. J. Platt, and F. Zhang, “Therapeutic genome editing: Prospects and challenges,” *Nature Medicine*, vol. 21, no. 2. pp. 121–131, 2015.

[347] A. Recchia, C. Bonini, Z. Magnani, F. Urbinati, D. Sartori, S. Muraro, E. Tagliafico, A. Bondanza, M. T. L. Stanghellini, M. Bernardi, A. Pescarollo, F. Ciceri, C. Bordignon, and F. Mavilio, “Retroviral vector integration deregulates gene expression but has no consequence on the biology and function of transplanted T cells,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 5, pp. 1457–1462, 2006.

[348] S. Hacein-Bey-Abina, C. von Kalle, M. Schmidt, F. Le Deist, N. Wulffraat, E. McIntyre, I. Radford, J.-L. Villeval, C. C. Fraser, M. Cavazzana-Calvo, and A. Fischer, “A Serious Adverse Event after Successful Gene Therapy for X-Linked Severe Combined Immunodeficiency,” *N. Engl. J. Med.*, vol. 348, no. 3, pp. 255–256, 2003.

[349] G. K. Lam, H. X. Liao, Y. Xue, S. M. Alam, R. M. Scearce, R. E. Kaufman, G. D. Sempowski, and B. F. Haynes, “Expression of the CD7 ligand K-12 in human thymic epithelial cells: Regulation by IFN-γ,” *J. Clin. Immunol.*, vol. 25, no. 1, pp. 41–49, 2005.

[350] K. A. Slentz-Kesler, L. P. Hale, and R. E. Kaufman, “Identification and characterization of K12 (SECTM1), a novel human gene that encodes a Golgi-associated protein with transmembrane and secreted isoforms,” *Genomics*, vol. 47, no. 3, pp. 327–340, 1998.

[351] T. Wang, C. Huang, A. Lopez-Coral, K. A. Slentz-Kesler, M. Xiao, E. J. Wherry, and R. E. Kaufman, “K12/SECTM1, an interferon- gamma regulated molecule, synergizes with CD28 to costimulate human T cell proliferation,” *J. Leukoc. Biol.*, vol. 91, no. 3, pp. 449–459, 2012.

[352] K. E. Pace, H. P. Hahn, M. Pang, J. T. Nguyen, and L. G. Baum, “CD7 Delivers a Pro-Apoptotic Signal During Galectin-1-Induced T Cell Death,” *J. Immunol.*, vol. 165, no. 5, pp. 2331–2334, 2000.

[353] U. Reinhold, L. Liu, J. Sesterhenn, and H. Abken, “CD7-negative T cells represent a separate differentiation pathway in a subset of post-thymic helper T cells.,” *Immunology*, vol. 89, no. 3, pp. 391–396, 1996.

[354] U. Reinhold, H. Abken, S. Kukel, M. Moll, R. Müller, I. Oltermann, and H. W. Kreysel, “CD7- T cells represent a subset of normal human blood lymphocytes.,” *J. Immunol.*, vol. 150, no. 5, p. 2081, 1993.

[355] A. Papadopoulou, U. Gerdemann, U. L. Katari, I. Tzannou, H. Liu, C. Martinez, K. Leung, G. Carrum, A. P. Gee, J. F. Vera, R. A. Krance, M. K. Brenner, C. M. Rooney, H. E. Heslop, and A. M. Leen, “Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT,” *Sci. Transl. Med.*, vol. 6, no. 242, 2014.

[356] A. Di Stasi, S.-K. Tey, G. Dotti, Y. Fujita, A. Kennedy-Nasser, C. Martinez, K. Straathof, E. Liu, A. G. Durett, B. Grilley, H. Liu, C. R. Cruz, B. Savoldo, A. P. Gee, J. Schindler, R. A. Krance, H. E. Heslop, D. M. Spencer, C. M. Rooney, and M. K. Brenner, “Inducible Apoptosis as a Safety Switch for Adoptive Cell Therapy,” *N. Engl. J. Med.*, vol. 365, no. 18, pp. 1673–1683, 2011.

[357] D. L. Porter, N. V. Frey, J. J. Melenhorst, W.-T. Hwang, S. F. Lacey, P. Shaw, A. Chew, S. A. Grupp, J. Capobianchi, J. Gilmore, M. Kalos, M. Litchman, L. Lledo, A. W. Loren, Y. Mahnke, K. T. Marcucci, H. McConville, A. Shen, P. A. Wood, Z. Zheng, B. L. Levine, and C. H. June, “Randomized, Phase II Dose Optimization Study of Chimeric Antigen Receptor Modified T Cells Directed Against CD19 (CTL019) in Patients with Relapsed, Refractory CLL,” *Blood*, vol. 124, no. 21, 2014.

[358] D. Gomes-Silva, M. Srinivasan, S. Sharma, C. M. Lee, D. L. Wagner, T. H. Davis, R. H. Rouce, G. Bao, M. K. Brenner, and M. Mamonkin, “CD7-edited T cells expressing a CD7-specific CAR for the therapy of T-cell malignancies,” *Blood*, vol. 130, no. 3, pp. 285–296, 2017.

[359] M. S. Kuhns and M. M. Davis, “Disruption of Extracellular Interactions Impairs T Cell Receptor-CD3 Complex Stability and Signaling,” *Immunity*, vol. 26, no. 3, pp. 357–

369, 2007.

[360] K. Midtvedt, P. Fauchald, B. Lien, A. Hartmann, D. Albrechtsen, B. L. Bjerkely, T. Leivestad, and I. B. Brekke, “Individualized T cell monitored administration of ATG versus OKT3 in steroid-resistant kidney graft rejection,” *Clin. Transplant.*, vol. 17, no. 1, pp. 69–74, 2003.

[361] M. Gramatzki, R. Burger, G. Strobel, U. Trautmann, C. R. Bartram, G. Helm, G. Horneff, S. Alsalameh, M. Jonker, and E. Gebhart, “Therapy with OKT3 monoclonal antibody in refractory T cell acute lymphoblastic leukemia induces interleukin-2 responsiveness.,” *Leukemia*, vol. 9, no. 3, pp. 382–390, 1995.

[362] F. M. Foss, P. L. Zinzani, J. M. Vose, R. D. Gascoyne, S. T. Rosen, and K. Tobinai, “Peripheral T-cell lymphoma,” *Blood*, vol. 117, no. 25. pp. 6756–6767, 2011.

[363] D. Gomes-Silva, M. Mukherjee, M. Srinivasan, G. Krenciute, O. Dakhova, Y. Zheng, J. M. S. Cabral, C. M. Rooney, J. S. Orange, M. K. Brenner, and M. Mamonkin, “Tonic 4-1BB Costimulation in Chimeric Antigen Receptors Impedes T Cell Survival and Is Vector-Dependent,” *Cell Rep.*, vol. 21, no. 1, pp. 17–26, 2017.

[364] a Hombach, a a Hombach, and H. Abken, “Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc ‘spacer’ domain in the extracellular moiety of chimeric antigen receptors avoids ‘off-target’ activation and unintended initiation of an innate immune response.,” *Gene Ther.*, vol. 17, no. 10, pp. 1206–1213, 2010.

[365] J. S. Bridgeman, R. E. Hawkins, S. Bagley, M. Blaylock, M. Holland, and D. E. Gilham, “The Optimal Antigen Response of Chimeric Antigen Receptors Harboring the CD3 Transmembrane Domain Is Dependent upon Incorporation of the Receptor into the Endogenous TCR/CD3 Complex,” *J. Immunol.*, vol. 184, no. 12, pp. 6938–6949, Jun. 2010.

[366] P. A. Carpenter, S. Pavlovic, J. Y. Tso, O. W. Press, T. Gooley, X.-Z. Yu, and C. Anasetti, “Non-Fc Receptor-Binding Humanized Anti-CD3 Antibodies Induce Apoptosis of Activated Human T Cells,” *J. Immunol.*, vol. 165, no. 11, pp. 6205–6213, 2000.

[367] K.-A. Sheppard, L. J. Fitz, J. M. Lee, C. Benander, J. A. George, J. Wooters, Y. Qiu, J. M. Jussif, L. L. Carter, C. R. Wood, and D. Chaudhary, “PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta.,” *FEBS Lett.*, vol. 574, no. 1–3, pp. 37–41, 2004.

[368] K. N. Balaji, N. Schaschke, W. Machleidt, M. Catalfamo, and P. A. Henkart, “Surface Cathepsin B Protects Cytotoxic Lymphocytes from Self-destruction after Degranulation,” *J. Exp. Med.*, vol. 196, no. 4, pp. 493–503, 2002.

[369] M. S. Buzza, C. E. Hirst, C. H. Bird, P. Hosking, J. McKendrick, and P. I. Bird, “The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses,” *Cell. Immunol.*, vol. 210, no. 1, pp. 21–29, 2001.

[370] K. H. Chen, M. Wada, A. E. Firor, K. G. Pinz, A. Jares, H. Liu, H. Salman, M. Golightly, F. Lan, X. Jiang, and Y. Ma, “Novel anti-CD3 chimeric antigen receptor targeting of aggressive T cell malignancies,” *Oncotarget*, vol. 7, no. 35, pp. 56219–56232, 2016.

[371] P. M. Maciocia, P. A. Wawrzyniecka, B. Philip, I. Ricciardelli, A. U. Akarca, S. C. Onuoha, M. Leguţ, D. K. Cole, A. K. Sewell, G. Gritti, J. Somja, M. A. Piris, K. S. Peggs, D. C. Linch, T. Marafioti, and M. A. Pule, “Targeting the T cell receptor β-chain constant region for immunotherapy of T cell malignancies,” *Nat. Med.*, vol. 23, no. 12, pp. 1416–1423, 2017.

[372] J. Vera, B. Savoldo, S. Vigouroux, E. Biagi, M. Pule, C. Rossig, J. Wu, H. E. Heslop, C. M. Rooney, M. K. Brenner, and G. Dotti, “T lymphocytes redirected against the κ light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived

malignant cells," *Blood*, vol. 108, no. 12, pp. 3890–3897, 2006.

[373] A. Mardiros, C. Dos Santos, T. McDonald, C. E. Brown, X. Wang, L. E. Budde, L. Hoffman, B. Aguilar, W. C. Chang, W. Bretzlaff, B. Chang, M. Jonnalagadda, R. Starr, J. R. Ostberg, M. C. Jensen, R. Bhatia, and S. J. Forman, "T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia," *Blood*, vol. 122, no. 18, pp. 3138–3148, 2013.

[374] S. Gill, S. K. Tasian, M. Ruella, O. Shestova, Y. Li, D. L. Porter, M. Carroll, G. Danet-Desnoyers, J. Scholler, S. A. Grupp, C. H. June, and M. Kalos, "Preclinical targeting of human acute myeloid leukemia and myeloablation using chimeric antigen receptor-modified T cells," *Blood*, vol. 123, no. 15, pp. 2343–2354, 2014.

[375] J. A. Westwood, M. J. Smyth, M. W. L. Teng, M. Moeller, J. A. Trapani, A. M. Scott, F. E. Smyth, G. A. Cartwright, B. E. Power, D. Honemann, H. M. Prince, P. K. Darcy, and M. H. Kershaw, "Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice," *Proc. Natl. Acad. Sci.*, vol. 102, no. 52, pp. 19051–19056, 2005.

[376] H. Tashiro, T. Sauer, T. Shum, K. Parikh, M. Mamonkin, B. Omer, R. H. Rouce, P. Lulla, C. M. Rooney, S. Gottschalk, and M. K. Brenner, "Treatment of Acute Myeloid Leukemia with T Cells Expressing Chimeric Antigen Receptors Directed to C-type Lectin-like Molecule 1," *Mol. Ther.*, vol. 25, no. 9, pp. 2202–2213, 2017.

[377] M. Casucci, B. N. Di Robilant, L. Falcone, B. Camisa, M. Norelli, P. Genovese, B. Gentner, F. Gullotta, M. Ponzoni, M. Bernardi, M. Marcatti, A. Saudemont, C. Bordignon, B. Savoldo, F. Ciceri, L. Naldini, G. Dotti, C. Bonini, and A. Bondanza, "CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma," *Blood*, vol. 122, no. 20, pp. 3461–3472, 2013.

[378] R. C. Lynn, M. Poussin, A. Kalota, Y. Feng, P. S. Low, D. S. Dimitrov, and D. J. Powell, "Targeting of folate receptor β on acute myeloid leukemia blasts with chimeric antigen receptor-expressing T cells," *Blood*, vol. 125, no. 22, pp. 3466–3476, 2015.

[379] S. Gill, "Chimeric antigen receptor T cell therapy in AML: How close are we?," *Best Pract. Res. Clin. Haematol.*, vol. 29, no. 4, pp. 329–333, 2016.

[380] K. Kita, H. Miwa, K. Nakase, K. Kawakami, T. Kobayashi, S. Shirakawa, I. Tanaka, C. Ohta, H. Tsutani, S. Oguma, T. Kyo, H. Dohy, N. Kamada, K. Nasu, and H. Uchino, "Clinical importance of CD7 expression in acute myelocytic leukemia," *Blood*, vol. 81, no. 9, pp. 2399–2405, 1993.

[381] G. Del Poeta, R. Stasi, A. Venditti, C. Cox, G. Aronica, M. Masi, A. Bruno, M. D. Simone, F. Buccisano, and G. Papa, "CD7 expression in acute myeloid leukemia," *Leuk. Lymphoma*, vol. 17, no. 1–2, pp. 111–119, 1995.

[382] A. Saxena, D. P. Sheridan, R. T. Card, A. M. McPeek, C. C. Mewdell, and L. F. Skinnider, "Biologic and clinical significance of CD7 expression in acute myeloid leukemia.," *Am. J. Hematol.*, vol. 58, no. 4, pp. 278–84, 1998.

[383] H. Chang, J. Yeung, J. Brandwein, and Q. long Yi, "CD7 expression predicts poor disease free survival and post-remission survival in patients with acute myeloid leukemia and normal karyotype," *Leuk. Res.*, vol. 31, no. 2, pp. 157–162, 2007.

[384] K. Ogata, N. Yokose, Y. Shioi, Y. Ishida, J. Tomiyama, H. Hamaguchi, F. Yagasaki, M. Bessyo, H. Sakamaki, K. Dan, and S. Kuriya, "Reappraisal of the clinical significance of CD7 expression in association with cytogenetics in de novo acute myeloid leukaemia," *Br. J. Haematol.*, vol. 115, no. 3, pp. 612–615, 2001.

[385] H. Chang, F. Salma, Q. Yi, B. Patterson, B. Brien, and M. D. Minden, "Prognostic relevance of immunophenotyping in 379 patients with acute myeloid leukemia.," *Leuk. Res.*, vol. 28, no. 1, pp. 43–8, 2004.

[386] Q. L. Hao, J. Zhu, M. A. Price, K. J. Payne, L. W. Barsky, and G. M. Crooks,

"Identification of a novel, human multilymphoid progenitor in cord blood," *Blood*, vol. 97, no. 12, pp. 3683–3690, 2001.

[387] A. Galy, M. Travis, D. Cen, and B. Chen, "Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset," *Immunity*, vol. 3, no. 4, pp. 459–473, 1995.

[388] S. Doulatov, F. Notta, K. Eppert, L. T. Nguyen, P. S. Ohashi, and J. E. Dick, "Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development," *Nat. Immunol.*, vol. 11, no. 7, pp. 585–593, 2010.

[389] M. L. Cooper, J. Choi, K. Staser, J. K. Ritchey, J. M. Devenport, K. Eckardt, M. P. Rettig, B. Wang, L. G. Eissenberg, A. Ghobadi, L. N. Gehrs, J. L. Prior, S. Achilefu, C. A. Miller, C. C. Fronick, J. O'Neal, F. Gao, D. M. Weinstock, A. Gutierrez, R. S. Fulton, and J. F. DiPersio, "An 'off-the-shelf' fratricide-resistant CAR-T for the treatment of T cell hematologic malignancies," *Leukemia*, pp. 1–14, 2018.

[390] C. Satoh, H. Tamura, T. Yamashita, T. Tsuji, K. Dan, and K. Ogata, "Aggressive characteristics of myeloblasts expressing CD7 in myelodysplastic syndromes," *Leuk. Res.*, vol. 33, no. 2, pp. 326–331, 2009.

[391] K. Ogata, K. Nakamura, N. Yokose, H. Tamura, M. Tachibana, O. Taniguchi, R. Iwakiri, T. Hayashi, H. Sakamaki, Y. Murai, K. Tohyama, S. Tomoyasu, Y. Nonaka, M. Mori, K. Dan, and Y. Yoshida, "Clinical significance of phenotypic features of blasts in patients with myelodysplastic syndrome," *Blood*, vol. 100, no. 12, pp. 3887–3896, 2002.

[392] F. Lo Coco, G. De Rossi, D. Pasqualetti, M. Lopez, D. Diverio, R. Latagliata, S. Fenu, and F. Mandelli, "CD7 positive acute myeloid leukaemia: a subtype associated with cell immaturity.," *Br. J. Haematol.*, vol. 73, no. 4, pp. 480–5, 1989.

[393] F. Ishikawa, S. Yoshida, Y. Saito, A. Hijikata, H. Kitamura, S. Tanaka, R. Nakamura, T. Tanaka, H. Tomiyama, N. Saito, M. Fukata, T. Miyamoto, B. Lyons, K. Ohshima, N. Uchida, S. Taniguchi, O. Ohara, K. Akashi, M. Harada, and L. D. Shultz, "Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region," *Nat. Biotechnol.*, vol. 25, no. 11, pp. 1315–1321, 2007.

[394] N. Goardon, E. Marchi, A. Atzberger, L. Quek, A. Schuh, S. Soneji, P. Woll, A. Mead, K. A. Alford, R. Rout, S. Chaudhury, A. Gilkes, S. Knapper, K. Beldjord, S. Begum, S. Rose, N. Geddes, M. Griffiths, G. Standen, A. Sternberg, J. Cavenagh, H. Hunter, D. Bowen, S. Killick, L. Robinson, A. Price, E. Macintyre, P. Virgo, A. Burnett, C. Craddock, T. Enver, S. E. W. Jacobsen, C. Porcher, and P. Vyas, "Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia," *Cancer Cell*, vol. 19, no. 1, pp. 138–152, 2011.

[395] E. M. Six, D. Bonhomme, M. Monteiro, K. Beldjord, M. Jurkowska, C. Cordier-Garcia, A. Garrigue, L. Dal Cortivo, B. Rocha, A. Fischer, M. Cavazzana-Calvo, and I. André-Schmutz, "A human postnatal lymphoid progenitor capable of circulating and seeding the thymus," *J. Exp. Med.*, vol. 204, no. 13, pp. 3085–3093, 2007.

[396] L. A. Kohn, Q. L. Hao, R. Sasidharan, C. Parekh, S. Ge, Y. Zhu, H. K. A. Mikkola, and G. M. Crooks, "Lymphoid priming in human bone marrow begins before expression of CD10 with upregulation of L-selectin," *Nat. Immunol.*, vol. 13, no. 10, pp. 963–971, 2012.

[397] G. E. Tjønnfjord, O. P. Veiby, R. Steen, and T. Egeland, "T lymphocyte differentiation in vitro from adult human prethymic CD34+ bone marrow cells.," *J. Exp. Med.*, vol. 177, no. 6, pp. 1531–9, 1993.

[398] B. F. Haynes, D. L. Mann, M. E. Hemler, J. A. Schroer, J. H. Shelhamer, G. S. Eisenbarth, J. L. Strominger, C. A. Thomas, H. S. Mostowski, and A. S. Fauci, "Characterization of a monoclonal antibody that defines an immunoregulatory T cell subset for immunoglobulin synthesis in humans.," *Proc. Natl. Acad. Sci. U. S. A.*, vol.

77, no. 5, pp. 2914–2918, 1980.

[399] P. Hanley, A. Leen, A. P. Gee, K. Leung, C. Martinez, R. A. Krance, H. Liu, M. K. Brenner, H. E. Heslop, C. M. Rooney, and C. M. Bollard, “Multi-Virus-Specific T-Cell Therapy For Patients After Hematopoietic Stem Cell and Cord Blood Transplantation,” *Blood*, vol. 122, no. 21, p. 140, 2013.

[400] A. M. Leen, G. D. Myers, U. Sili, M. H. Huls, H. Weiss, K. S. Leung, G. Carrum, R. A. Krance, C. C. Chang, J. J. Molldrem, A. P. Gee, M. K. Brenner, H. E. Heslop, C. M. Rooney, and C. M. Bollard, “Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals,” *Nat. Med.*, vol. 12, no. 10, pp. 1160–1166, 2006.

[401] J. N. Kochenderfer, M. E. Dudley, S. H. Kassim, R. P. T. Somerville, R. O. Carpenter, S. S. Maryalice, J. C. Yang, G. Q. Phan, M. S. Hughes, R. M. Sherry, M. Raffeld, S. Feldman, L. Lu, Y. F. Li, L. T. Ngo, A. Goy, T. Feldman, D. E. Spaner, M. L. Wang, C. C. Chen, S. M. Kranick, A. Nath, D. A. N. Nathan, K. E. Morton, M. A. Toomey, and S. A. Rosenberg, “Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor,” *J. Clin. Oncol.*, vol. 33, no. 6, pp. 540–549, 2015.

[402] S. S. Neelapu, F. L. Locke, N. L. Bartlett, L. J. Lekakis, D. B. Miklos, C. A. Jacobson, I. Braunschweig, O. O. Oluwole, T. Siddiqi, Y. Lin, J. M. Timmerman, P. J. Stiff, J. W. Friedberg, I. W. Flinn, A. Goy, B. T. Hill, M. R. Smith, A. Deol, U. Farooq, P. McSweeney, J. Munoz, I. Avivi, J. E. Castro, J. R. Westin, J. C. Chavez, A. Ghobadi, K. V. Komanduri, R. Levy, E. D. Jacobsen, T. E. Witzig, P. Reagan, A. Bot, J. Rossi, L. Navale, Y. Jiang, J. Aycock, M. Elias, D. Chang, J. Wiezorek, and W. Y. Go, “Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma,” *N. Engl. J. Med.*, p. NEJMoa1707447, 2017.

[403] N. Ali, B. Flutter, R. Sanchez Rodriguez, E. Sharif-Paghaleh, L. D. Barber, G. Lombardi, and F. O. Nestle, “Xenogeneic Graft-versus-Host-Disease in NOD-scid IL-2Rγnull Mice Display a T-Effector Memory Phenotype,” *PLoS One*, vol. 7, no. 8, 2012.

[404] M. A. King, L. Covassin, M. A. Brehm, W. Racki, T. Pearson, J. Leif, J. Laning, W. Fodor, O. Foreman, L. Burzenski, T. H. Chase, B. Gott, A. A. Rossini, R. Bortell, L. D. Shultz, and D. L. Greiner, “Human peripheral blood leucocyte non-obese diabetic-severe combined immunodeficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex,” *Clin. Exp. Immunol.*, vol. 157, no. 1, pp. 104–118, 2009.

[405] J. N. Brudno, R. P. T. Somerville, V. Shi, J. J. Rose, D. C. Halverson, D. H. Fowler, J. C. Gea-Banacloche, S. Z. Pavletic, D. D. Hickstein, T. L. Lu, S. A. Feldman, A. T. Iwamoto, R. Kurlander, I. Maric, A. Goy, B. G. Hansen, J. S. Wilder, B. Blacklock-Schuver, F. T. Hakim, S. A. Rosenberg, R. E. Gress, and J. N. Kochenderfer, “Allogeneic T cells that express an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem-cell transplantation without causing graft-versus-host disease,” *J. Clin. Oncol.*, vol. 34, no. 10, pp. 1112–1121, 2016.

[406] A. Ghosh, M. Smith, S. E. James, M. L. Davila, E. Velardi, K. V. Argyropoulos, G. Gunset, F. Perna, F. M. Kreines, E. R. Levy, S. Lieberman, H. V. Jay, A. Z. Tuckett, J. L. Zakrzewski, L. Tan, L. F. Young, K. Takvorian, J. A. Dudakov, R. R. Jenq, A. M. Hanash, A. C. F. Motta, G. F. Murphy, C. Liu, A. Schietinger, M. Sadelain, and M. R. M. Van Den Brink, “Donor CD19 CAR T cells exert potent graft-versus-lymphoma activity with diminished graft-versus-host activity,” *Nat. Med.*, vol. 23, no. 2, pp. 242–249, 2017.

[407] P. Klenerman and A. Oxenius, “T cell responses to cytomegalovirus,” *Nature Reviews Immunology*, vol. 16, no. 6. pp. 367–377, 2016.

[408] M. J. Frigault, J. Lee, M. C. Basil, C. Carpenito, S. Motohashi, J. Scholler, O. U. Kawalekar, S. Guedan, S. E. McGettigan, A. D. Posey, S. Ang, L. J. N. Cooper, J. M. Platt, F. B. Johnson, C. M. Paulos, Y. Zhao, M. Kalos, M. C. Milone, and C. H. June, "Identification of Chimeric Antigen Receptors That Mediate Constitutive or Inducible Proliferation of T Cells.," *Cancer Immunol. Res.*, vol. 3, no. 4, pp. 356–367, 2015.

[409] O. U. Kawalekar, R. S. O'Connor, J. A. Fraietta, L. Guo, S. E. McGettigan, A. D. Posey, P. R. Patel, S. Guedan, J. Scholler, B. Keith, N. Snyder, I. Blair, M. C. Milone, and C. H. June, "Distinct Signaling of Coreceptors Regulates Specific Metabolism Pathways and Impacts Memory Development in CAR T Cells," *Immunity*, vol. 44, no. 2, pp. 380–390, 2016.

[410] I. K. Jang, Z. H. Lee, Y. J. Kim, S. H. Kim, and B. S. Kwon, "Human 4-1BB (CD137) signals are mediated by TRAF2 and activate nuclear factor-κB," *Biochem. Biophys. Res. Commun.*, vol. 242, no. 3, pp. 613–620, 1998.

[411] A. Roulston, R. Lin, P. Beauparlant, M. A. Wainberg, and J. Hiscott, "Regulation of human immunodeficiency virus type 1 and cytokine gene expression in myeloid cells by NF-kappa B/Rel transcription factors.," *Microbiol. Rev.*, vol. 59, no. 3, pp. 481–505, 1995.

[412] J. Hiscott, H. Kwon, and P. Génin, "Hostile takeovers: Viral appropriation of the NF-κB pathway," *Journal of Clinical Investigation*, vol. 107, no. 2. pp. 143–151, 2001.

[413] G. Herbein and K. A. Khan, "Is HIV infection a TNF receptor signalling-driven disease?," *Trends Immunol.*, vol. 29, no. 2, pp. 61–67, 2008.

[414] J. L. Pomerantz and D. Baltimore, "NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase.," *EMBO J.*, vol. 18, no. 23, pp. 6694–704, 1999.

[415] M. Muzio, A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit, "FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex," *Cell*, vol. 85, no. 6, pp. 817–827, 1996.

[416] A. Kunkele, A. J. Johnson, L. S. Rolczynski, C. A. Chang, V. Hoglund, K. S. Kelly-Spratt, and M. C. Jensen, "Functional Tuning of CARs Reveals Signaling Threshold above Which CD8+ CTL Antitumor Potency Is Attenuated due to Cell Fas-FasL-Dependent AICD," *Cancer Immunol. Res.*, vol. 3, no. 4, pp. 368–379, 2015.

[417] D. R. Green, N. Droin, and M. Pinkoski, "Activation-induced cell death in T cells," *Immunological Reviews*, vol. 193. pp. 70–81, 2003.

[418] M. R. Alderson, T. W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K. A. Schooley, R. G. Goodwin, C. A. Smith, F. Ramsdell, and D. H. Lynch, "Fas ligand mediates activation-induced cell death in human T lymphocytes.," *J. Exp. Med.*, vol. 181, no. 1, pp. 71–7, 1995.

[419] N. P. Singh, M. Nagarkatti, and P. S. Nagarkatti, "Role of dioxin response element and nuclear factor-kappaB motifs in 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated regulation of Fas and Fas ligand expression," *Mol. Pharmacol.*, vol. 71, no. 1, pp. 145–157, 2007.

[420] B. Lin, C. Williams-Skipp, Y. Tao, M. S. Schleicher, L. L. Cano, R. C. Duke, and R. I. Scheinman, "NF-κB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type," *Cell Death Differ.*, vol. 6, no. 6, pp. 570–582, 1999.

[421] H. Chan, D. P. Bartos, and L. B. Owen-Schaub, "Activation-dependent transcriptional regulation of the human Fas promoter requires NF-κB p50-p65 recruitment," *Mol Cell Biol*, vol. 19, no. 3, pp. 2098–2108, 1999.

[422] K. A. Roebuck and A. Finnegan, "Regulation of intercellular adhesion molecule-1 (CD54) gene expression.," *J. Leukoc. Biol.*, vol. 66, no. 6, pp. 876–88, 1999.

[423] P. Melotti, E. Nicolis, A. Tamanini, R. Rolfini, A. Pavirani, and G. Cabrini, “Activation of NF-kB mediates ICAM-1 induction in respiratory cells exposed to an adenovirus-derived vector,” *Gene Ther.*, vol. 8, no. 18, pp. 1436–1442, 2001.

[424] N. A. Zumwalde, E. Domae, M. F. Mescher, and Y. Shimizu, “ICAM-1-Dependent Homotypic Aggregates Regulate CD8 T Cell Effector Function and Differentiation during T Cell Activation,” *J. Immunol.*, vol. 191, no. 7, pp. 3681–3693, 2013.

[425] J. A. Hernandez-Chacon, Y. Li, R. C. Wu, C. Bernatchez, Y. Wang, J. S. Weber, P. Hwu, and L. G. Radvanyi, “Costimulation through the CD137/4-1BB pathway protects human melanoma tumor-infiltrating lymphocytes from activation-induced cell death and enhances antitumor effector function,” *J. Immunother.*, vol. 34, no. 3, pp. 236–250, 2011.

[426] T. M. Baetu, H. Kwon, S. Sharma, N. Grandvaux, and J. Hiscott, “Disruption of NF-kappaB signaling reveals a novel role for NF-kappaB in the regulation of TNF-related apoptosis-inducing ligand expression.,” *J. Immunol.*, vol. 167, no. 6, pp. 3164–3173, 2001.

[427] M. J. Sánchez, M. O. Muench, M. G. Roncarolo, L. L. Lanier, and J. H. Phillips, “Identification of a common T/natural killer cell progenitor in human fetal thymus.,” *J. Exp. Med.*, vol. 180, no. 2, pp. 569–576, 1994.

[428] E. Liu, Y. Tong, G. Dotti, H. Shaim, B. Savoldo, M. Mukherjee, J. Orange, X. Wan, X. Lu, A. Reynolds, M. Gagea, P. Banerjee, R. Cai, M. H. Bdaiwi, R. Basar, M. Muftuoglu, L. Li, D. Marin, W. Wierda, M. Keating, R. Champlin, E. Shpall, and K. Rezvani, “Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity,” *Leukemia*, p. doi:10.1038/leu.2017.226, Aug. 2017.

[429] L. Yang, “Is CAR-T Cell Immunotherapy a Good Strategy for T-Cell Acute Lymphoblastic Leukemia?,” *Clin. Oncol.*, vol. 1, no. 1169, pp. 1–2, 2016.

[430] P. K. Mandal, L. M. R. Ferreira, R. Collins, T. B. Meissner, C. L. Boutwell, M. Friesen, V. Vrbanac, B. S. Garrison, A. Stortchevoi, D. Bryder, K. Musunuru, H. Brand, A. M. Tager, T. M. Allen, M. E. Talkowski, D. J. Rossi, and C. A. Cowan, “Efficient Ablation of Genes in Human Hematopoietic Stem and Effector Cells using CRISPR/Cas9,” *Cell Stem Cell*, vol. 15, no. 5, pp. 643–652, Nov. 2014.

[431] M. Mamonkin, M. Mukherjee, M. Srinivasan, S. Sharma, D. Gomes-Silva, F. Mo, G. Krenciute, J. S. Orange, and M. K. Brenner, “Reversible Transgene Expression Reduces Fratricide and Permits 4-1BB Costimulation of CAR T Cells Directed to T-cell Malignancies,” *Cancer Immunol. Res.*, vol. 6, no. 1, pp. 47–58, 2018.

[432] L. C. Fleischer, S. S. Raikar, R. Moot, K. A. Knight, C. B. Doering, and H. T. Spencer, “Engineering CD5-Targeted Chimeric Antigen Receptors and Edited T Cells for the Treatment of T-Cell Leukemia,” *Blood*, vol. 130, no. Suppl 1, 2017.

[433] Z. Zhu, B. Ma, R. J. Homer, T. Zheng, and J. A. Elias, “Use of the Tetracycline-controlled Transcriptional Silencer (tTS) to Eliminate Transgene Leak in Inducible Overexpression Transgenic Mice,” *J. Biol. Chem.*, vol. 276, no. 27, pp. 25222–25229, 2001.

[434] S. F. Lacey, J. Xu, M. Ruella, D. M. Barrett, I. Kulikovskaya, D. E. Ambrose, P. R. Patel, T. Reich, J. Scholler, F. Nazimuddin, J. A. Fraietta, S. L. Maude, S. I. Gill, B. L. Levine, C. L. Nobles, F. D. Bushman, E. Orlando, S. A. Grupp, C. H. June, and J. J. Melenhorst, “Cars in Leukemia: Relapse with Antigen-Negative Leukemia Originating from a Single B Cell Expressing the Leukemia-Targeting CAR,” *Blood*, vol. 128, no. 22, 2016.

[435] C. R. Y. Cruz, K. P. Micklethwaite, B. Savoldo, C. a Ramos, S. Lam, S. Ku, O. Diouf, E. Liu, a J. Barrett, S. Ito, E. J. Shpall, R. a Krance, R. T. Kamble, G. Carrum, C. M. Hosing, A. P. Gee, Z. Mei, B. J. Grilley, H. E. Heslop, C. M. Rooney, M. K. Brenner, C. M. Bollard, and G. Dotti, “Infusion of donor-derived CD19-redirected-virus-specific

T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase I study.," *Blood*, vol. 122, no. 17, pp. 2965–2973, 2013.