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Car Drivers and Fuel Sources: How Distinct Signaling Domains in Chimeric Antigen Receptors Reprogram T Cells

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Abstract

With breakthroughs in synthetic biology, improved cell culture techniques and advanced genetic engineering, it has now become possible to generate bi-specific primary human T cells with desired specificities. One mode of redirecting specificity is the modification of T cells to express chimeric antigen receptors (CARs). Recent studies indicate that natural T cells have distinct biochemical and metabolic features that endow them with short lived effector or long lived memory fates. The central objective of this thesis was to investigate whether the signaling endodomain of CARs could reprogram T cells with pre-specified effector and memory fates. This thesis describes a novel technique that allows for detailed investigation of the impact of CAR design on the fate of T cells. Specifically, it compares the short-term and long-term signaling effects of CD28 and 4-1BB costimulatory domains in the CAR architecture. These two signaling domains have been most extensively employed in CAR therapy trials against a wide variety of malignancies. Incorporation of 4-1BB signaling domain imparts superior proliferative and survival benefits as compared to the CD28-containing CAR T cells. This increased persistence correlates with clinical observations. 4-1BB CARs T cells show an enrichment of central memory phenotype along with relative increase in fatty acid based metabolism. This is accompanied by a relative increase in mitochondrial mass, upregulation of key metabolic enzymes and increased spare respiratory capacity. Furthermore, stimulation of CD28-containing CARs promotes rapid induction of biochemical signaling events that are associated with T cell activation. Specifically, the phosphorylation of key proximal and distal signaling proteins between the two CAR models have been compared. Inclusion of CD28 domain in the CAR structure dramatically reduces activation threshold and leads to increased and sustained calcium flux. Taken together, this thesis work uncovers some key differences triggered by the different costimulatory domains. This thesis establishes that the choice of CAR signaling domain can be used to dictate the fate of engineered T cells. Moving forward, the ability of CARs to reprogram T cell metabolism and induce differential activation patterns will need to be considered when designing future CAR trials.

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CAR DRIVERS AND FUEL SOURCES: HOW DISTINCT SIGNALING DOMAINS IN CHIMERIC
ANTIGEN RECEPTORS REPROGRAM T CELLS

Omkar Uday Kawalekar

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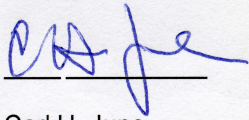
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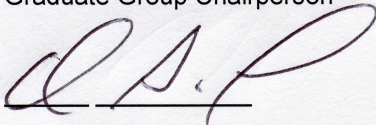
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DEDICATION

To my grandmother, Meenaxi Narayan Kawalekar and my parents Uday N. Kawalekar and Ujwala N. Kawalekar – I dedicate this work to you for the immeasurable and innumerable sacrifices you have made in your lives to get me to where I am today.

Thank you

*One of Khalil Gibran's quotes compared parents to bows,
and children to arrows.*

*The more the bow bent and stretched, the farther the arrow could fly.
I fly, not because I am special, but because they have stretched for me.*

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ABSTRACT

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Omkar Uday Kawalekar

Carl H. June

With breakthroughs in synthetic biology, improved cell culture techniques and advanced genetic engineering, it has now become possible to generate bi-specific primary human T cells with desired specificities. One mode of redirecting specificity is the modification of T cells to express chimeric antigen receptors (CARs). Recent studies indicate that natural T cells have distinct biochemical and metabolic features that endow them with short lived effector or long lived memory fates. The central objective of this thesis was to investigate whether the signaling endodomain of CARs could reprogram T cells with pre-specified effector and memory fates. This thesis describes a novel technique that allows for detailed investigation of the impact of CAR design on the fate of T cells. Specifically, it compares the short-term and long-term signaling effects of CD28 and 4-1BB costimulatory domains in the CAR architecture. These two signaling domains have been most extensively employed in CAR therapy trials against a wide variety of malignancies. Incorporation of 4-1BB signaling domain imparts superior proliferative and survival benefits as compared to the CD28-containing CAR T cells. This increased persistence correlates with clinical observations. 4-1BB CARs T cells show an enrichment of central memory phenotype along with relative increase in fatty acid based metabolism. This is

accompanied by a relative increase in mitochondrial mass, upregulation of key metabolic enzymes and increased spare respiratory capacity. Furthermore, stimulation of CD28-containing CARs promotes rapid induction of biochemical signaling events that are associated with T cell activation. Specifically, the phosphorylation of key proximal and distal signaling proteins between the two CAR models have been compared. Inclusion of CD28 domain in the CAR structure dramatically reduces activation threshold and leads to increased and sustained calcium flux. Taken together, this thesis work uncovers some key differences triggered by the different costimulatory domains. This thesis establishes that the choice of CAR signaling domain can be used to dictate the fate of engineered T cells. Moving forward, the ability of CARs to reprogram T cell metabolism and induce differential activation patterns will need to be considered when designing future CAR trials.

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Chapter 1

INTRODUCTION

Cancer and the Immune system

Cancer – a single word that represents over 200 diseases, manifests as an uncontrolled proliferation of cells with the potential to invade, disrupt bodily processes and destroy tissues. Despite its pervasiveness, each form of the disease shares several common characteristics. The complex interplay of six biological capabilities has been described as the logical framework based on which cancer develops (Hanahan and Weinberg, 2000). These include sustained proliferative signaling, escaping growth suppressors, evading cell death, acquisition of replicative immortality, promotion of angiogenesis and metastasis.

The mechanisms of cancer development have not yet been fully understood, but it has been shown that this is a multistep process (Farber, 1984). Genomic instability, a basic hallmark of cancer underlies many of the phenotypic features attributed to almost all cancer cells (Negrini et al., 2010). The most common genetic alteration in cancer - mutation - arises from defects in DNA damage repair systems of the cancer cell. Mutations in the coding regions of genes promoting cell proliferation, “oncogenes” underlie the sustained proliferative rates of cancer cells.

Cancer cells continue to grow, often unrecognized by the immune system; a phenomenon strikingly distinct from the immune response generated against microorganisms. The immune system, including the innate and the adaptive arms, is composed of cells, tissues and organs that monitor the host organism for foreign

molecules or aberrantly growing cells. Researchers have hypothesized that this process of immunosurveillance is only a part of the process that host immune system utilizes to constantly identify developing tumors. A theory termed as immunoediting evolved, which proposed a dynamic process that shapes the immunogenicity of tumors as they develop. Three steps, popularly referred to as the 3 Es of cancer immunoediting were proposed - elimination, equilibrium and escape(Dunn et al., 2004). Elimination refers to the successful rejection of evolving tumors by the host innate and adaptive arms of the immune system. However, if all the tumor cells are not eradicated, they may enter into the equilibrium phase where the tumor cells can enter a prolonged undisturbed dormant state. Ultimately, the tumor cells can escape this immune control and progress uncontrollably, leading to a full-fledged clinical progression of cancer. One form of immunoediting involves modulation of cancer-specific antigens. Cancer cells express normal self-antigens in addition to specific cancer-associated antigens. By rendering their cancer-specific antigens inaccessible or through genetic modifications leading to loss of these antigens, cancer cells evade/escape the cytolytic arm of the immune system, which is mainly comprised of lymphocytes.

Lymphocytes are responsible for the specificity of the adaptive immune system. They play an important role in both natural and therapeutically induced immunoediting. T lymphocytes develop in the thymus and comprise the main cytolytic arm of the immune system to orchestrate cell-mediated responses against foreign insults to the body. However, due to various evasive strategies employed by tumor cells, T cells are often non-responsive to tumor cells(Mapara and Sykes, 2004). In some cases, the natural state

of endogenous tumor-reactive T lymphocytes is characterized by anergy, a phase of unresponsive quiescence. Various other mechanisms such as reduced immune recognition, increased resistance to attack and/or the development of an immunosuppressive tumor microenvironment often mediate tumor escape (Mapara and Sykes, 2004). In this manner, cancer cells induce tolerance. These hurdles coupled with other factors such as the non-conductive tumor microenvironment with limited oxygen availability and nutrient depletion, inhibitory chemical cytokines secreted by the cancer cells, make therapeutic interventions by the components of the immune system increasingly difficult.

Recent work has identified additional features shared by cancer cells. Cross talk between oncogenic signaling pathways and metabolic pathways leads to an increased reliance on conventional and unconventional nutrient sources, specifically glucose and glutamine (Eagle, 1955; Som et al., 1980). Tumorigenesis-associated metabolic adaptations affect metabolic influx and an increased ability to acquire nutrients. These alterations also shape the way the nutrients are allocated to different pathways that contribute to tumorigenic properties. From a therapeutic perspective, such metabolic modifications exert long-ranging effects on the components of the tumor microenvironment. There, thus exists a fierce competition between cells in the tumor microenvironment, as the demand for resources in this niche can be very high (Hanahan and Coussens, 2012). Taken together, this reciprocal interaction between tumor targets and therapeutic agents has been the focus of thorough investigation in the recent years.

Thus far, the first line of treatment for cancer involves surgical resection, chemotherapy or radiation either alone or in combination. However, there are inherent limitations with these forms of treatment. They may damage healthy surrounding cells and may have little influence on metastasized tumor masses, thereby yielding unsatisfactory results. Another line of treatment has been the application of cancer vaccines. So far, there have been only two preventive cancer vaccines approved by the FDA against HPV that confer protection against cervical cancer in woman (Group, 2007; Paavonen et al., 2009); and one therapeutic vaccine called sipuleucel-T (Higano et al., 2010), for use in men with metastatic prostate cancer. However, many of these approaches can affect normal cells and side effects can limit treatment options. A rapidly increasing knowledge base of the immune system and technology to allow genetic engineering and propagation of immune cells has motivated much interest in the development of anti-cancer immunological strategies. The status of the field and the prospects for clinical translation warranted the need for the development of targeted therapeutic interventions such that the normal tissues are left unharmed. One such advancement in the field of cancer therapeutics has been immunotherapy, which aims to fortify the body's own immune system to target non-self agents.

Cancer Immunotherapy

Until a few decades ago, the possibility that the immune system could mount a response to and eradicate cancer was met with severe skepticism. However, scattered anecdotes and evidence of spontaneous tumor regression have been documented in certain patients before the turn of the last century. In 1891, William Coley administered

an attenuated form of streptococcus as an immune therapy to cancer patients. Similar case studies that followed lent support to the idea that an antitumor immune response showed therapeutic potential in some cancer patients. Some of the most promising evidence for the existence of an immune response against tumors was a series of clinical trials in the late 1980s using the cytokine interleukin IL-2(Atkins et al., 1999; Fyfe et al., 1995; Lotze and Rosenberg, 1986). These trials reported dramatic tumor regression in patients with metastatic melanoma and renal cell carcinoma. Interestingly, IL-2 alone has no cytotoxic capacity, but is a potent activator of an important arm of the immune system - cytotoxic T cells (CTLs). These results later convinced the US FDA to approve IL-2 as the first bona fide immunotherapy treatment for cancer. Since then, the tumoricidal capacity of the cytolytic weapons of the immune system has been widely explored and the field of cancer immunotherapy has undergone a surge of enthusiasm as an approach to fight cancer.

One such strategy to harness and enhance the innate power and specificity of the immune system to target cancer is cancer immunotherapy. It represents a promising cancer treatment strategy since the evolution of the first chemotherapy in the late 1940s(DeVita and Chu, 2008). Cancer immunotherapy broadly comes in two forms – active and passive. Active immunotherapy induces long-lasting tumor antigen-specific responses, which may be preventive or therapeutic. This can be done using vaccinations or materials obtained from biopsies of the tumor(Giarelli, 2007). Such vaccines aim to prime a cancer patient's immune system to control and/or eradicate the disease, much like conventional vaccines. Despite their efficacy in murine models, the clinical benefit of cancer vaccines in cancer patients has been modest, with Sipuleucel-T, a dendritic cell-

based vaccine, and Gardasil and Cervarix against HPV, being the only FDA approved cancer vaccines to date(Kantoff et al., 2010). Additionally, the non-specific stimulation of the immune system involving administration of cytokines such as interleukin(Yang et al., 2003) and interferons can further enhance immune responses against tumors, posing as an advancement of the traditional active form of immunotherapy.

Passive immunotherapy, on the other hand provides a tumor-specific immune response with the aid of effector molecules such as antibodies or effector cells. Antibody based therapy refers to the use of monoclonal antibodies that bind specifically to cancer cells and ultimately lead to the death of cancer cells(Mellstedt, 2003). One method is to block the growth factor molecules with their receptors using mAbs in order to prevent the growth of cancer cells. Another such death mechanism is to block key cancer specific cell receptors. The hypothesis for this treatment is that blocking immunosuppressive receptors, such as CTLA-4 and PD-1, would potentiate an antitumor response. The recently FDA-approved agent, ipilumimab, an antibody that blocks CTLA-4, among many others, exemplifies the success of this approach(Hodi et al., 2010). As acknowledged as the scientific breakthrough of 2014, recent studies highlight the therapeutic promise of checkpoint inhibitors in the particularly challenging solid tumor environment.

More recently, the transfusion of lymphocytes as a therapeutic approach against cancer has come into the limelight. This strategy, called as Adoptive Cell Therapy (ACT) introduces another promising class of immunotherapy-based treatments against cancer

and certain viral infections. Encouraging data regarding clinical efficacy of ACT using T cell lymphocytes has spiked growing interest in this field.

Adoptive T Cell Therapy

The transfusion of T cells, a technique referred to as Adoptive T Cell Therapy involves the infusion of mature T cell subsets aimed to eliminate tumor and prevent regression (Figure 1.1). The basis for this idea stems from a study by Southam and colleagues (Southam et al., 1966). They observed regression in subcutaneous growth of tumor cells in almost half of the 41 patients evaluated, when mixed with the patient's own leukocytes. This provided evidence that lymphocytes potentially had a detrimental effect on the growth of cancer cells. These findings coupled with the extended clonal expansion life span, inherently high specificity and the ability to genetically manipulate their targeting capability laid the groundwork for considering T cells as prime candidates for adoptive immunotherapy.

Researchers took advantage of the plethora of cancer-specific antigens that have already been identified, to improve the targeting power of ACT. In one approach, autologous T cells were isolated from fresh patient biopsy samples and progressively expanded ex vivo to obtain a concentrated culture of antigen-specific T cells, known as tumor-infiltrating lymphocytes (TILs). These TILs were then adoptively transferred into the patient in the hope of selectively homing to and targeting tumor cells. However, the success of this technique has been restricted to melanoma patients (Dreno et al., 2002; Figlin et al., 1999; Rosenberg et al., 1994). MHC down regulation or the loss of tumor specific antigen could be possible reasons behind the limited efficacy. Although recent

studies have shown improved responses to TIL therapy, the challenge of improving tumor targeting of adoptively transferred cells remains unresolved. This could be a major factor leading to the modest clinical benefit observed. Therefore, genetic modification of T cells was proposed to circumvent the issues plaguing anti-tumor efficacy in many cancer settings.

Engineering T cells for gain-of-function as a cancer therapeutic has become an increasingly popular strategy in the field of synthetic biology to overcome the issue of tolerance, tumor targeting and engraftment of adoptively transferred T cells (Ho et al., 2003). The feasibility of this approach has been shown in multiple studies where genetically modified T cells have been shown to persist for years in humans following adoptive transfer (Mitsuyasu et al., 2000; Scholler et al., 2012). Additionally, since most tumors are poorly antigenic and after heavy chemotherapy, T cells with desired specificities may not remain functional, the art of genetic engineering allows one to overcome these limitations. Two major approaches on introducing an antigen-specific receptor have been examined. One approach is to express natural $\alpha\beta$ TCR heterodimers of known specificity and avidity for tumor antigens (Schumacher, 2002). This redirects the T cell to recognize cancer-specific intracellular antigens on tumor cells. However, this technique does not come without its drawbacks. It runs the potential risk of mispairing of transgenic $\alpha\beta$ chains with endogenous TCR chains, thereby revealing novel receptors with unknown specificities. Another major consideration of this approach is the requirement for the antigen to be expressed on the tumor surface in context of the MHC-complex. This is especially concerning because tumors often down-regulate MHC

molecules or surface peptides for evasion from immune surveillance. Among attempts to work around these issues, Eshhar and colleagues performed pioneering work to overcome these hurdles in the late 1980s (Gross et al., 1989). They engineered T cells to express an antibody-based artificial receptor, which is now known as a chimeric antigen receptor (CAR).

Chimeric Antigen Receptors (CARs)

In principle, a CAR is an engineered fusion molecule that combines any ligand-binding domain with T cell signaling protein(s). These can be introduced into a T cell as cDNA to be expressed on the cell surface allowing them to recognize its cognate antigen of interest. This strategy allows redirection of the cells against suitable targets, while its endogenous (TCR mediated) specificity remains intact. These also permit MHC-independent recognition of surface antigens. Although there have been numerous variations of CARs under investigation, the basic design remains fairly consistent consisting of a binding moiety, an extracellular hinge and spacer element, a transmembrane region and the intracellular signaling domains (Figure 1.2).

The binding moiety commonly consists of a single-chain fragment (scFv), comprising the light (V_L) and heavy (V_H) variable fragments of a tumor-associated antigen (TAA)-specific antibody, linked together by a flexible linker. Using a range of different scFvs with varying affinities and specificities to TAAs, CARs against numerous malignancies have been designed and tested (Sadelain et al., 2009). Connected to the scFv is a hinge region that links the scFv to the transmembrane domain embedded in the surface membrane of the T cell. This hinge promotes flexibility and accessibility for the

binding moiety. In its simplest form, this structure is then connected intracellularly to known signaling units of the TCR complex such as CD3 ζ or to Fc-gamma chain domains. These are referred to as the first generation of CARs(Eshhar et al., 1993; Kuwana et al., 1987). Although these receptors could initiate antitumor cytotoxicity, the ability of such CAR-grafted cells to proliferate and persist was suboptimal(Eshhar et al., 1993; Kuwana et al., 1987; Wilkie et al., 2008). Following the two-signal hypothesis for complete activation of T cells, investigators thought to provide a costimulatory signaling domain in cis with the CD3 ζ domains to give rise to the second generation of CARs. Various costimulatory domains including CD28(Finney et al., 1998; Maher et al., 2002), 4-1BB(Carpenito et al., 2009; Imai et al., 2004; Milone et al., 2009), CD27(Song et al., 2012), ICOS(Finney et al., 2004) and OX40(Hombach et al., 2012) have undergone thorough investigation in the preclinical settings. Combining two such costimulatory domains in cis with the CD3 ζ domain led to the development of the third generation of CARs(Carpenito et al., 2009; Tammana et al., 2010; Zhong et al., 2010). Notably, inclusion of costimulatory domains has significantly improved the proliferative capacity, cytokine secretion, cytotoxic potential, and thus the overall effectiveness of CAR therapy in the clinics. Although almost all of these clinical trials have revealed that this strategy is feasible and safe, the outcomes of these trials have been largely distinct. The disparity is likely due to the different CARs developed by each investigatory center, each using different scFv's for the same antigen, different methods of genetically modifying T cells, different culture system and conditioning regimen or different post-therapy interventions. Among all the possible combinations of CAR endo-domains, the second generation of

CARs using either CD28 or 4-1BB signaling domains (hereon referred to as 28 ζ or BB ζ CARs) has undergone the most rigorous clinical investigation. Preclinical murine models using CARs directed against a leukemic cell line (Milone et al., 2009) or a CAR against a solid tumor line (Carpenito et al., 2009) showed the differential induction of tumor regression by 28 ζ and BB ζ CAR T cells. Importantly, there was a striking difference in the levels of persistence of T cells containing the BB ζ CAR, as compared to the 28 ζ CAR. Understanding the mechanistic differences in activation of T cells mediated by these two costimulatory domains continues to be an unaddressed challenge.

CARs in the clinic

Clinical use of redirected T cells with CARs has overcome many of the barriers that tumors employ to evade immune surveillance, including down-regulation of cancer-specific peptides, down-regulation of major histocompatibility complex molecules and immunosuppressive factors that dampen T-cell signaling and function. At the time when this thesis project started, there were only a handful of clinical studies that were published (Table 1.1). These trials used only the first-generation CARs with modest activation, survival and cytolytic activity. To discuss the prospects of improving the effectiveness of CAR therapies, a committee of CAR experts, including principal investigators of several clinical trial centers, project officers from the National Heart, Lung and Blood Institute and the National Cancer Institute convened for a small workshop in May 2010 preceding the annual meeting of the American Society of Gene and Cell Therapy.

Following thorough discussions regarding the elements needed for a CAR trial against B-cell malignancies, these investigators revealed promising clinical data. By 2011, there were several sites in the United States where patients were given infusions with T cells modified to express CARs against CD19 antigen (Table 1.2). CD19 presents itself as an excellent tumor-associated target for immunotherapy, as it is expressed at high levels on essentially all B-lineage cells, including B-cell leukemias and lymphomas. Importantly, CD19 is not expressed on hematopoietic stem cells or on other tissues, thereby significantly lowering the risks of on-target off-tumor toxicities. At the time, only one study performed with infusion of anti-CD19 CAR T cells reported a serious adverse event resulting in the patient death (Brentjens et al., 2010). However, it was concluded that the patient's death was not directly caused by the cellular product, but was likely due to an inflammatory cascade of cytokines resulting from the lymphodepletion regimen that was administered prior to the infusion. The trial was thus reopened.

The question still remained— which CAR design is the best, and is it context-dependent i.e. blood-based versus solid tumor? The various components of the CAR architecture allowed for various permutations of CAR design. The dense grid of variables including choice of the monoclonal antibody from which the scFv was derived, type and length of extracellular hinge and the nature of the transmembrane domain, made the comparison of CAR structures very complicated. Each center's proposal also differed in terms of which T cell population to employ: bulk peripheral blood mononuclear cells (PBMCs), CD8⁺ (CD4-depleted PBMCs), PBMCs depleted of T-regulatory cells, central memory T cells (T_{CM}) or EBV-specific T cells (Berger et al., 2008; Heslop et al., 2010;

June et al., 2009; Micklethwaite et al., 2010). A highly debated factor in determining the CAR design focused on the intracellular cytoplasmic domains, which, as discussed earlier, provide the appropriate signals to activate and define the fate of the infused cellular product. Since different centers, till date, have continued to use different signaling domains, it became imperative to understand how these domains operated at a molecular level. More specifically, the signaling cascades initiated by each domain in context of the CAR remain to be delineated.

Outline of Thesis

This thesis investigates the effects of using two different signaling domains in the CAR structure – 28 ζ and BB ζ , on the activation and survival of primary human T cells. Also described is the development of a novel in vitro technique used to assay the intricate differences in the activation signaling pathways of T cells as well as differences in calcium influx. The potential applications of this technique for ex vivo expansion and manipulation have also been discussed briefly. Most importantly, the effects of these two signaling domains on defining the metabolic fates and survival of CD8⁺ T cells have been investigated in great detail. These studies provide valuable insights to the growing body of knowledge about the influence of CAR signaling domains and how the appropriate choice and incorporation of these domains could be critical to the ultimate clinical success of CAR T therapies.

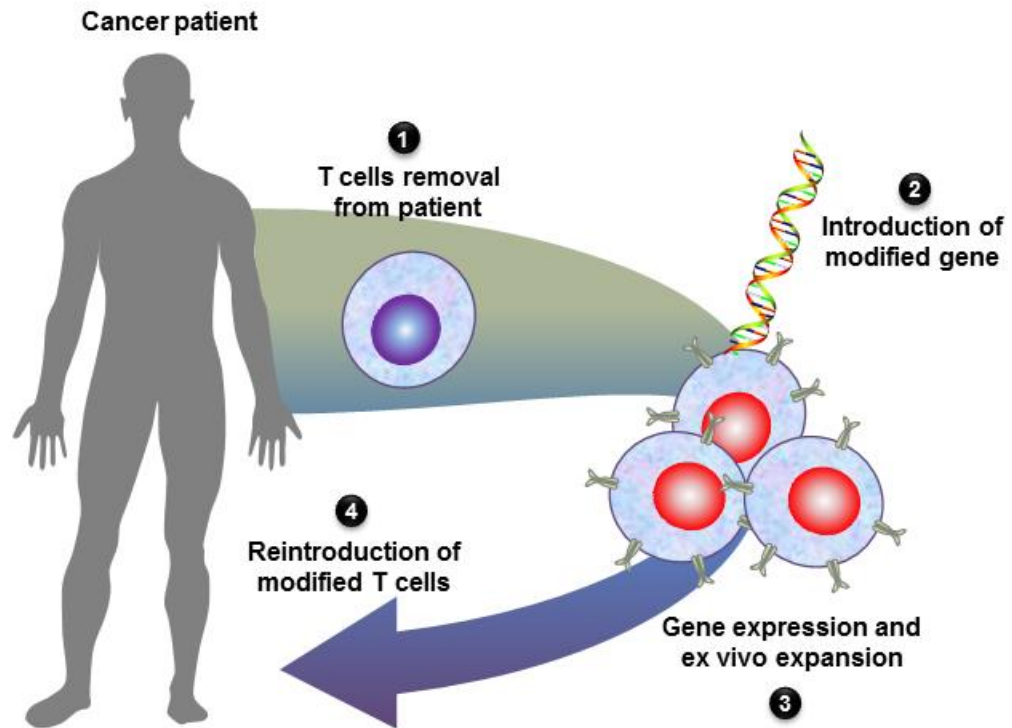


Figure 1.1 Principles of Adoptive T Cell Therapy.

This method of adoptive transfer of modified T cells involves (1) harvesting T cells from the patient by apheresis, followed by (2) activation and genetic modification by transgenesis. (3) The gene-modified cells are expanded with antibodies and/or cytokines and then (4) reinfused into the patient. The premise is that the engineered T cells can now traffic to and target the tumor.

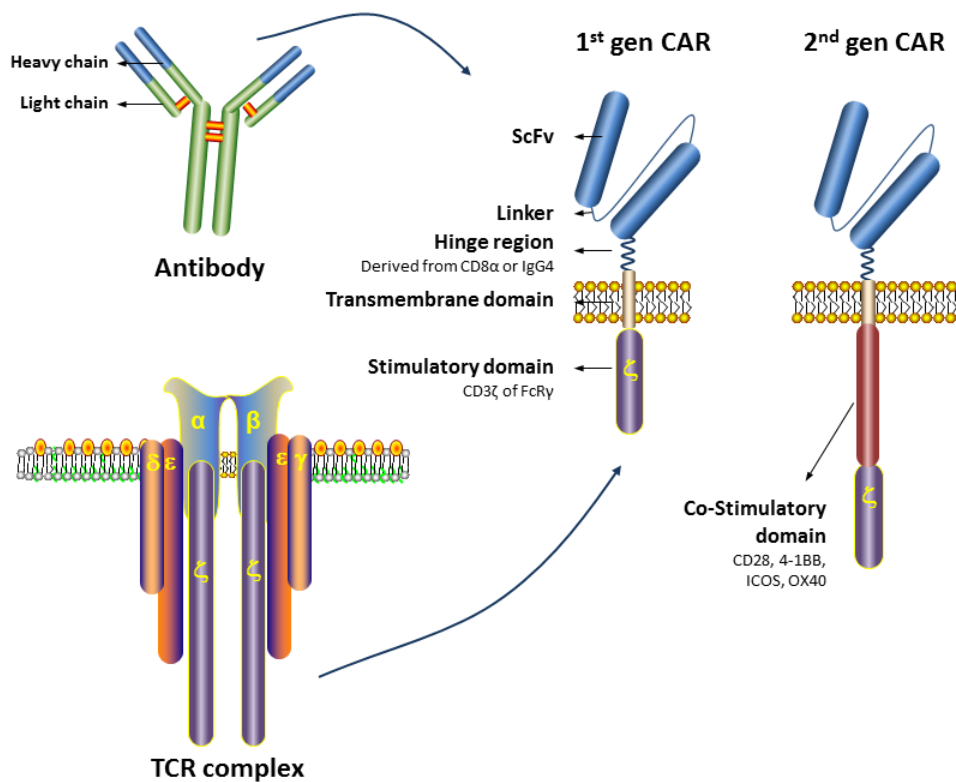


Figure 1.2 Structure of a Chimeric Antigen Receptor.

A CAR is a synthetic transmembrane protein designed to have desired specificity. The specificity is designated by an antigen recognition domain that comprises of the variable regions of the light and heavy chain molecules of an immunoglobulin (scFv). It is linked together to form a single polypeptide chain using a short-chain peptide linker. Through a hinge, the scFv is held up on the cell surface by a transmembrane domain. The latter is stitched known signaling domains on the TCR complex to form the 1st generation CARs. Addition of co-stimulatory domains constitutes a 2nd generation CAR.

Table 1.1 Clinical trials using CARs published until 2010

<i>Target Antigen</i>	<i>Disease</i>	<i>Reference</i>
CD20	Follicular lymphoma	(Wang et al., 2004)
CD20	NHL/mantle cell lymphoma	(Till et al., 2008)
CD171	Neuroblastoma	(Park et al., 2007)
Folate Receptor	Ovarian cancer	(Kershaw et al., 2006)
GD2	Neuroblastoma	(Pule et al., 2008)
gp100	AIDS	(Mitsuyasu et al., 2000)

Table 1.1 Clinical trials published up until December 2010 using CARs for the treatment of specified diseases. All these trials used the first-generation of CARs. (NHL: Non-Hodgkin's Lymphoma; AIDS: Acquired Immunodeficiency Syndrome)

Table 1.2 Clinical trials using CAR-modified T cells against B-cell malignancies until June 2011

<i>Disease</i>	<i>CAR signaling domain</i>	<i>Center</i>	<i>ClinicalTrials.gov NCT number</i>
Relapsed/refractory CLL	4-1BB + CD3 ζ	UPenn	NCT01029366
Relapsed/refractory ALL	4-1BB + CD3 ζ	UPenn	NCT01626495
B NHL and CLL	CD28 + CD3 ζ	BCM	NCT00586391
B NHL and CLL	CD28 + CD3 ζ vs EBV + CD3 ζ	BCM	NCT00608270
B ALL, S/P HSCT	CD28 + CD3 ζ	BCM	NCT00709033
Lymphoma, CLL	CD28 + CD3 ζ	NCI	NCT00924326
B-NHL, S/P autologous HSCT	CD28 + CD3 ζ	MDACC	NCT00968760
Relapsed/refractory F-NHL	CD3 ζ	COH	NCT00182650
CLL-refractory	CD28 + CD3 ζ	MSKCC	NCT00466531
B ALL-relapsed	CD28 + CD3 ζ	MSKCC	NCT01044069

Table 1.2 Clinical trials using CD19-targeted CARs directed against specified B cell malignancies. (CLL: Chronic lymphocytic leukemia; ALL: Acute lymphoblastic leukemia; B NHL: B lineage Non-Hodgkin’s Lymphoma; S/P HSCT: status post-hematopoietic stem cell transplant; NHL: Non-Hodgkin’s Lymphoma; UPenn: The University of Pennsylvania; BCM: Baylor College of Medicine; NCI: National Cancer Institute; MDACC: MD Anderson Cancer Center; COH: The City of Hope National Medical Center; MSKCC: Memorial Sloan-Kettering Cancer Center)

Chapter 2

DEVELOPMENT OF AN IN VITRO MODEL TO INVESTIGATE CAR-MEDIATED SIGNALING IN T CELLS

Summary

The recognition bestowed upon T lymphocytes as key mediators of cellular immunity has been further attested by recent successful clinical studies using genetically modified T cells. With an ever-growing interest in the application of T cells to treat human malignancies, studying the molecular mechanisms of T cell activation, signaling and function has become imperative. This, therefore, calls for the development of new easy-to-use and accurate models to investigate the biological phenomena that begins at the synaptic levels of T cell and antigen interactions to the ultimate exhaustion and death of the T cell. Here, we describe an approach to transiently express a chimeric molecule on the cell surface that permits activation and expansion of T cells, thereby providing a model to study T cells signaling.

Introduction

Deciphering the detailed cascade of events that initiate the earliest biochemical events ultimately leading to T cell activation has come a long way in the past few decades(Norcross, 1984; Smith-Garvin et al., 2009). More recently, the advent of synthetic biology has enabled easy genetic manipulation of T cells permitting the expression of chimeric molecules to enhance functions of T cells. One such method of genetic engineering is the engraftment of chimeric antigen receptors (CARs) on the T cell surface. CARs are synthetic molecules that contain a single chain variable fragment

(scFv) obtained from the variable chains of a monoclonal antibody with desired specificity, which is fused to intracellular domains that provide T-cell activation and costimulatory signals(Gross et al., 1989). CARs allow T cells to recognize pre-determined targets, which would otherwise escape immune recognition, thereby making this technology an attractive tool in the combating various cancers and infections(June and Levine, 2015). Despite extensive clinical investigation of genetically engineered T cells, the intricate signaling mechanisms that occur downstream of such chimeric molecules is highly understudied. One major obstacle that has limited this research is the lack of an accurate model to study the antigen-receptor synapse as well as signaling downstream of it.

Current in vitro models for activation and expansion to study T cell signaling and function pose several limitations. Until about a decade ago, mitogenic lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) were being used for stimulation and expansion of polyclonal T cell population(Kay, 1991). These mitogenic molecules bind to glycoproteins on the cell surface. Another such stimulant is phorbol 12-myristate 13-acetate (PMA), which activates T cells by direct stimulation of protein kinase C(Kay, 1991). To achieve T cell receptor (TCR) complex-specific stimulation, antibodies specific to such molecules, including CD2, CD3, CD28 and CD45 have being used. These antibodies provide the required co-stimulatory signal to trigger complete activation and proliferation of T cells in culture(Frauwirth and Thompson, 2002). The field has since progressed to immobilizing these antibodies to accessory cells, beads or a solid surface for robust expansion of T lymphocytes(Trickett and Kwan, 2003).

Requirement of a functional TCR, reliance on commercial vendors for production, procurement and application of TCR-antagonizing antibodies and the additional costs of acquiring two different antibodies (primary and secondary stimulants) for complete T cell activation, all contribute to the myriad of limitations and drawback of these methods. Prolonged stimulation of with such antibodies could provide “excess” activation signal, which, in naïve T cells for example, has been shown to be detrimental (Collette et al., 1998; Noel et al., 2001). There is thus, a clear need for an improved model to study T cell signaling.

Described below is refined method that can be employed for the activation and/or expansion of immune cells. Briefly, the technique involves transient expression of a CAR molecule of the T cell surface and subsequent activation via a ligand specific to the CAR molecule. The transient mode of gene delivery allows CAR expression on over 95% of the cells, thereby allowing activation of almost the entire cell population.

Materials

Production of in vitro transcribed (IVT) mRNA

1. The IVT mRNA encoding the CAR can be manufactured using a polymerase chain reaction (PCR)-generated template. This template is the DNA sequence of the CAR of interest obtained from any appropriate source such as plasmid DNA, cDNA, or synthetic DNA sequence.
2. The template must contain appropriate promoters and a corresponding RNA polymerase. For example, to use the T7 mScript™ RNA system (Catalog no. C-MS11610, Cellscript, WI, USA) requires the T7 bacteriophage promoter

(TAATACGACTCACTATAG) upstream of the double-stranded DNA template. Other RNA production kits using different promoter systems, such as SP6 and T3 are also available and can be used for synthesis of mRNA to be used for this protocol.

3. Follow the manufacturer's instruction for mRNA production. Purify IVT mRNA products using an RNeasy Mini Kit (Qiagen Inc, Valencia, CA, USA) as per manufacturer's specifications. Elute purified mRNA in sterile RNase-free water at a concentration of 1mg/ml. Prepare aliquots of 10 μ l each in RNAase-free tubes and store at -80°C until further use.

Components for electroporation

1. ECM830 Electro Square Wave Porator (Harvard Apparatus BTX, MA, USA)
2. 2mm cuvette (Catalog no. 1652086, Biorad, Hercules, CA, USA)

Components for cell culture and CAR expression analysis

1. Opti-MEM I: Reduced serum medium (Catalog no. 31985, Gibco, Grand Island, NY, USA)
2. R10: RPMI 1640 medium (Catalog no. 11875, Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum.
3. One T25 culture flask for every 1x10⁷ cells electroporated.
4. FACS tubes: 5ml round-bottom tubes for flow cytometry analysis.
5. FACS buffer: Phosphate buffered saline with 1% fetal calf serum.
6. CAR detection antibodies: Biotin-labeled polyclonal anti-mouse F(ab)₂ (Catalog no. 115-006-072, Jackson ImmunoResearch, PA, USA) or any other antibody that

would detect the CAR scFv. If it is not pre-conjugated with a flurochrome, then use phycoerythrin-labeled streptavidin (Catalog no. 554061, BD pharmingen, CA, USA)

Antigens and coating of stimulation beads

1. Antigen: Purified antigenic protein or an anti-idiotypic specific to the scFv of the CAR. This cognate antigenic molecule should specifically bind to and stimulate the distinctive sequence of the scFv.
2. Stimulation beads: Cognate antigen needs to be coupled with magnetic tosylactivated beads, such as Dynabeads. M-450 (Catalog no. 14103, Life Technologies, Grand Island, NY, USA). The coupling procedure needs to be followed as per the manufacturer's instructions. Briefly, the coupling is performed overnight by co-incubation of the antigenic molecule with the Dynabeads at a high pH (8.5-9.5) and at 37°C. The coated beads should be stored at 4°C at a desired concentration in the bead-storage buffer as specified in the manufacturer's protocol. Suggested concentration for long-term bead storage is 3×10^7 beads/ml.

Methods

Carry out all procedures at room temperature and in sterile conditions unless otherwise specified.

Electroporation of mRNA into T cells

1. Obtain live T cells from any source (human peripheral blood, human umbilical cord blood, etc) and count cells while ensuring good cell viability.

2. Centrifuge cells at 300xg for 5 minutes at 4°C. Carefully discard supernatant and resuspend cell pellet in fresh Opti-MEM media.
3. Centrifuge again and repeat wash steps for a total of three washes.
4. Count and resuspend cells in fresh Opti-MEM media at 1×10^8 cells/ml. For each electroporation, aliquot 1×10^7 cells in a 100 μ l of Opti-MEM. Keep cells on ice until use.
5. Pre-configure the electroporator by setting the voltage to 500V and time to 1000 μ -seconds. Prewarm R10 to 37°C and add 10ml of the media to a T25 flask.
6. In a separate tube, combine 10 μ g of RNA (stock concentration of 1mg/ml) with the 100 μ l aliquot of cells. Uniformly mix by gentle pipetting. Immediately empty the entire content into a 2mm cuvette. (See notes for manipulation of RNA quantity to modulate CAR expression levels).
7. Place the cuvette into the electroporator cassette, tighten the electrodes around the metal plates of the cuvette and initiate the electric pulse.
8. Immediately transfer the contents of the cuvette into the T25 flask containing R10. Rinse the cuvette once with fresh R10 to maximize recovery of electroporated cells.
9. Place the cells in a 37°C CO₂ incubator until further use.

Surface detection of CAR on electroporated T cells

1. Allow cells to rest for at least 3-4 hours before analyzing surface expression.
2. Count and collect an aliquot of about 150,000 cells in a FACS tube in a total of 3ml. Add additional FACS buffer if needed.

3. Centrifuge cells at 300xg for 5 minutes at 4°C, discard supernatant and carefully resuspend cell pellet in 3ml FACS buffer. Centrifuge the tube again and repeat this wash step one more time with fresh FACS buffer.
4. Resuspend cell pellet in 10µg of primary antibody diluted in a total of 100µl FACS buffer. Incubate on ice for 45 minutes.
5. After the incubation period, add 3ml FACS buffer and centrifuge the tube to wash off unbound antibody. Repeat this wash one more time with fresh FACS buffer.
6. If the primary antibody was pre-conjugated to a flurophore, skip to step 8. If using a non-conjugated primary antibody, resuspend cell pellet in 1µg of secondary antibody diluted in a total of 100µl FACS buffer and incubate on ice for 15 minutes.
7. Following the incubation, repeat washes twice as performed earlier.
8. Finally resuspend the cells in a desired volume and analyze samples on a flow cytometer to check surface expression of CARs (Figure 2.1).

CAR T cell stimulation

1. After verifying CAR expression and cell viability, collect the desired number of cells to be stimulated. Add R10 if required to bring the final cell concentration of $0.8-1 \times 10^6$ cells/ml.
2. Typical bead to cell ratio for optimal stimulation is 3:1. This ratio may vary based on the affinity and activation threshold of the scFv used in the CAR. Calculate the total number of beads required for the desired number of CAR-positive T cells

and collect it in an appropriately sized tube. . (See notes for different stimulation conditions)

3. Wash off any bead-storage buffer by applying the beads against a magnet and rinsing the beads with fresh R10. At least three rinses are recommended.
4. Finally add the beads to the cells.
5. Culture the cells in a 37°C CO₂ incubator for desired time-periods. For long-term cultures, certain cell types may require exogenous supply of growth cytokines.

Notes

1. RNA introduction into target cells can be carried out using other available electroporation instruments that are commercially available, including, but not limited to Amaxa Nucleofactor-II (Amaxa Biosystems, Cologne, Germany), Gene Pulser Xcell (Biorad, Denver, CO, USA) or Multiporator (Eppendorf, Hamburg, Germany).
2. RNA transfection can also be carried out using other methods of gene transfer, including, but not limited to lipofection, polymerase encapsulation, peptide mediated transfection or gene guns.
3. The level of CAR expressed on the surface can be titrated by varying the amount of mRNA used in the gene transfer protocol (Figure 2.2).
4. Transfection efficiency and expression of CAR mRNA can be measured by any other method including northern analysis, western blot or quantitative real time PCR.

5. In vitro culture of certain cell types may require culture media supplemented with cytokines such as IL2, IL7, IL15, etc.
6. For short-term signaling analysis, stimulate cells for desired time-periods with antigen-coated beads, collect and lyse cell pellet for western blot analysis (Figure 2.3). Alternatively, signaling events in stimulated cells can be monitored by flow cytometry-based methods.
7. Signaling events and analysis of antigen-receptor synapses can also be studied by coating antigen on the surface of a culture plate.
8. This method can be utilized for long-term expansion of T cells in culture. Below is a growth curve of CD8⁺ T cells electroporated with a CD19-BBζ CAR cultured with anti-idiotypic beads against CD19 and in the presence of 10ng/ml of IL7 and IL15 cytokines each (Figure 2.4).
9. This protocol can be extended to study signaling and perform in vitro expansion of other T cell subset including CD4⁺ T cells, naïve T cells, T-regulatory cells, Th-17 cells, as well as anergized T cells and stem cells.
10. This protocol can also be applied to other lymphocytes including, but not limited to NK, NKT and B cells.
11. This protocol can be used for in vivo expansion of lymphocytes.

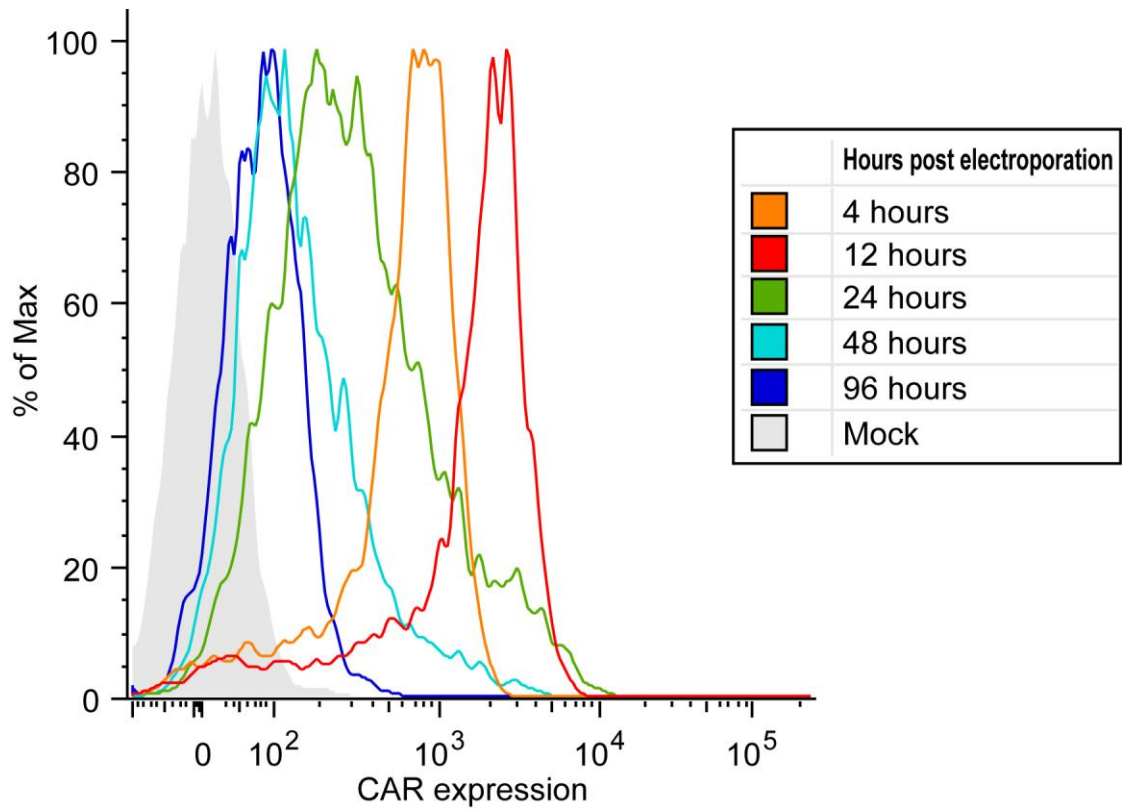


Figure 2.1 CAR surface expression.

CAR expression on T cell surface as measured at different time points post gene transfer. Cells electroporated without any mRNA (mock) serve as a staining control.

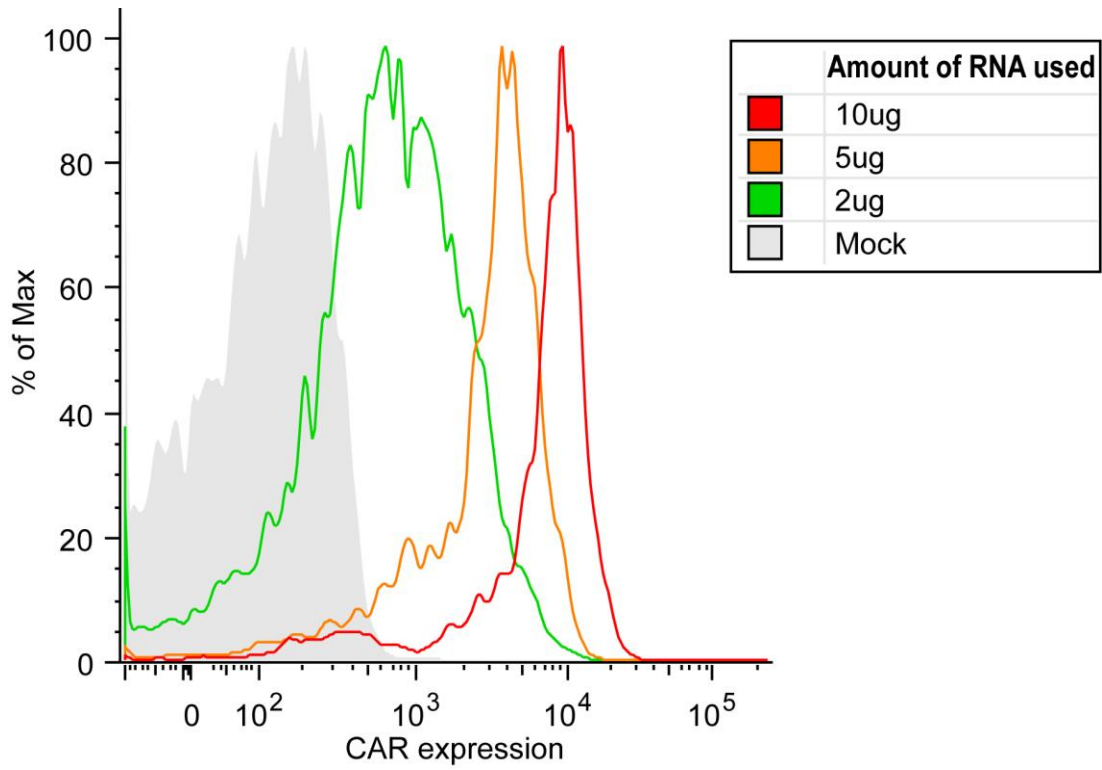


Figure 2.2 Titration of CAR densities.

Surface expression of electroporated CAR mRNA showing gradual increase of mean fluorescence intensities with corresponding increase in mRNA amounts.

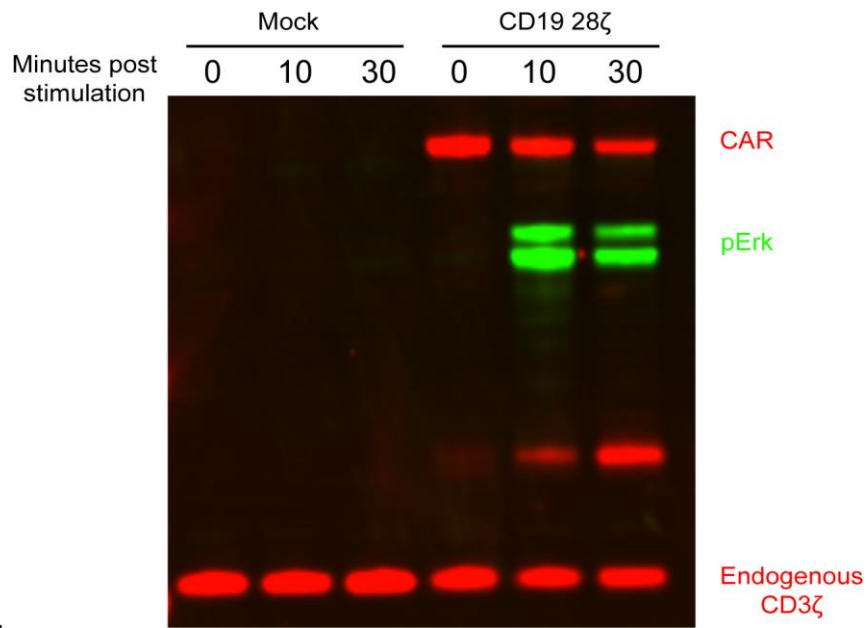


Figure 2.3 CAR-specific signals induced in CAR-T cells.

Phosphorylation of a distal signaling protein (Erk) following stimulation with an anti-idiotypic against the CAR scFv at specified time points. T cells electroporated without any mRNA (mock) serve as a stimulation control.

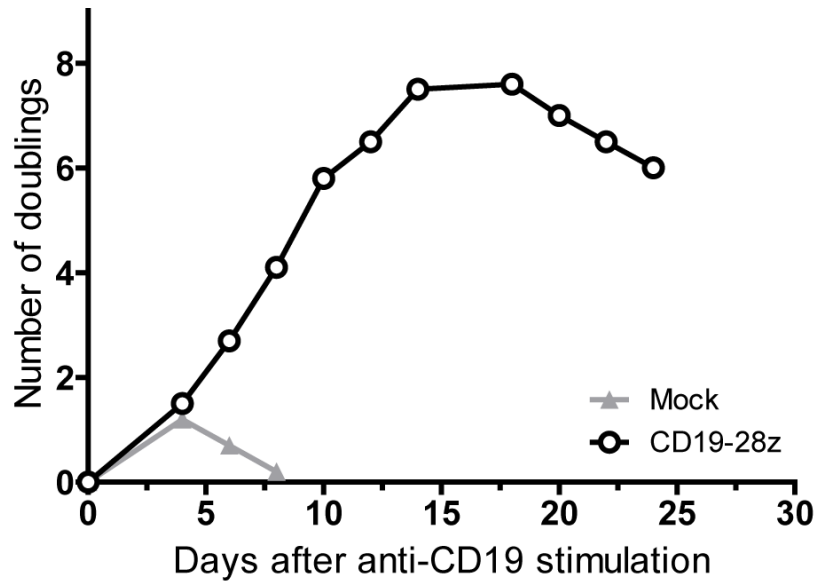


Figure 2.4 Expansion profile of CAR T cells.

CD19 28 ζ CAR T cell growth recorded post stimulation with an anti-idiotype against the anti-CD19 scFv and cultured in the presence of IL7 and IL15. T cells not expressing CARs (mock) serve as a stimulation control.

Chapter 3

REPROGRAMMING THE METABOLIC FATE OF T CELLS WITH DISTINCT SIGNALING DOMAINS IN CHIMERIC ANTIGEN RECEPTORS

Summary

Chimeric antigen receptors (CAR) redirect T cell cytotoxicity against cancer cells, providing a promising new approach to cancer immunotherapy. Despite extensive clinical use, the attributes of CAR co-stimulatory domains that impact persistence and resistance to exhaustion of CAR-T cells remain largely undefined. Here we report the influence of CD28 and 4-1BB signaling domains on the metabolic characteristics of human CAR T cells. Inclusion of 4-1BB in the CAR architecture promoted the outgrowth of CD8⁺ central memory T cells that had significantly enhanced respiratory capacity, increased fatty acid oxidation and enhanced mitochondrial biogenesis. In contrast, CAR T cells with CD28 signaling domains yielded effector memory cells with a genetic signature consistent with enhanced glycolysis. These results provide, at least in part, a mechanistic insight into the differential persistence of CAR-T cells expressing 4-1BB or CD28 signaling domains in clinical trials and inform the design of future CAR T cell therapies.

Introduction

Adoptive immunotherapy based on the infusion of genetically redirected autologous T cells has demonstrated promise for the treatment of both hematologic malignancies and solid tumors. Accordingly, multiple gain-of-function strategies to endow T cells with desired antigen receptors, based on either T cell receptors (TCRs) or

chimeric antigen receptors (CARs) have been described (June et al., 2015). Among several proposed strategies, the use of CARs has shown potent effects in augmenting the immune response to cancers, particularly B cell malignancies (Brentjens et al., 2013; Grupp et al., 2013; Kalos et al., 2011b). Although CAR T cell therapy can have a significant impact on disease clearance, the essential components of a clinically successful CAR, and how they influence therapeutic efficacy, remain largely undefined (Kalos and June, 2013).

CARs are synthetic molecules that combine the effector functions of T cells with the exquisite specificity of antibody-binding domains. In their simplest form, these receptors consist of the TCR grafted to extracellular variable regions of an antibody (Eshhar et al., 1993; Kuwana et al., 1987). One advantage of antibody-based receptors is that they can recognize pre-defined tumor targets independent of antigen processing and MHC-restricted presentation, rendering a single design applicable to a wide range of patients. First generation CARs consisting of the cytoplasmic domain of the Fc receptor γ chain or the CD3 ζ signaling modules alone often become anergic and do not elicit potent T cell antitumor effects (Brocker, 2000; Kershaw et al., 2006; Lamers et al., 2006). This led to the development of second and third generation CARs that incorporate additional costimulatory cytoplasmic domains such as CD28, 4-1BB (CD137), ICOS, and OX40, either individually or in combination (Dotti et al., 2014; Sadelain et al., 2013). This modular design successfully recapitulates many aspects of natural costimulation and enhances proliferation and function of CAR T cells (Maus et al., 2014).

CD19-specific CAR T cells have shown encouraging clinical responses against various hematological malignancies, including chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma. The success rates however, have been difficult to compare due to several variations in study design, as well as differences in the single chain variable antibody fragment (scFv), costimulatory domains, gene-transfer protocols and interventions following CAR T cell infusion, among others. Trials conducted with CARs incorporating CD28 or 4-1BB costimulatory domains have shown similar initial response rates in patients with ALL (Brentjens et al., 2013; Lee et al., 2015; Maude et al., 2014). However, in CLL the clinical efficacy of CAR T cells with 4-1BB costimulatory domains (Porter et al., 2015) appears superior to CD28 domains (Brentjens et al., 2011). The reported persistence of CD28-based CAR T cells in vivo is about 30 days (Brentjens et al., 2013; Lee et al., 2015) in contrast to the sustained expression and effector function of 4-1BB CAR T cells, which may exceed 4 years in some patients (Porter et al., 2015). In addition, the incorporation of 4-1BB signaling domains in certain CARs ameliorates exhaustion (Long et al., 2015). Another important consideration is that endogenous CD28 and members of the TNF Receptor family (TNFR) such as 4-1BB invoke distinct signaling cascades in T cells. CD28 leads to activation of the P13K/Akt pathway with downstream effects on glucose metabolism and increased glycolysis (Frauwirth et al., 2002). In contrast, endogenous 4-1BB signaling has been implicated in imparting long term survival benefits to T cells (Sabbagh et al., 2008) and signaling pathways used by 4-1BB are distinct from CD28 (Martinez-Forero et al., 2013). Enhanced fatty acid oxidation (FAO) contributes to T cell

memory (Pearce et al., 2009). Thus a thorough understanding of the molecular signaling effects of CARs may in part, explain the observed differences in clinical efficacy for CLL.

A challenge for the identification of optimal CAR designs has been the lack of a physiological in vitro model investigating the impact of CAR-based stimulation. Moreover, current gene transfer protocols using retroviruses require concomitant activation of T cells via its endogenous TCR, potentially obscuring effects due to signaling through the CAR per se. In this report, we describe an approach enabling CAR expression in over 90% of the T cells without the need to activate the endogenous TCR. Stimulating the CAR T cells with cognate antigen permitted identification of distinct effects on the differentiation and metabolism of primary human T cells. Interestingly, we find that CAR signaling domains can mediate metabolic reprogramming, while modifying bioenergetics and mitochondrial biogenesis. We found that 4-1BB ζ CAR T cells demonstrate enhanced survival associated with an increased frequency of central memory T cells, mitochondrial biogenesis and greater oxidative metabolism. In contrast antigen stimulation of CD28 ζ CAR T cells promoted effector memory differentiation and led to enhanced aerobic glycolysis.

Results

BB ζ CAR T cells show increased expansion and survival ex vivo

We initially compared two CAR designs (Figure 3.1A) specific for either CD19 or mesothelin. The CARs were equipped with signaling domains comprised of either CD28 (Kochenderfer et al., 2009) or 4-1BB (Milone et al., 2009). These CARs were

chosen because they have been tested extensively in clinical trials (Beatty et al., 2014; Kochenderfer et al., 2012; Lee et al., 2015; Maude et al., 2014; Maus et al., 2013; Porter et al., 2015). Both CAR constructs were expressed on >90% of CD4⁺ and CD8⁺ T cells at comparable MFIs (Figure 3.1B). We compared the effects of the CD28 and 4-1BB (referred to as 28 ζ and BB ζ) signaling domains on the differentiation and metabolic fate of T cells following the protocol described in Figure 3.1C. CD4⁺ T cells were cultured in medium supplemented with 30U/ml of human IL2. CD8⁺ T cells were cultured in medium supplemented with either 100U/ml of human IL2 or 10ng/ml IL7 and 10ng/ml IL15, as indicated. Approximately 24-hours post electroporation, CAR-T cells were stimulated with a bead-bound anti-idiotypic-Fc to the FMC-63 scFv, which serves as a surrogate for cognate CD19 antigen. To ensure that the CAR T cells received uniform stimulation, we analyzed the surface expression of the activation molecule CD69 on day 1 post activation. CD69 is an inducible cell surface glycoprotein that is a sensitive indication of lymphoid activation (Hara et al., 1986). Cells that received CAR-specific stimulation showed elevated levels of CD69 on Day 1 that was similar on 28 ζ and BB ζ CAR T cells (Figure 3.2A). However, the proliferative potential of both CD4⁺ and CD8⁺ T cells bearing the BB ζ CAR was extended through to at least Day 20. In contrast, the proliferative phase of 28 ζ CAR T cells was limited to 14 days (Figure 3.2B, p<0.01). CAR surface expression rapidly decreased following stimulation with cognate antigen. In one donor, we observed over 10 population doublings in the BB ζ CAR T cell culture, expanding the starting culture of 4x10⁶ cells to a calculated yield of over 5x10⁹ in less than four weeks (Table 3.1). Interestingly, the BB ζ CAR T cells persisted in culture

for over 4 weeks in cytokine-supplemented medium following a single stimulation. In contrast, the proliferation and survival of the 28 ζ CAR T cells was lower. Although proliferative capacity varied among donors, the trend remained consistent, in that BB ζ CAR T cells displayed a higher proliferative capacity and persistence compared to the 28 ζ CAR T cells. Similar results were obtained using CARs directed against mesothelin (Figure 3.2C and Table 3.1). For the remainder of this study, we have focused on the effect of CAR design in CD8⁺ T cells.

BB ζ CAR signaling promotes enhanced central memory T cell (T_{CM}) subset

We hypothesized that the enhanced persistence of BB ζ T cells was due to a relative preservation of cells with a more extensive proliferative capacity. To test the differentiation status of BB ζ and 28 ζ CAR-T cells, we used a standard panel of cell surface markers associated with T cell differentiation. We assessed expression of CD45RO and CCR7, which are associated with T_{CM}. All cultures contained the same heterogeneous population of T cell subsets at day 0. After stimulation through the CAR, the proportion of CD45RO⁺CCR7⁺ cells was progressively enriched (Figure 3.3A). Notably, the enrichment of this T_{CM} population was higher in the BB ζ CAR group compared to the 28 ζ group (p<0.01), and persisted through the end of culture (Figure 3.3B). In contrast, the 28 ζ CAR cultures consistently yielded a higher proportion of effector-memory phenotype (T_{EM}), identified as CD45RO⁺CCR7⁻ cells.

CAR signaling domains reprogram T cell metabolism

Upon stimulation, CD8⁺ T cells undergo an ordered process involving proliferation and differentiation into effector and memory cells. Activation is associated

with a biosynthetic and bioenergetics flux required to support T cell proliferation and function (Pearce and Pearce, 2013; Wang and Green, 2012). For example, naïve and memory T cells rely primarily on the mitochondrial oxidation of free fatty acids for development and persistence (Pearce et al., 2009; van der Windt et al., 2012). In contrast, activated effector T cells shift to glycolysis or concurrently upregulate oxidative phosphorylation and aerobic glycolysis to fulfill the metabolic demands of proliferation (van der Windt et al., 2012).

Based on the distinct growth rates and differentiation of 28 ζ and BB ζ CAR T cells, we sought to explore the interconnection of cellular metabolism and CAR signaling. We first examined the metabolic profiles of T cells expressing the two CARs at different time points after stimulation. Cell volume, a surrogate for cell mass, was found to be comparable after cognate antigen stimulation (Figure 3.4A). We measured the oxygen consumption rate (OCR) of 28 ζ and BB ζ CAR T cells before and 7 and 21 days after antigenic stimulation during log-phase proliferation. Basal OCR was measured, followed by serial additions of oligomycin (inhibitor of ATP synthesis), FCCP (Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, an uncoupling ionophore) and rotenone with antimycin A (blocking agents for complex I and III of the electron transport chain, respectively) to discern the relative contributions of mitochondrial and non-mitochondrial mechanism of oxygen consumption (van der Windt et al., 2012). The OCR profiles were similar before antigen stimulation on day 0 (Figure 3.4B). After antigen stimulation, there was a ~10-fold increase in basal OCR in both groups of T cells on days 7 and 21 (Figure 3.4C). However, there was a robust increase in maximal respiratory capacity that was

specific to the 19-BB ζ CAR T cells, following decoupling of the mitochondrial membrane using FCCP on both days 7 and 21 (Figure 3.4D). In contrast the maximal respiratory capacity of the 28 ζ CAR T cells on days 7 and 21 was similar to day 0. To confirm that these differences in OCR were due to the signaling domains of the receptor, similar experiments were performed using mesothelin-specific CAR T cells. The mesothelin-BB ζ CAR T cells exhibited an elevated basal and maximal respiratory capacity compared to the 28 ζ CAR T cells on day 7 and 21 after stimulation with mesothelin. We also measured the extracellular acidification rate (ECAR) as a measurable surrogate for lactic acid production during glycolysis. Glycolysis involves a series of enzyme-catalyzed reactions culminating in the production of lactic acid. At physiologic pH, lactic acid dissociates into lactate and H⁺, which are exported extracellularly. ECAR levels were elevated in 28 ζ cells as compared to BB ζ CAR T cells on days 7 and 21 (Figure 3.4E).

Several reports have shown that natural central memory differentiated T cells have basal OCR and spare respiratory capacity (SRC), likely reflecting a metabolic switch to FAO (Pearce et al., 2009; van der Windt et al., 2012). Since we saw a differential enrichment of memory phenotypes in the two CAR T cell groups in culture, we proceeded to investigate how the metabolic profiles are distinct within these subpopulations. Again, using CCR7 and CD45RO as phenotypic markers, we sorted the populations into CCR7⁺CDRO45⁻, CCR7⁺CDRO45⁺ and CCR7⁻CDRO45⁺ to define naïve-like (N), central memory (T_{CM}) and effector memory (T_{EM}) subpopulations, respectively. Performing the seahorse assay on these cells revealed higher basal OCR and

maximum respiratory capacity of the BB ζ in the T_{CM} and T_N memory subtypes as compared to 28 ζ CAR T cells (Figure 3.5A and 3.5B). As observed in past reports concerning effector cells, the basal OCR as well as the maximum respiratory levels remained low for the T_{EM} subpopulations for both CAR groups (Figure 3.5C). The ECAR levels, on the other hand remained higher for T_{CM} and T_{EM} subpopulations of cells obtained from the 28 ζ CAR T cell culture (Figure 3.5D).

The supplementation of culture medium with exogenous cytokines is necessary to support the long term proliferation of primary human CD8 T cells. This is a well-established practice routinely adopted by all labs studying human CD8 lymphocytes. To ensure that the cytokines used are not responsible for driving the metabolic responses, we compared the growth patterns of the CAR T cells in media supplemented with either IL2 or a combination of IL7 and IL15. We found no difference in the exponential growth of T cells within the same CAR group when compared with either cytokine cocktail (Figure 3.6A). The relative trends in OCR remained similar irrespective of the cytokine used (Figure 3.6B and C). Importantly, cytokine receptor expression was comparable in both CAR groups, indicating that the proliferative differences between the different CAR T cells are not due to differences in cytokine receptor expression (Figure 3.6D).

To analyze whether there is a dominant effect of one signaling domain over the other, we included the cytoplasmic domain of CD28 in the SS1-BB ζ CAR to yield the third generation of CAR, referred to as SS1-28BB ζ . After antigenic stimulation, we observed similar growth patterns between these three CAR groups (Figure 3.7A). However, the metabolic profile, as assayed using the seahorse analyzer differed (Figure

3.7B). Particularly the basal OCR and maximal OCR were lowered with the inclusion of the CD28 domain in the BB ζ CAR structure (Figure 3.7 C and D). This suggests that the CD28 signaling may be dominating in the context of a CAR. Although tested in only a single donor so far, this experiment serves as a preliminary finding that needs further investigation.

In aggregate, these studies show that BB ζ CAR T cells are metabolically distinct from 28 ζ CAR T cells with the former displaying greater capacity for oxidative metabolism that might contribute to the enhanced central memory differentiation and persistence of BB ζ CAR T cells.

28 ζ and BB ζ CAR T cells have distinct glycolytic and fatty acid metabolism

To investigate if the differences in the basal OCR in CAR T cells altered the fuel sources by which these cells satisfy their bioenergetic appetite, we proceeded to measure glucose uptake and fatty acid utilization rates in CAR T cells. At day 7 post stimulation, the cells were replated in fresh media. At different points (as indicated in Figure 3.8A), we measured the amount of residual glucose in the media and the lactate produced. 28 ζ CAR T cells consumed glucose at a relatively quicker rate along with production of lactic acid. This is consistent with the greater ECAR we observed in 28 ζ CAR T cells in Figure 3.4E and 3.5D.

The increased OCR in BB ζ CAR T cells prompted us to examine the fatty acid consumption rate in these cells. Using a heavy carbon labeled long-chain fatty acid, palmitic acid, we analyzed its uptake rate by measuring the levels of heavy-carbon labeled acetyl-CoA. The catabolic process of beta-oxidation breaks down fatty acid

molecules into acetyl-CoA in the mitochondria to feed the citric acid cycle. We found that BB ζ showed a higher percentage of labeled acetyl-CoA pool as compared to 28 ζ CAR T cells (Figure 3.8B). This data suggests that BB ζ CAR T cells, similar to CD8 T_{CM} cells extensively rely on catabolic pathways such as FAO to fuel their bioenergetic demands.

To gain insight into the mechanism leading to the metabolic differences conferred by distinct CAR signaling domains, we measured the expression of candidate genes that are implicated in glycolytic and lipid metabolism. We initially focused on two main enzymes implicated in glucose metabolism, Glut1 and PDK1. The cell surface expression of Glut1, the transporter involved in glucose uptake is induced following CD28 activation (Frauwirth et al., 2002). In certain contexts, including hypoxia, PDK1, inhibits the decarboxylation of pyruvate and entry of glucose derivatives into the TCA cycle (Duvell et al., 2010). Both Glut1 and PDK1 are induced to significantly higher levels in 28 ζ cells relative to BB ζ cells at day 7 (Figure 3.8C). Increased expression levels of Glut1 and PDK1, coupled with our earlier finding of increased ECAR is consistent with enhanced glycolysis in 28 ζ CAR T cells, as compared to their BB ζ counterparts.

Two critical enzymes involved in the breakdown of glucose during the ATP-generating step of the glycolytic pathway are Phosphoglycerate kinase (PGK) and Glucose-6-phosphate dehydrogenase (G6PD). PGK transfers a phosphate group to ADP to facilitate ATP generation, while G6PD, a NADP⁺-dependent enzyme catalyzes the oxidative phase of the pentose phosphate pathway. Since these enzymes have an important role in glycolysis, we investigated their expression levels in CAR T cells on

day 7. Interestingly, their levels were elevated in 28 ζ CAR T cells relative to BB ζ CAR T cells. Finally, we also examined the levels of Solute carrier family 16 (SLC16A3), an exporter of the glycolysis byproducts lactic acid and pyruvate. 28 ζ CAR T cells showed higher levels of SLC16A3 mRNA as compared to BB ζ T cells, consistent with our hypothesis that 28 ζ CAR T cells use increased glycolysis as a means to meet their metabolic demands. We also detected increased expression of VEGFA in 28 ζ CAR T cells, which is an established target of the hypoxia-inducible factors (HIF). Several genes involved in glycolysis are targets of HIF1 α (Finlay et al., 2012) including Glut1 and PFK. Others have shown that *HIF1 α* ^{-/-} knockout T cells display impaired autoreactivity (Dang et al., 2011). Our findings add to the growing body of evidence implicating costimulation through CD28 and glycolytic reprogramming in effector differentiation.

To investigate the reliance of CAR T cells on fatty acids, we took advantage of a pharmacological inhibitor of an important mitochondrial transporter of fatty acids, known as carnitine palmitoyl transferase (CPT1A). CPT1A is a metabolic enzyme that controls a rate-limiting step in mitochondrial FAO as well as promoting mitochondrial biogenesis. Etomoxir is used to disrupt the function of CPT1A. During the stimulation period of CAR T cells in culture, we added 50 μ M of Etomoxir on Day 3 and every 48 hours thereafter. Although the disruption of CPT1A showed no effects on the growth and survival of 28 ζ CAR T cells, there was a detrimental effect on the exponential growth of BB ζ CAR T cells, which stopped proliferating after Day 7 (Figure 3.9). We further sought to investigate genes associated with mitochondrial FAO after triggering CAR T cells. Increasing evidence has demonstrated a role for CPT1A in regulating oxidative metabolism in CD8+

memory T cells (van der Windt et al., 2012). We observed significantly higher levels of *CPT1A* mRNA in BB ζ CAR T cells as compared to 28 ζ CAR T cells. Additionally, mRNA levels of fatty acid binding protein (FABP5) that plays a critical role in long-chain fatty acid uptake, transport and metabolism were significantly upregulated in BB ζ CAR T cells compared to 28 ζ (Figure 3.8C). These findings suggest that 28 ζ CAR T cells rely more on a glycolytic based metabolism whereas BB ζ programs T cells to use fatty acids as the predominant energy source, characteristics of natural effector and memory T cells, respectively.

BB ζ CAR T cells have increased Spare Respiratory Capacity

Mitochondrial spare respiratory capacity (SRC) is a measure of how effectively protons can be shuttled into the mitochondrial intermembrane space upon cellular or mitochondrial stress (Mookerjee et al., 2010; Nicholls, 2009). SRC enhances survival and function of memory T cells by providing a contingency source of energy for cells exposed to metabolic stress including nutrient depletion, oxygen deprivation or under conditions of increased cellular activity. Increased SRC likely supports T cell function in a hostile tumor environment (Ferrick et al., 2008; Nicholls, 2009; Yadava and Nicholls, 2007). Memory CD8⁺ T cells, unlike effectors, maintain a substantial SRC (van der Windt et al., 2012). When comparing the SRC of the two CAR groups, we observed that BB ζ CAR T cells maintained higher levels of SRC as compared to 28 ζ CAR T cells (Figure 3.10A). This is consistent with the metabolic characteristics of long-lived CD8⁺ memory cells, lending further support to the hypothesis that BB ζ signals support a metabolic reprogramming that contributes to long-lived memory-like T cells.

Given the role of mitochondrial density in oxidative metabolism (van der Windt et al., 2012), we next explored the possibility whether the increased SRC in BBζ CAR T cells was associated with an increase in mitochondrial mass. Using electron microscopy, we measured similar mitochondrial density between 28ζ and BBζ CAR-T cells at day 7 (Figure 3.10B and 3.10C). However, there was a substantial increase in mitochondrial mass in BBζ CAR T cells at days 14 and 21 (Figure 3.10B and 3.10C) after antigen stimulation. Despite similar cell volumes (Figure 3.4A), we observed a significantly ($p < 0.001$) increased density of mitochondria in BBζ CAR-T cells. We also measured mitochondrial density using confocal microscopy (Figure 3.11A). BBζ CAR T cells showed an increased ratio of mitochondrial mass to total cell mass on days 14 and 21 (Figure 3.11B).

BBζ CAR T cells show enhanced mitochondrial biogenesis

We speculated that specific signals from the 4-1BB signaling domain in the CAR structure supported mitochondrial biogenesis, thus endowing these cells with greater mitochondrial mass. However, in addition to quantitative differences in mitochondrial content, we examined if qualitative differences in mitochondria might contribute to the differences in metabolic profiles between these CAR cells. We examined levels of certain mitochondrial genes encoded by the nuclear and the mitochondrial genome, namely TFAM and MTCO-1, respectively. Notably, BBζ cells had significantly enhanced mRNA expression of mitochondrial transcription factor A (TFAM) and mitochondrially encoded cytochrome c oxidase 1 (MT-CO1), the main subunit of the cytochrome c oxidase complex (Figure 3.12A).

To explore the role of 28 ζ and BB ζ costimulatory domains on the mitochondrial function in the context of CAR T cells, we measured gene expression of two transcription factors of mitochondrial genes, namely nuclear respiratory factor-1 (NRF1) and GA-binding protein (also known as NRF2). While NRF1 regulates the expression of TFAM and coordinates mitochondrial DNA replication and expression, NRF2 has a role in the transcription of the OXPHOS components, mitochondrial import and TFAM. Consistent with its enhanced oxidative features as seen by metabolic flux analyses and mitochondrial density, we found that BB ζ CAR T cells had significantly higher expression of NRF1 and NRF2 as compared to the 28 ζ CAR T cell group (Figure 3.12B). We further substantiated the role of TFAM, by performing knock-down studies. Day 7 post stimulation with the CAR-cognate antigen, we knocked TFAM using a targeted siRNA pool. Two days following introduction of the siRNA, we confirmed knock down of TFAM and analyzed the cells on the Seahorse flux analyzer. We observed significant decrease in the basal and maximum OCR levels in both CAR groups when treated with TFAM siRNA (Figure 3.13A). The decrease in both these parameters was even more drastic in BB ζ CAR T cells (Figure 3.13B and C), suggesting a major role of TFAM in shaping the oxidative features of these CAR T cells.

Taken together, these findings suggest increased mitochondrial content in BB ζ CAR T cells compared to 28 ζ CAR T cells, which strongly correlates with the increased SRC observed in these cells. Our findings are consistent with a model in which BB ζ CAR signaling reprograms transcriptional networks supporting mitochondrial biogenesis and oxidative metabolism. Given the role of metabolic adaptation in enabling T cell memory

and effector functions, the aforementioned oxidative features in BB ζ CAR T cells likely support central memory differentiation and T cell persistence.

Discussion

We have uncovered significant differences in the differentiation and metabolic profiles of CAR T cells using CD28 or 4-1BB signaling domains. The predominant metabolic program in 28 ζ CAR T cells is aerobic glycolysis and in BB ζ CAR T cells it is oxidative breakdown of fatty acids. Our studies provide evidence for plasticity in T cell metabolic reprogramming and further, that the choice of CAR signaling domain can impact the subsequent fate of the T cells. The enhanced proliferation and persistence of BB ζ over 28 ζ CAR T cells observed in our studies mirrors the outcomes of CAR persistence observed in clinical studies (Brentjens et al., 2013; Brentjens et al., 2011; Lee et al., 2015; Porter et al., 2015). Our studies suggest that one mechanism for the differential persistence may be the metabolic reprogramming of the CAR T cells to enhance either oxidative phosphorylation that is characteristic of memory cells or aerobic glycolysis that is characteristic of effector cells (MacIver et al., 2013; van der Windt et al., 2012).

Previous studies have shown that CD28 signaling initiates a cascade leading to enhanced surface expression of Glut1 and increased reliance on aerobic glycolysis (Frauwirth et al., 2002). In contrast a TNFR pathway is required for the initiation of mitochondrial FAO and T cell memory development (Pearce et al., 2009). While IL2 promotes effector differentiation and glycolysis in CD8⁺ T cells (Finlay et al., 2012; Liao et al., 2013; Pipkin et al., 2010), IL7 and IL15 have been implicated in the maintenance

of memory T cells and increased mitochondrial biogenesis (Ku et al., 2000; Schluns and Lefrancois, 2003; van der Windt et al., 2012). As human CD8+ T survival is impaired in the absence of exogenous cytokines, IL7 and IL15 are necessarily present in our culture system. Although these extrinsic factors may play a significant role in stabilizing the metabolic profiles of T cells, we hypothesize that our system is largely governed by cell-intrinsic factors influenced by the two unique intracellular CAR signaling domains. This is further corroborated by the lack of differences in the cell surface expression of these cytokine receptors, suggesting that the relative distinction in metabolic reprogramming between the two CARs cannot be solely mediated by the supplemented cytokines. Thus, our studies suggest that the ectopic expression of CD28 or 4-1BB signaling domains in CARs leads to a phenocopy of the natural T cell activation process. By extension, our studies suggest that the incorporation of various signaling modules may biosynthetically reprogram T cells to desired effector or regulatory functions. For example, we have recently found that the incorporation of the ICOS signaling domain in CARs promotes a Th17 differentiation program (Guedan et al., 2014).

One clinical application of our findings is either that short-lived or long-lived CAR T cells can be created “at will”. This could extend the range of targets, depending on certain surface molecules where long-term CAR effects may not be tolerable due to potential off-tumor toxicity. In this case, a CD28 signaling domain would be expected to be superior. Another implication from our studies is that a mixture of CAR T cells expressing 4-1BB and CD28 domains may be superior to either CAR as a single population. We speculate this because the combination of CAR T cells would be

expected to more completely mimic a natural immune response comprised of an early dominance of T effector cells, achieved with CD28 CARs having enhanced aerobic glycolysis in the cytoplasm, and T memory cells, achieved with 4-1BB CARs having enhance mitochondrial oxidative phosphorylation.

Apart from cell intrinsic factors, there has been substantial interest in understanding the effects of nutrient consumption on T cell survival in the tumor microenvironment. T cells have substantial bioenergetic and biosynthetic challenges to survive and conduct effector functions. Our results indicate that BB ζ CAR T cells have an increased capacity to generate mitochondrial mass. This increase in mitochondrial mass provides a survival advantage (van der Windt et al., 2013). We consistently saw a higher SRC in BB ζ CAR T cells, and this mitochondrial respiratory capacity has been shown to be an important characteristic of natural CD8⁺ T cell memory development (van der Windt et al., 2012). The increased basal oxygen consumption of BB ζ cells also suggests a preferential reliance on oxidative phosphorylation as the predominant energy generating mechanism to account for the metabolic demands required for enhanced CAR T cell proliferation. Our data further suggests that metabolism is a key mediator of CAR T cell survival, and is influenced by the signaling induced by the costimulatory domain included in the CAR.

In summary, our results reveal a new role for CAR T cell engineering to control T cell metabolism as a key determinant of T cell effector and memory responses. Using synthetic biology it is possible to shape the immune response to a desired balance of long-lived memory cells and short-lived effector cells. By extension, our studies should

influence the design engineered T effector or engineered T regulatory cells that resist exhaustion or have enhanced survival in hostile tumor and inflammatory microenvironments.

Experimental Procedures

CAR constructs and generation of CAR-encoding in vitro transcribed (IVT) RNA

For the purpose of these studies, CARs specific to the human CD19 or mesothelin antigen were used. Figure 3.1A shows the schematic of the CARs used in this study. All CARs contained the single-chain variable fragment (scFv) against human CD19 (clone FMC-63), or the SS1 scFv against human mesothelin protein, wherever indicated (Hassan et al., 2002; Nicholson et al., 1997). The mesothelin CAR was previously described (Carpenito et al., 2009). The CD28 ζ CAR consisted of the scFv linked *in cis* to the intracellular domains of CD28 and CD3 ζ through the CD8 α hinge and a CD28-transmembrane domain, as described previously (Milone et al., 2009). Similarly the BB ζ CAR contained the scFv linked to the 4-1BB intracellular portion and the CD3 ζ domain through a CD8 α hinge and transmembrane domain (Milone et al., 2009). For preparation of IVT RNA, the CAR-encoding gene constructs were subcloned into the pGEM.64A based vector, as described previously (Zhao et al., 2010).

Isolation, electroporation and expansion of primary human T lymphocytes

Primary human T lymphocytes were obtained from anonymous healthy donors at the University of Pennsylvania Apheresis Unit. Using the BTX CM380 (Harvard Apparatus BTX, Holliston, MA USA) electroporation machine, the IVT RNA was introduced into

the T cells at a ratio of 1 μ g RNA/10⁶ cells. This technique was optimized to promote uniform CAR expression on the cell surface (Figure 3.1B). T cells were stimulated using magnetic beads coated with a recombinant anti-CD19 idiotype or mesothelin-Fc.

Flow cytometry analysis

Live cells were gated on Live/Dead Aqua-negative and then gated for CD3, CD4 and CD8 positive events. Using markers for memory, CCR7 and CD45RO, cells in culture were analyzed and sorted for the three different memory phenotypes using the BD FACSCalibur. Absolute T cell counts were determined with the aid of CountBright Absolute Counting Beads (Life Technologies) using the formula:

(Number of T cells events/number of bead events) X number of beads used

Analysis of metabolic parameters

Mitochondrial function was assessed using an extracellular flux analyzer (Seahorse Bioscience). Individual wells of an XF24 (for Figure 3.4B, C, D and E) or XF96 (for Figure 3.5A, B, C and D) cell culture microplate were coated with CellTak in accordance with the manufacturer's instructions. The matrix was adsorbed overnight at 37°C, aspirated, air dried, and stored at 4°C until use. Mitochondrial function was assessed on days 0, 7, and 21. To assay mitochondrial function, T cells were centrifuged at 1200g for 5 minutes. Cell pellets were resuspended in XF assay medium (non-buffered RPMI 1640) containing 5.5 mM glucose, 2mM L-glutamine, 1mM sodium pyruvate and seeded at 1x10⁶ cells/well. The microplate was centrifuged at 1000g for 5 minutes and incubated in standard culture conditions for 60 minutes. During instrument calibration (30 mins), the

cells were switched to a CO₂-free, 37°C, incubator. XF24/XF96 assay cartridges were calibrated in accordance with the manufacturer's instructions. Cellular oxygen consumption rates (OCR) were measured under basal conditions and following treatment with 1.5µM oligomycin, 1.5µM fluoro-carbonyl cyanide phenylhydrazine (FCCP) and 40nM rotenone + 1µM antimycin A (XF Cell Mito Stress kit, Seahorse Bioscience).

Gene expression analysis by RT-PCR

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to quantify expression levels of certain candidate genes. Total RNA from cells was used as templates to synthesize cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). qRT-PCR was performed in triplicates using Taqman Universal Master Mix on a ViiA 7 Real Time PCR system as per manufacturer's instructions. mRNA levels of each candidate gene as quantified by the PCR system were normalized to a housekeeping gene, *GAPDH*. All probes used are commercially available (Applied Biosystems).

Glucose uptake assay

Cells at Day 7 post stimulation were starved in PBS at room temperature for 30 minutes followed by incubation at 37°C in regular RPMI culture media supplemented with 11mM glucose, 10% FCS, 100-U/ml penicillin, 100µg/ml streptomycin sulfate and 2mM glutamax. 500uL aliquots of cell culture was collected at indicated time points, spun down and the supernatants were analyzed for glucose and lactate concentrations using the Nova BioProfile Analyzer, Nova Biomedical, MA.

Palmitic acid uptake assay

[¹³C₁₆] palmitic acid was purchased from Sigma-Aldrich. All solvents for LC-MS were Optima grade purchased from Fisher Scientific. For palmitic acid-labeled isotope experiments, cells were cultured overnight in RPMI 1640 w/o D-glucose, w/o L-glutamine (Biological Industries) supplemented with 10% charcoal-stripped FBS (Gibco), 2mM L glutamine (Life technologies), 5.0mM glucose, and 100µM [¹³C₁₆] palmitic acid.

Short-chain Acyl-CoA Extraction

Extractions were performed as described previously (Basu and Blair, 2012; Worth et al., 2014). Briefly, lymphocytes were centrifuged at 1200 rcf for 5 mins. Cell pellets were resuspended in 750µl of ice-cold 10% trichloroacetic acid and pulse-sonicated using a sonic dismembrator (Fisher Scientific). The samples were centrifuged at 15,000 rcf for 15 minutes and the supernatants were purified by solid phase extraction. Briefly, Oasis HLB 1-ml (30mg) solid-phase extraction columns were conditioned with 1ml methanol, followed by 1ml of H₂O. The supernatants were applied to the column and washed with 1ml of H₂O. The analytes were eluted in methanol containing 25mM ammonium acetate. The eluates were dried overnight in N₂ gas and resuspended in 50µl of 5% 5-sulfosalicylic acid. 10µl injections were applied in LC/ESI/MS/MS analysis.

LC/MS Analysis of Acyl-CoA thioesters

Acyl-CoA's were separated using a Phenomenex Luna C₁₈ reversed-phase HPLC column (2.0×150 mm, 5 µm pore size) with 5mM ammonium acetate in water as solvent A, 5mM

ammonium acetate in ACN/water (95:5, v/v) as solvent B, and ACN/water/formic acid (80:20:0.1, v/v) as solvent C as described previously (Basu et al., 2011; Worth et al., 2014). A linear gradient was run as follows: 2% solvent B for 1.5 mins, increased to 25% over 3.5mins, increased to 100% over 0.5mins, held for 8.5mins, and washed with 100% solvent C for 5 mins before equilibration for 5 mins. The flow rate was 200 μ l/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive electrospray ionization (ESI) mode. Samples (10 μ l) were injected using a LEAP autosampler (CTC Analytics AG, Zwingen, Switzerland) and maintained at 4°C. Data were analyzed using Analyst Version 1.4.1 software (AB SCIEX). The column effluent was diverted to the mass spectrometer from 8-23 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0kV), nitrogen as curtain gas (15 units), ion source gas 1 (8 units), ion source gas 2 (15 units) and collision-induced dissociation (CID) gas (5 units). The ESI probe temperature was 450°C, the declustering potential was 105V, the entrance potential was 10V, the collision energy was 45eV, and the collision exit potential was 15V. A loss of 507Da was monitored for each acyl-CoA.

Microscopy

Cells at different time points were stained with DiI, Mitotracker green and DAPI (Life Technologies) and fixed with 4% PFA before imaging on the Leica TSC SP8 Confocal microscope. Captured images were analyzed using Fiji (ImageJ) and fluorescence emission was quantified as mean fluorescence intensity (MFI). For transmission electron

microscopy, the cells were prepared by Penn's Electron Microscopy Resource Laboratory and imaged using the Jeol-1010 microscope.

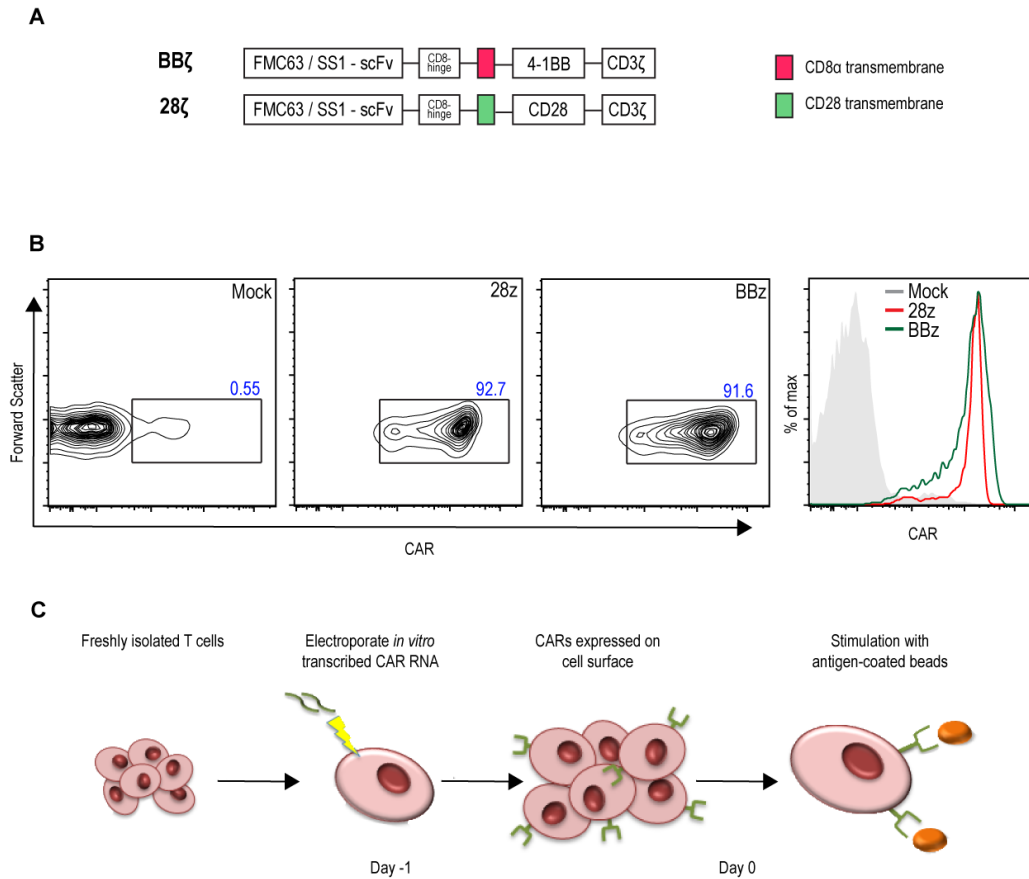


Figure 3.1 CAR constructs and study design.

(A) Schematics of the CAR constructs compared in this study. CARs contain a single-chain variable fragment of the FMC63 antibody that binds human CD19 or the SS1 scFv that binds human mesothelin. The transmembrane (TM) and intracellular domains are indicated.

(B) Flow cytometric analysis of cell surface expression of the CARs on day 1 after electroporation as compared to an electroporation only (Mock) control. The right panel shows the mean fluorescence intensities of the CARs detected with an anti-idiotypic reagent.

(C) CD8⁺ T cells are electroporated with *in vitro* transcribed CAR RNA. After the cells are rested overnight, the CAR expression is confirmed, and the *in vitro* culture commences with the addition of cognate antigen-coated beads and cytokines.

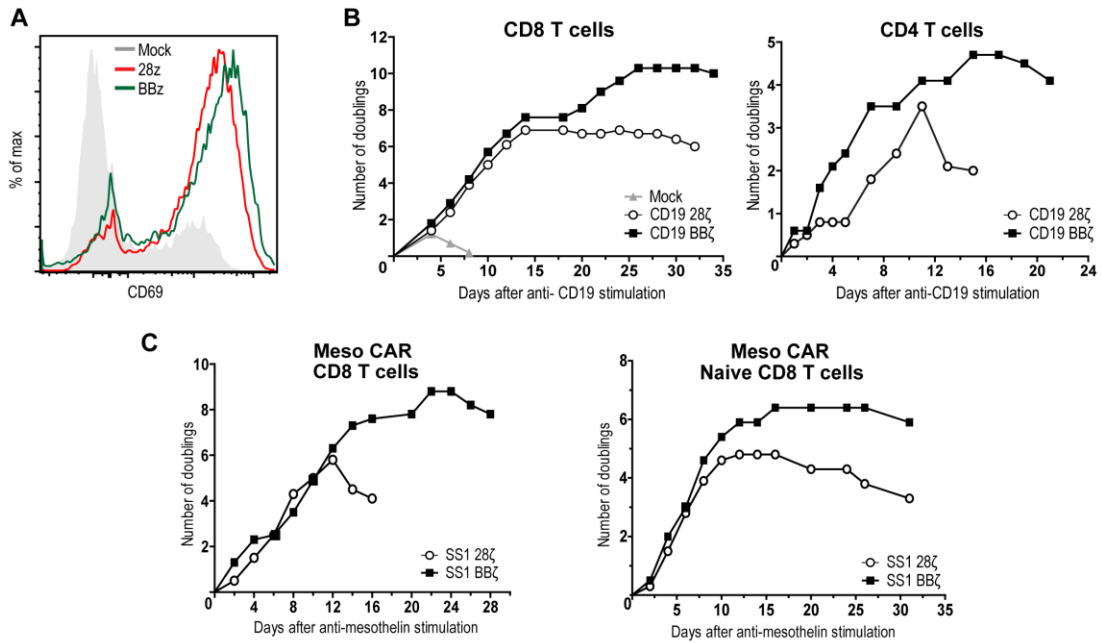


Figure 3.2 4-1BB ζ signaling domain provides a survival and proliferative advantage to CD8 $^+$ T cells in vitro.

(A) CD69 levels measured on cell surface 24 hours post co-culture with cognate antigen.

(B) CD19 CAR T cell growth. CD4 $^+$ and CD8 $^+$ T cells were stimulated as in panel A. Data are representative of at least 10 different healthy donors.

(C) Mesothelin CAR T cell growth of bulk CD8 $^+$ T cells (top) or naïve (CD45RO $^-$ CD62L $^+$ CD8 $^+$) T cells (bottom). CAR T cells were stimulated using beads coated with mesothelin-Fc.

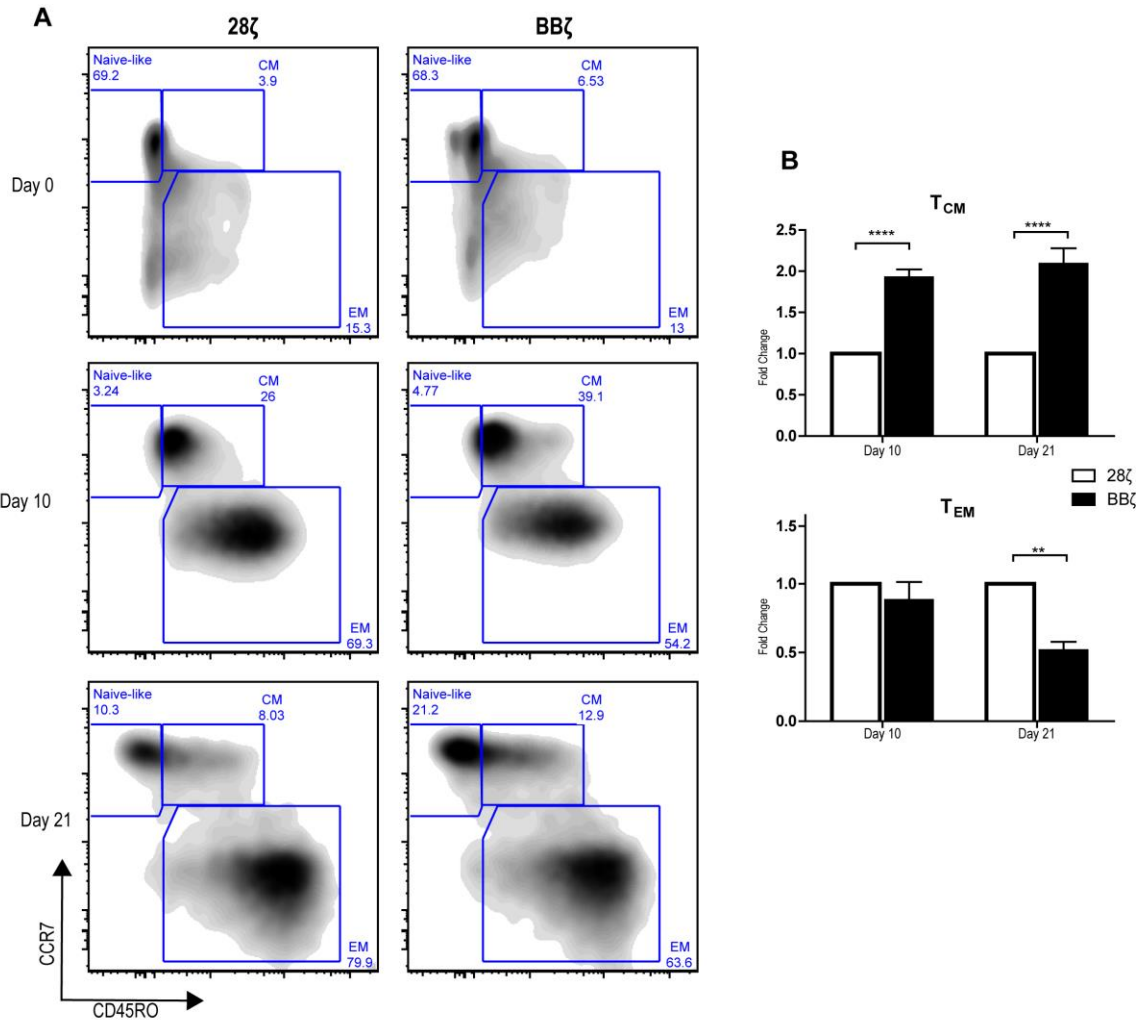


Figure 3.3 Enrichment of T_{CM} in 4-1BBζ containing CAR T cells

(A) Representative plots (from at least 6 donors) of cell surface expression of CCR7 and CD45RO on CAR T cells at specified time points during culture. Cells shown have been pre-gated for live CD3⁺CD8⁺ T cells. Numbers shown are percentages of cells detected in each gate.

(B) Relative change of T_{CM} and T_{EM} subsets in 28ζ and BBζ CD19 CAR T cell cultures. Absolute numbers of live cells were calculated for each population at the specified time points. The graphs show relative fold change of T_{CM} or T_{EM} in BBζ CAR T cells normalized to 28ζ CAR T cells. Data plotted as mean±SEM (p=****<0.0001, **=<0.01).

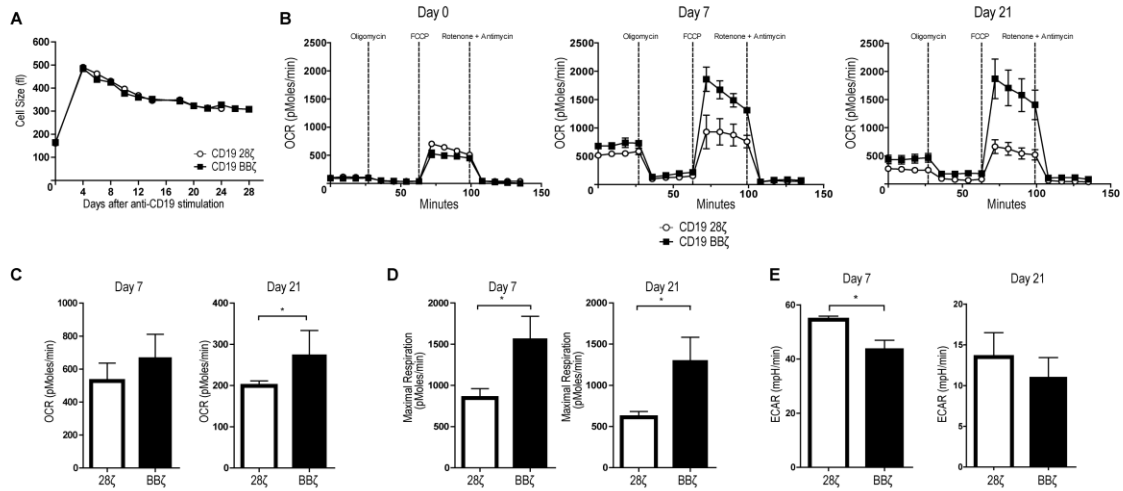


Figure 3.4 Effects of CAR signaling domain on cellular metabolism.

(A) Effects of antigen stimulation on mean cell volume after stimulation of CD19 CAR CD8⁺ T cells expressing 28 ζ and BB ζ signaling domains with anti-idiotype.

(B) The oxygen consumption rates (OCR) of 28 ζ and BB ζ CAR T cells at baseline (after electroporation of CAR mRNA and before stimulation) on day 0, and after stimulation on days 7 and 21 in culture, under basal metabolic conditions and in response to mitochondrial inhibitors as specified in the methods.

(C, D and E) Basal OCR levels **(C)**, maximum respiratory levels **(D)** and basal ECAR levels **(E)** measured at days 7 and 21. Data representative of at least 5 independent experiments performed using at least 5 healthy human donor cells, plotted as mean \pm SEM ($p=^* < 0.05$).

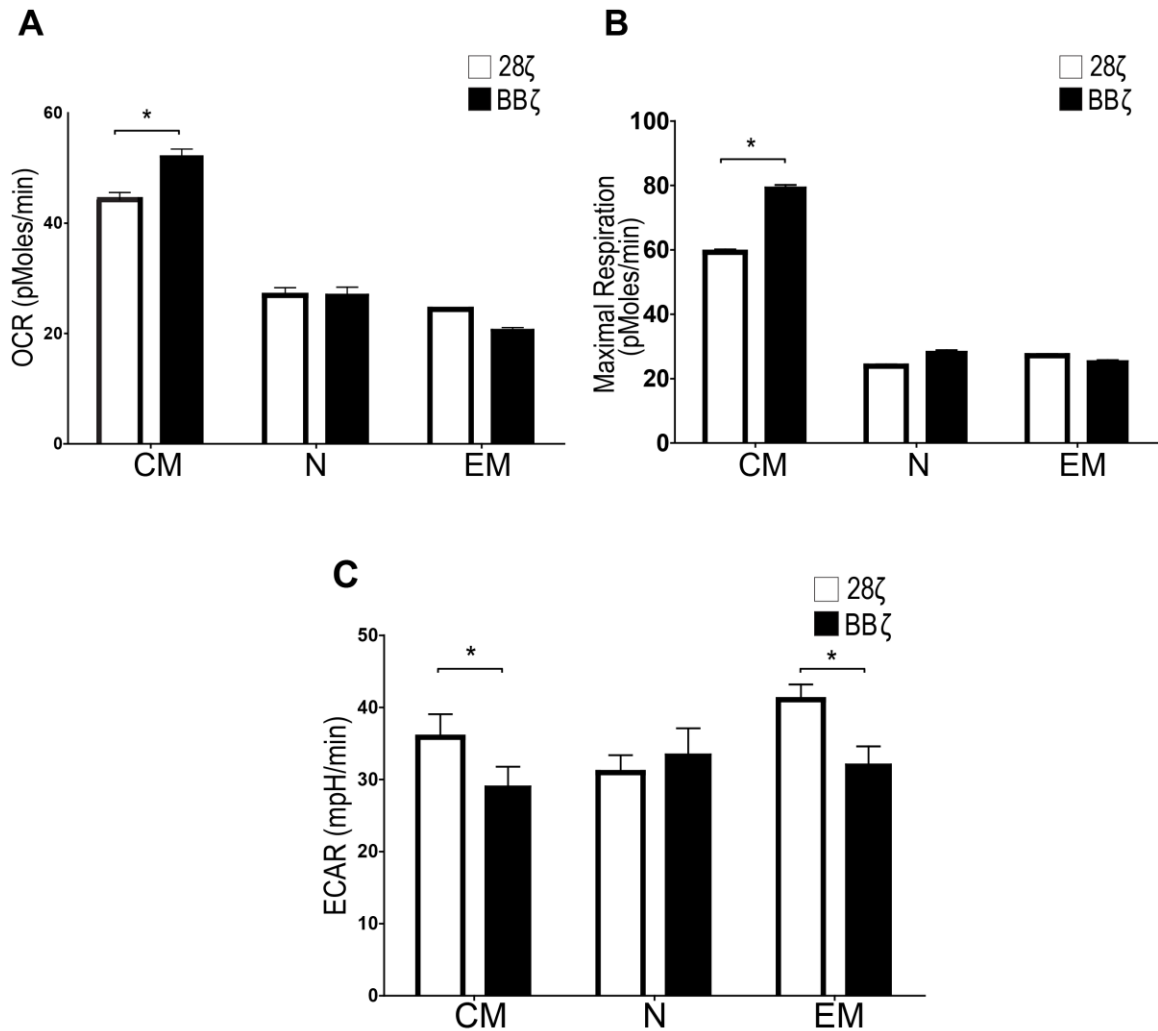


Figure 3.5 Metabolic profiles of T cell memory subsets.

(A and B) Basal OCR and maximum respiratory levels measured for CAR T cells sorted for different memory phenotypes - central memory (CM), naïve (N) and effector memory (EM). Data representative of at least 3 independent experiments performed using at least 3 healthy human donor cells, plotted as mean±SEM

(C) Basal ECAR levels measured for the three different sorted memory subsets. Data representative of at least 3 independent experiments performed using at least 3 healthy human donor cells, plotted as mean±SEM ($p=*<0.05$).

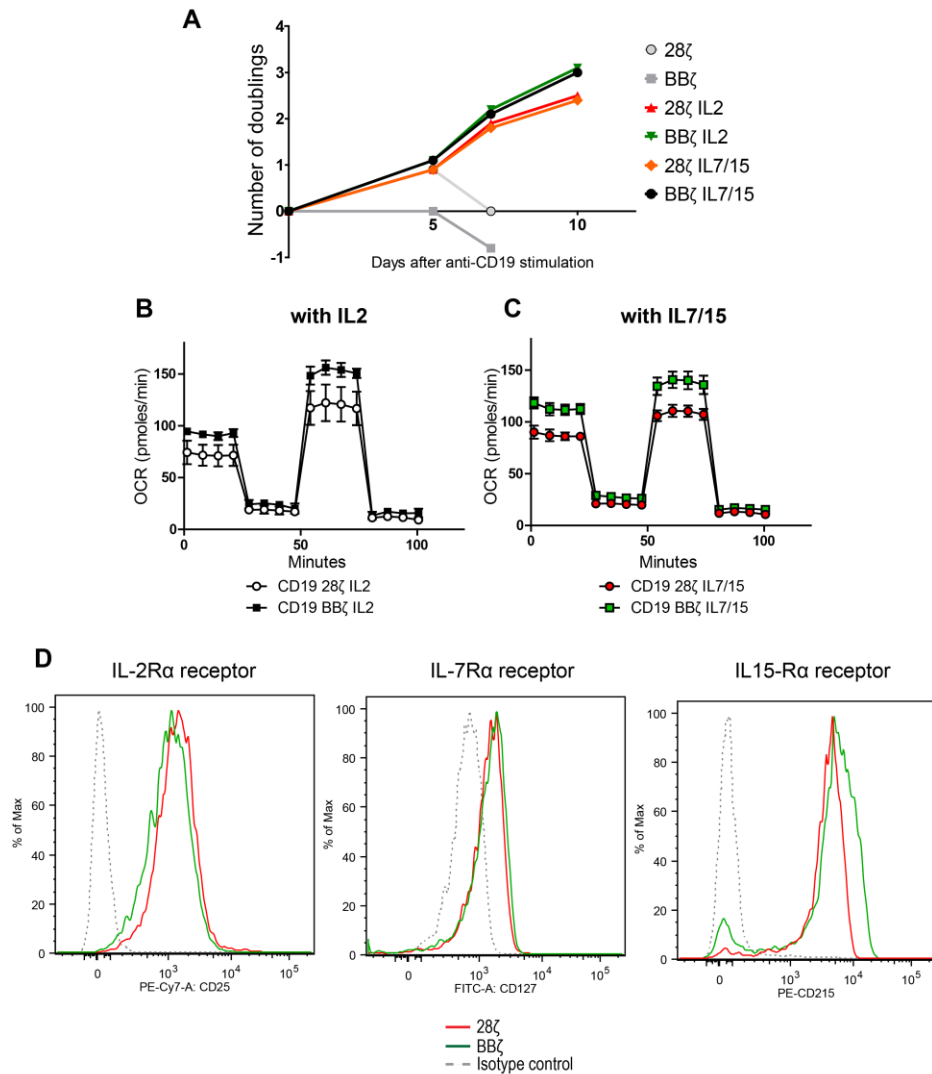


Figure 3.6 Distinct metabolic profiles observed is not influenced by cytokine cocktail used.

(A) Growth curves of CAR T cells grown in media supplemented without cytokine or with either IL2 alone or IL7 and IL15. Data are representative of two different healthy donors.

(B and C) The OCR of 28ζ and BBζ CAR T cells stimulated and cultured in media supplemented with IL2 alone or IL7+15, respectively. OCR measurements were performed on day 10 post CAR T cell stimulation.

(D) Levels of cytokine receptors, IL-2Rα, IL-7Rα and IL-15Rα on cell surface as assayed by flow cytometry

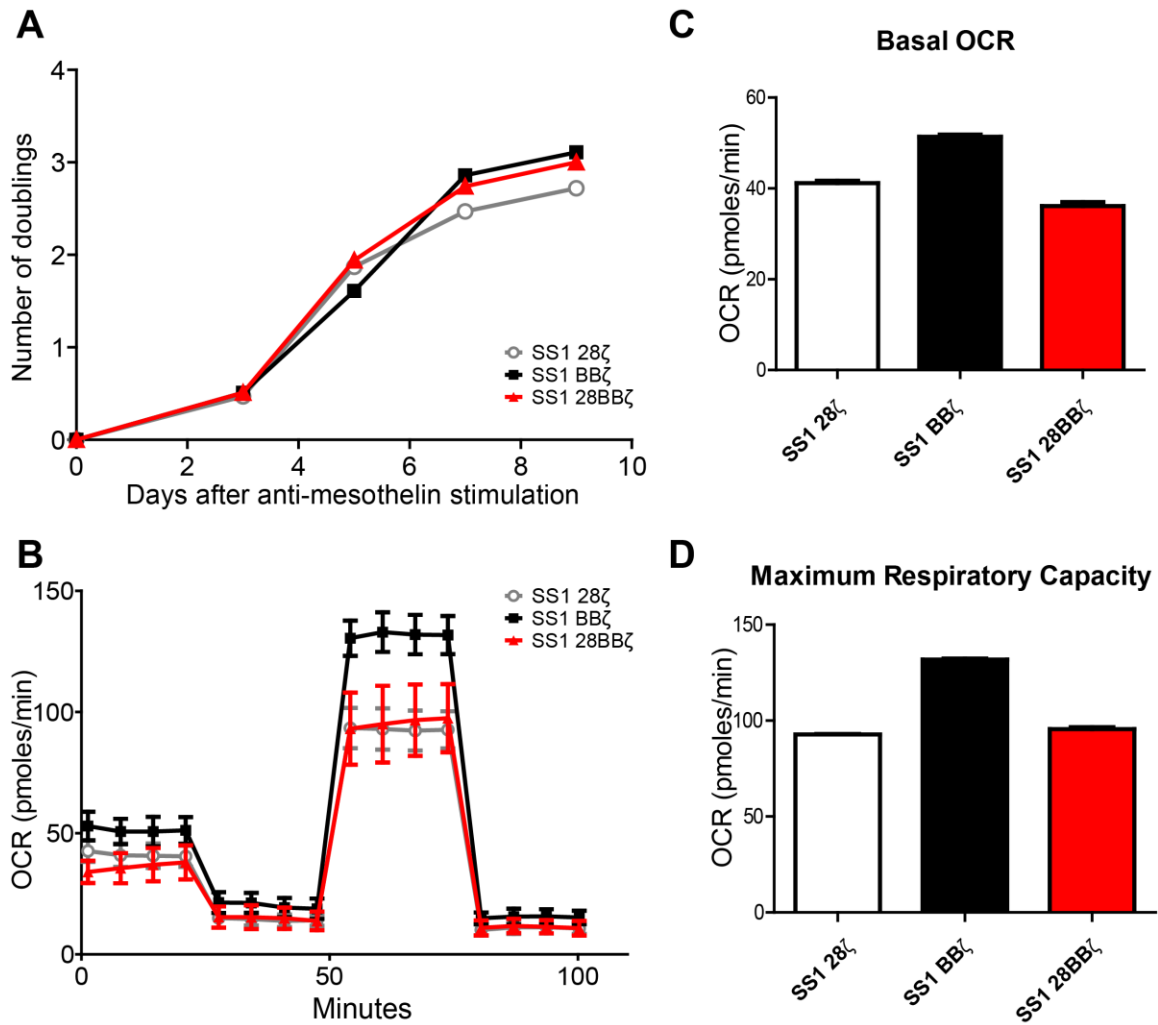


Figure 3.7 Dominant effect of CD28 signaling over 4-1BB.

(A) Schematic of CD19-28BB ζ CAR used to compare growth profiles and metabolic reprogramming of T cells.

(B) OCR profile, (C) basal OCR levels, (D) maximum respiratory levels of the three different CAR constructs compared. Seahorse analysis was performed on day 10 following CAR stimulation.

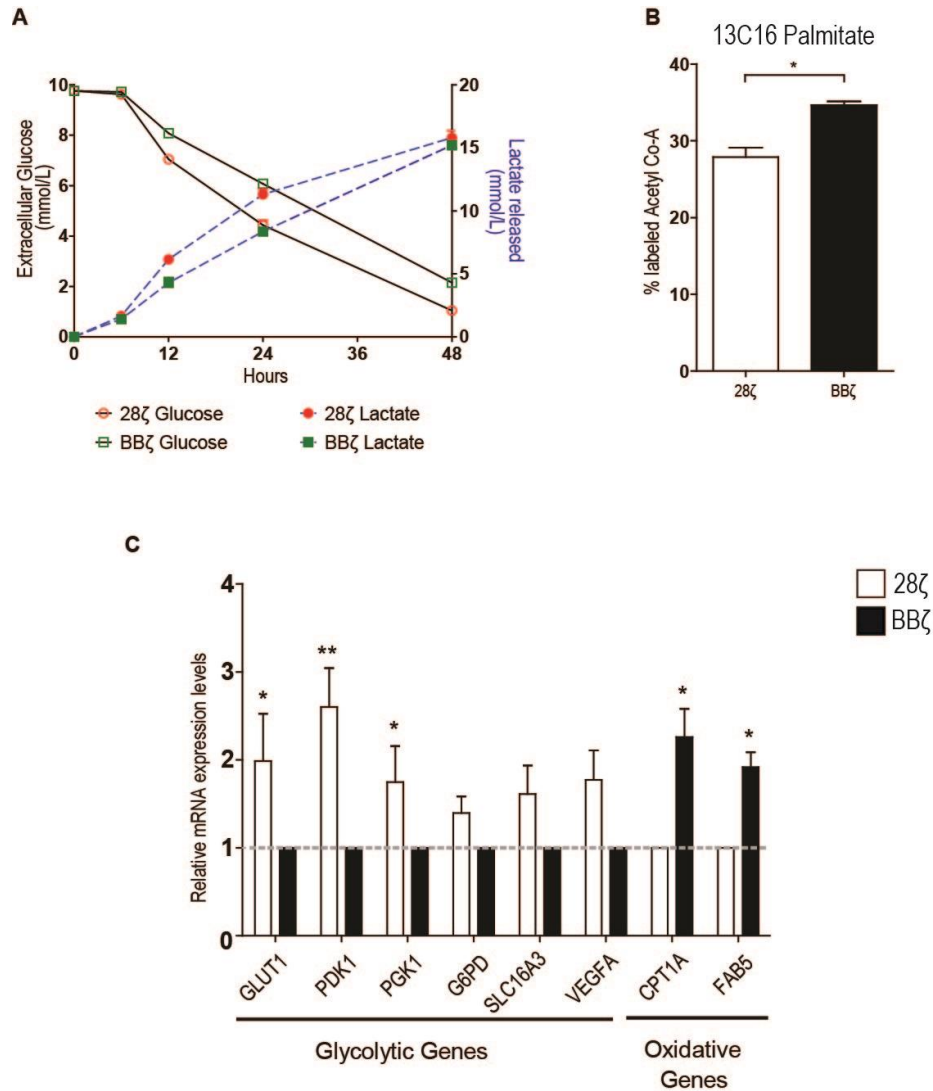


Figure 3.8 Preferential reliance on glycolysis or fatty acid oxidation by CAR T cells.

(A) Measurement of glucose uptake from extracellular media and lactate release into the media over a course of 48 hours.

(B) Percentage of labeled acetyl-CoA measured in T cells cultured with [$^{13}\text{C}_{16}$] palmitic acid to assess fatty acid uptake and breakdown.

(C) Relative mRNA expression levels of genes involved in glycolytic metabolism and lipid oxidation assessed in 28ζ and BBζ CAR T cells. Plot represents data from at least 3 independent experiments using 4 independent donors ($p=**<0.01$; $*<0.05$). Data represented as mean \pm SEM.

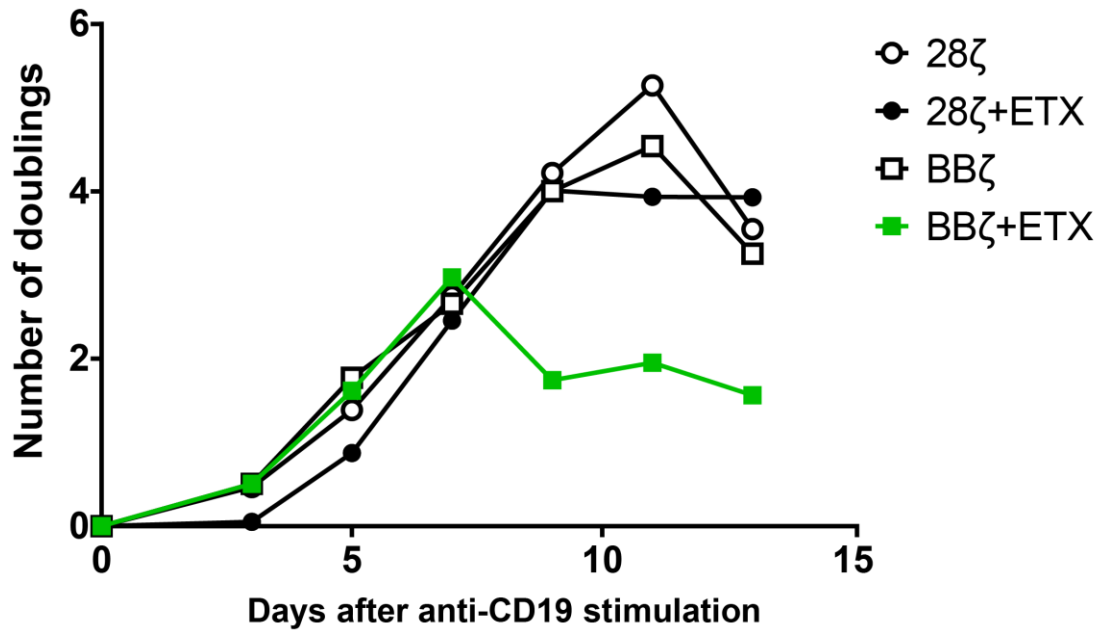


Figure 3.9 Pharmacological inhibition of CPT1A has a detrimental effect on the growth profiles of 4-1BBζ CAR T cells but not CD28ζ CAR T cells.

Growth curve showing population doublings of CAR T cells cultured for 14 days in the presence or absence of Etomoxir, an inhibitor of CPT1A.

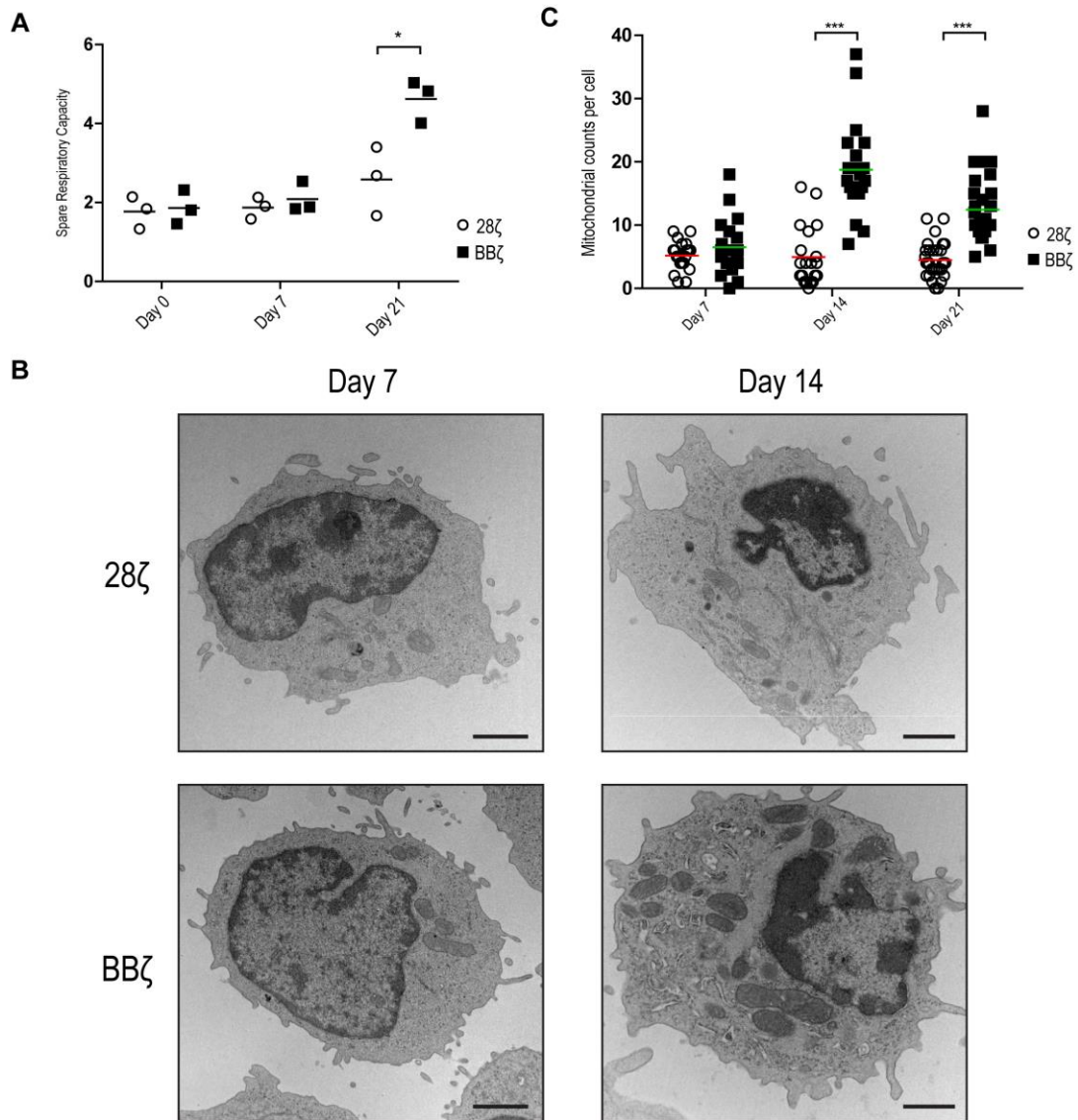


Figure 3.10 Enhanced spare respiratory capacity (SRC) in 4-1BB ζ CAR T cells.

(A) SRC measured as the ratio between the maximum OCR levels after treating cells with FCCP to the basal OCR levels while in culture. Data represents three independent donors tested ($p=*<0.05$).

(B) Transmission electron microscopy of 28 ζ and BB ζ CAR CD8⁺ T cells imaged at three different time points. Scale bars represent 2 μ m.

(C) Enumeration of the individual mitochondrion per cell. Data shown represent 20 individual randomly chosen cells (out of at least 75 cells analyzed per condition), represented as mean \pm SEM ($p=***<0.001$).

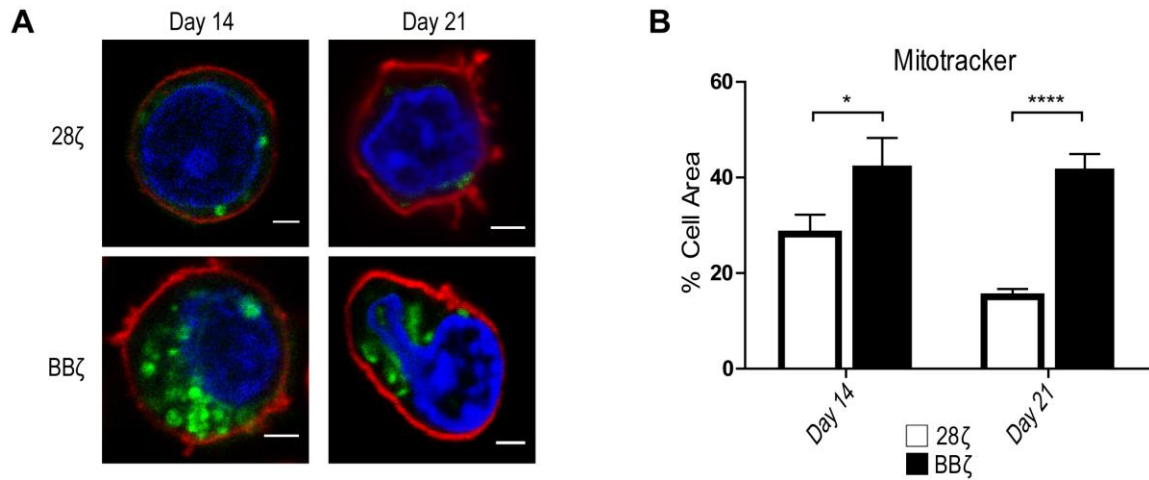


Figure 3.11 Enhanced mitochondrial biogenesis in 4-1BB ζ CAR T cells.

(A) Confocal images stained with Mitotracker (green), DAPI (blue) and a cell-membrane dye DiI (red). Scale bars represent 2 μ m.

(B) Quantification of the percentage of cytoplasm occupied by mitochondria, measured as percentage of Mitotracker (green) within area enclosed by the cell membrane (red). Data represented as mean \pm SEM from at least 3 images each at specified time points with at least 15 independent cells scored per image. ($p=****<0.0001$).

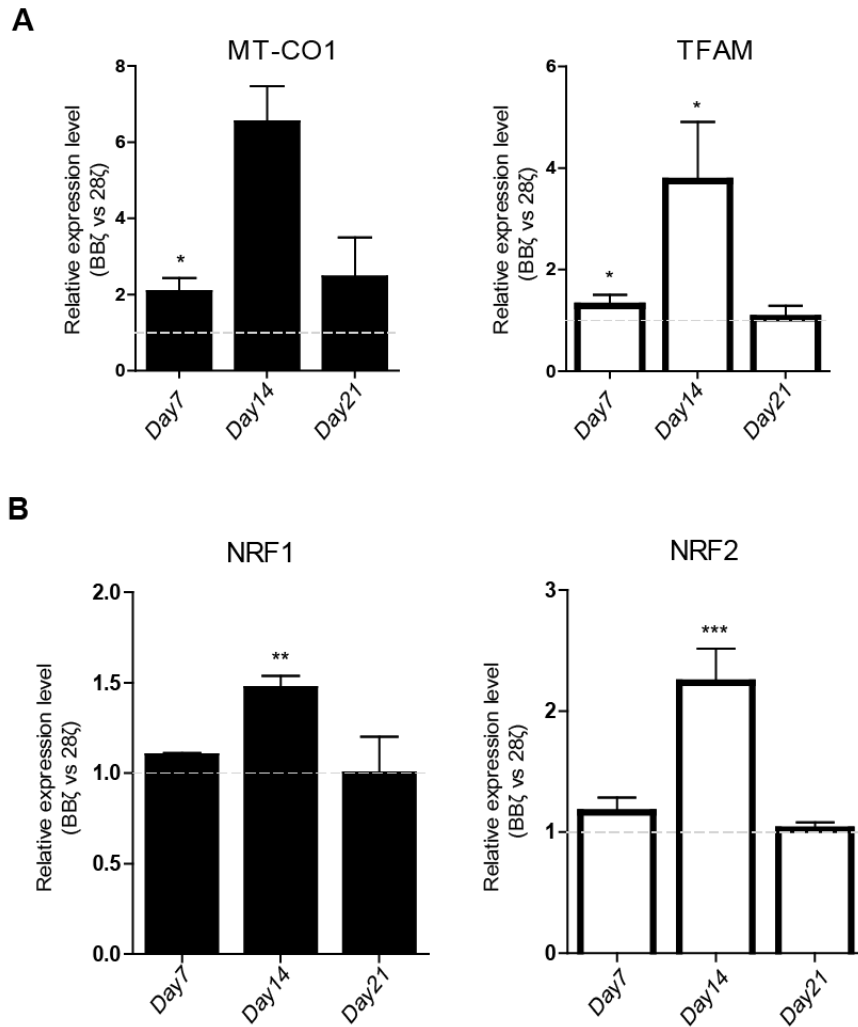


Figure 3.12 Mitochondrial biogenesis in CAR T cells is regulated at a genetic level.

(A) Relative mRNA expression of mitochondrial cytochrome c oxidase 1 (MT-CO1) and mitochondrial transcription factor A (TFAM) in BBζ CAR T cells normalized to expression levels of 28ζ CAR T cells at specified time points. Data generated from at least 3 independent experiments using 4 independent donors (*, $p < 0.05$), represented as mean \pm SEM.

(B) Normalized mRNA expression levels of Nuclear Respiratory factor-1 (NRF1) and GA-binding protein (NRF2) in BBζ CAR T cells as compared to 28ζ CAR T cells at specified time points. Data generated from at least 3 independent experiments using 4 independent donors (*, $p < 0.05$), represented as mean \pm SEM.

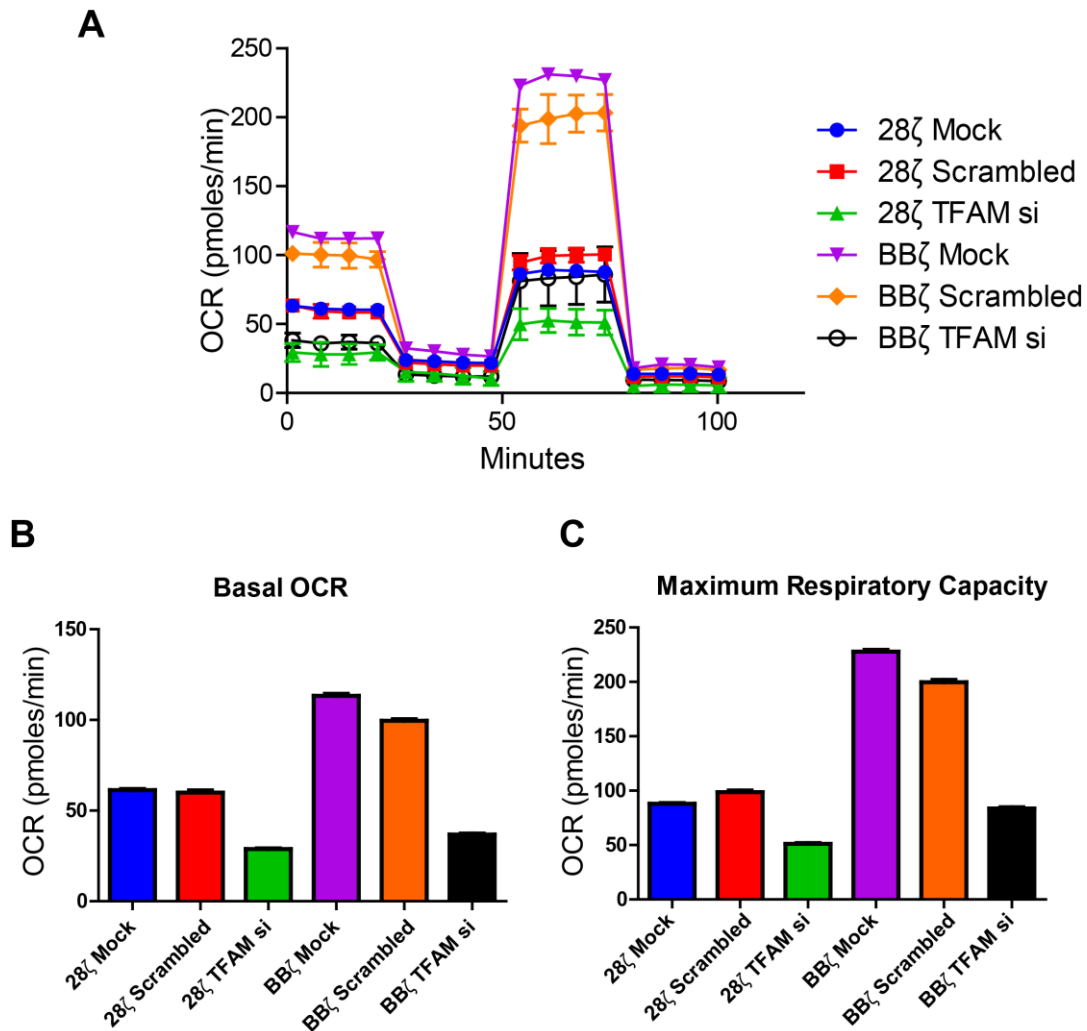


Figure 3.13 Marginal knock down of TFAM significantly lowers oxygen consumption rates of BBζ CAR T cells.

(A) Oxygen consumption rates of CAR T cells electroporated with scramble siRNA or siRNA pool specific for TFAM. siRNA electroporation was done on day 7 post CAR stimulation and seahorse analysis was performed 3 days later. Unelectroporated control referred to as Mock.

(B) Basal OCR and (C) maximum respiratory capacity of CAR T cells as specified in the graphs.

Table 3.1 Population doublings and cell yield of T cells expressing a CD19- or mesothelin-specific CAR coupled to CD28 or 4-1BB signaling domain.

Donor #	CAR	Number of days in culture	Total population doublings	Cell yield (x10 ⁶ cells)
1	CD19 28ζ	20	4.3	78.8
	CD19 BBζ	22	5.0	128.0
2	CD19 28ζ	22	6.0	256.0
	CD19 BBζ	28	7.2	588.1
3	CD19 28ζ	24	6.9	477.7
	CD19 BBζ	30	10.3	5,042.8
4	SS1 28ζ	12	5.8	222.9
	SS1 BBζ	24	8.8	1,782.9
5	SS1 28ζ	16	6.9	477.7
	SS1 BBζ	24	8.4	1,351.2
6	SS1 28ζ	14	6.0	256.0
	SS1 BBζ	22	8.4	1,351.2

Table 3.1 Healthy donor T cells were electroporated with the indicated CAR, and stimulated with anti-idiotypic (CD19) or recombinant mesothelin Fc. The cells were counted and maintained in culture until at least two consecutive declines in cell numbers were observed. The last column shows the total number of cells obtained at the end of expansion, starting with 4x10⁶ cells in each group. SS1 is a mesothelin-specific scFv.

Chapter 4

DISTINCT SIGNALING PATTERNS BETWEEN FIRST AND SECOND GENERATION OF CARs IN PRIMARY HUMAN LYMPHOCYTES

Summary

Chimeric antigen receptors (CARs) endow T cells with antibody-like specificity for cell surface antigens and promote the activation and differentiation of antigen-stimulated T cells. CARs targeting the pan-B cell marker CD19 have advanced to phase 2 clinical trials against B-cell acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL). CAR T cells with the 4-1BB signaling domain have demonstrated relatively higher clinical success in terms of tumor clearance, T cell persistence, and sustained remission in patients as compared to CD28 containing CAR T cells. Despite extensive clinical use, the molecular mechanisms involved in the activation of CAR T cells remain elusive, and it is unclear how the choice of CAR costimulatory molecule influences those mechanisms. We hypothesized that CARs take advantage of the endogenous T cell receptor (TCR) signaling pathways in a manner unique to their analogous intracellular domains. Using CARs specific for mesothelin and CD19, we compared the activation states of CAR T cells following cognate antigenic stimulation *in vitro*. We report that CARs with the CD28 intracellular domain (ICD) exhibit stronger T cell activation when compared to CARs with the 4-1BB ICD as measured by analysis of certain kinases and proximal signaling proteins. Stimulation of different CAR variants revealed that the antigen-specific activation threshold for CAR T cells is greatly reduced when the CD28 ICD is included in the CAR architecture. T cell activation state,

measured by the activation of proximal signaling proteins, as well as the MAPK and Akt signaling pathways, demonstrated increased and sustained stimulation in T cells with the CD28 ICD. Additionally, T cells with CARs containing CD28 ICD showed a high and sustained level of calcium flux in comparison to CAR T cells with the 4-1BB ICD, although no significant difference in NFAT activation was detected between these constructs. Taken together, these studies link the strong stimulation of CD28 CAR T cells to the reduced persistence, which adheres to the signal strength theory. These findings should have significant impact on the future design of CARs and adoptive immunotherapy.

Introduction

T cells, when activated through recognition of foreign peptide sequences proliferate and exert cytotoxic functions in the human body. These peptides can only be recognized in the context of Major Histocompatibility Complex (MHC) molecules on the surface of antigen presenting cells. MHC complexes, classified as I and II, specifically engage with CD8 or CD4 T cells, respectively. In cancer, presented MHC-peptide complexes are usually recognized by the immune system as self-peptides and fail to activate a large proliferative or cytotoxic response. Tumor infiltrating lymphocytes (TILs) that have demonstrated promise in melanoma and personalized TIL therapy should and will be explored further (Alexandrov et al., 2013). However, TILs for other tumor histotypes have not shown significant promise, either because the tumors are not immunogenic or the TILs expand poorly ex-vivo. In contrast, antibodies against tumor-associated antigens are abundant and novel antibodies are continuously generated. Combining the

cytotoxic power of T cells with the abundance of tumor antigen specific antibodies led to the development of chimeric antigen receptors (CARs).

CARs were developed to significantly increase the repertoire of potential tumor-targeting T cell therapies by taking advantage of the large number of anti-tumor antibodies. These antibody-based immune receptors were built in the first generation model as fusions of tumor-targeting scFvs to the FcR γ or CD3 ζ subunits of the Ig or T cell receptor. These chimeric receptors redirect T cells to scFv-specified antigens in a MHC-independent fashion (Eshhar et al., 1993). Second-generation CARs included the intracellular domains of costimulatory molecules in cis with the CD3 ζ domain and membrane proximal into the structure (Marin et al., 2007). CARs only require interaction with cognate antigen for T cell activation, in contrast to the MHC-peptide complex and co-receptor required by the TCR.

Phase I and Phase II clinical trials have explored the use of CAR T cells against B cell malignancies, and now against solid tumors as well. Using the FMC63 scFv, which targets the extracellular domain of the pan-B cell marker CD19, multiple groups have demonstrated tumor rejection in acute lymphoblastic leukemia and chronic lymphocytic leukemia (Brentjens et al., 2011; Grupp et al., 2013; Porter et al., 2011b). However, there are differences that exist between CAR usage in the second-generation costimulatory domain, the method of genetic introduction of the CAR, and in the T cell expansion protocol (Gill and June, 2015). There are also differences in the response of CAR therapy, measured by persistence of such genetically modified cells, patients' overall

survival, and patients' relapse-free survival. In particular, CARs containing the 4-1BB signaling domain demonstrate long-term persistence, which in some patients has been up to four years (Porter et al., 2015). On the contrary, CARs with CD28 signaling domain have not shown such superior persistence (Brentjens et al., 2013; Lee et al., 2015).

One important question to answer within the CAR field is how the CAR functions. Since their inception, it has been assumed that CARs employ the endogenous proximal and distal signaling machinery of the TCR. Here we investigate this assumption, measuring the immediate signaling events that occur during CAR activation, and we compare the signaling cascades of CD28 and 4-1BB costimulated CARs to understand why 4-1BB costimulation provides enhanced T cell survival.

Results

28 ζ CAR shows robust signal transduction in primary human T cells as compared to BB ζ or ζ alone.

We investigated the signaling proteins involved during the activation of T cells via stimulation through the CAR specifically. A schematic of the CAR constructs used in the study is shown in Figure 4.1A. By electroporation of *in vitro* transcribed RNA, surface expression of CARs on primary human T cells was measured (Figure 4.1B). Cells with no CAR (labeled as Mock) served as a negative control. Cell lysates from these stimulated cells were analyzed for proximal signaling proteins, such as ZAP70. ZAP70 is a member of the Syk family of kinases, which play an important role in activation of lymphocytes and mediate subsequent signaling events. Stimulation with cognate antigen, using beads coated with an anti-idiotypic to the FMC-63 scFv beads, generated high-level

phosphorylation of ZAP70, an important proximal signaling initiator of the TCR signaling pathway, in the 28 ζ CAR versus the ζ or BB ζ CARs (Figure 4.2A). Analysis of distal signaling proteins in the MAPK pathway revealed high levels of phosphorylated Erk in the 28 ζ CARs (Figure 4.2B). All cells were capable of being activated as shown by CD3/28 and PMA/Ionomycin stimulation controls. Analysis of unstimulated cells served as a negative control showing no basal levels of activation in these T cells.

Rapid and sustained signal transduction in 28 ζ CAR T cells.

To assess the stimulation threshold of the different CAR signaling domains, we performed a time course of stimulation of these CAR T cells. The cells were co-cultured with antigen-coated beads *in vitro* for a period of 1, 5, 10, 15, 30, 60 and 120 minutes. The cell lysates were analyzed for proximal and distal signaling proteins as performed above. The 28 ζ CAR showed rapid and intense signs of activation (Figure 4.3). ZAP70 was phosphorylated immediately even after 1 minute of CAR-specific stimulation in the 28 ζ CAR, whereas the ζ and the BB ζ CARs did not show such activation until 5 minutes or later (Figure 4.3). Phosphorylation of phospholipase C gamma (PLC γ), a crucial signaling mediator of the calcium-signaling pathway was also high in the 28 ζ CAR T cells, as compared to BB ζ or ζ . Analysis of distal signaling proteins Erk and Akt in the MAPK and Akt pathway, respectively revealed similar rates of phosphorylation events (Figure 4.4A and 4.4B). This suggests that the 28 ζ CAR transduces the initial activation signals more rapidly as compared to the BB ζ CAR.

28 ζ shows intense calcium flux upon stimulation in primary human T cells.

Changes in free intracellular calcium (Ca^{2+}) play a key role in the activation and homeostasis of T cells. Engagement of TCR/MHC complexes triggers the phosphorylation and activation of PLC γ , which ultimately leads to Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry from the extracellular space (Ledbetter et al., 1987). The dynamics of this Ca^{2+} signal (i.e. duration, amplitude, and frequency) determine the pattern of transcription factor activation and gene expression that ultimately influences T cell activation, proliferation, and apoptosis (Lewis, 2001). Since strength of proximal signaling varied between the CAR groups, we proceeded to explore how this may have an effect on calcium mobilization in T cells post CAR stimulation. We observed that SS1-28 ζ CARs produce an intense, detectable calcium flux response in T cells, similar to stimulation of the TCR by OKT3 (Figure 4.5). SS1BB ζ and SS1 ζ did not demonstrate a detectable calcium flux by flow cytometry with soluble surrogate antigen. To assess the difference in calcium flux in individual cells, we labeled CAR-T cells with the ratiometric calcium-binding dye Fura-2 and stimulated with either mesothelin-Fc coated beads or K562 cells overexpressing mesothelin. After perfusing Ca^{2+} containing media on the cells, SS1-28 ζ CAR-T cells demonstrated a stronger and more prolonged calcium flux response than SS1-BB ζ (Figure 4.6A and 4.6B). Taken together, these results demonstrate that SS1-28 ζ CAR-T cells exude a pronounced calcium signal with surrogate antigen stimulation not observed with SS1-BB ζ CAR-T cell stimulation. In addition, CD19-28 ζ CAR Jurkat T cells exhibited a similar increase in calcium flux amplitude when stimulated with anti-idiotypic coated beads compared to

CD19-BB ζ CAR Jurkat T cells (Figure 4.6C). This difference in calcium flux intensity was not ameliorated in JRT3 Jurkat T cells, which lack the beta chain of the TCR and thus a membrane associated TCR.

Point mutations in cytoplasmic tails of second generation CARs reveal interesting results

To extend our studies to other CAR models currently being tested in the clinic, we compared signaling through a CAR containing the ICOS costimulatory domain. ICOS (inducible costimulator) is a member of the CD28 family and is constitutively expressed on T_H17 cells (Hutloff et al., 1999). Preclinical studies with ICOS signaling domains in CARs have indicated extensive proliferative and cytotoxic capacity (Guedan et al., 2014; Paulos et al., 2010). We compared the effects of ICOS signaling domains on the metabolic fates of CAR T cells. Using a mesothelin-specific CAR containing the 28 ζ , BB ζ , ICOS ζ and 28BB ζ we analyzed the mitochondrial OCR using the seahorse flux analyzer on day 7 post CAR stimulation. We noticed that the basal OCR for ICOS ζ was higher than its CD28 counter-part, much like that of BB ζ (Figure 4.7A). The maximum respiratory level of ICOS ζ was also elevated compared to the other three CARs compared, signifying increased oxidative-based metabolism by these cells. To further investigate the significance of 28 ζ intracellular domain, we made point mutations in various tyrosine residues that are known to be important phosphorylation sites for the docking of known signaling molecules. Mutation of the prolines in the distal SH3 domain (PYAP) that bind Lck, Grb2 and filamin-A resulted in increased basal and maximum OCR (Figure 4.7B). This increase was even more significant when the tyrosine residues

in that distal motif were substituted (Y188F and Y200F). Mutation in the Itk binding site (PRRP) yielded no such changes. Substitution of the tyrosine residue (Y170F) in the proximal YMN motifs that is the docking site for p85, a subunit of PI3K, also increased the basal and maximum OCR, but changing the asparagine residue (N172Q) had no effect at all signifying a possible role of the PI3K-mediated signaling in modeling the metabolic profile of CAR T cells.

The native ICOS cytoplasmic domain contains the YMF motif in the corresponding region of CD28's YMN motif that binds to PI3K but not Grb2 (Yoshinaga et al., 1999). We made two mutant CARs with substituted tyrosine and phenylalanine residues. Mutation of phenylalanine (F181N) resulted in decreased oxidative consumption both at the basal and maximum levels as compared to wild-type ICOS ζ CAR (Figure 4.7C). On the contrary, abrogating the PI3K binding site at the tyrosine residue (Y179F), significantly enhanced the basal OCR as well as the maximum respiratory levels. These data are representative of analyses done using 2 independent healthy human donors. These promising results however must be looked into further to tease out the important signaling cascades initiated by these novel CAR constructs.

Discussion

The functional efficacy of CAR T cells has been shown in multiple preclinical and clinical studies using various tumor models in recent years. It therefore stands to reason that understanding the true mechanism that dictates CAR T cell effector function and survival in vivo is vital. Although various combinations of co-stimulatory signaling domains have been tested in CAR designs, CARs evaluated preclinically and clinically

have predominantly focused on two protein families – the CD28 superfamily, including CD28 and ICOS, or the tumor necrosis factor receptor superfamily, including 4-1BB, CD27, and OX40. Here, we systematically evaluated the functioning of the two most commonly employed co-stimulatory domains – CD28 and 4-1BB. We used a RNA electroporation platform to obtain CAR transgene expression greater than 95% in primary human T cells. By in vitro assays, we identified some signaling differences in 28 ζ and BB ζ CARs, which remained consistent across the scFv's tested. Stimulation of primary human T cells expressing 28 ζ CARs induces rapid and robust initiation of signaling cascades. These results provide valuable insights to the differences in clinical functionality observed between these CAR models.

Endogenous 4-1BB signaling appears to play a key role during secondary T cells responses and is important for T cells sustenance, more so than CD28-mediated stimuli (Bertram et al., 2004; Dawicki et al., 2004). Further supporting this notion, reactivation of tumor-infiltrating lymphocytes with 4-1BB has shown a fair decrease in AICD (Hernandez-Chacon et al., 2011). Conversely, secondary stimulation through CD28 alone, in the absence of CD3 does not yield great T cell proliferation and survival results (Siefken et al., 1998). Notably, studies have shown that that a secondary stimulation of pre-activated T cells through CD28 alone induced Fas-dependent (Collette et al., 1998) or Bax-mediated (Boussiotis et al., 1997) T cell apoptosis due to increased activation induced cell death. One could postulate that the percentage of pre-activated cells surviving and proliferating in response to a second costimulatory signal alone may be higher for 4-1BB as compared to CD28. Data from Chapter 3 also supports the

differences in survival of T cells stimulated through CARs containing either of these two costimulatory domains. This observation, at least in part, also explains the difference in longevity of CD28 (Davila et al., 2014) and 4-1BB-based (Grupp et al., 2013; Kalos et al., 2011a; Porter et al., 2011b) CAR T cells in treated ALL and CLL patients. Since T cells undergo an initial activation step *ex vivo* before the gene-transfer step, the CAR-mediated signaling provides a secondary signal. Combining the effects of endogenous CD28 signaling referred above to our findings, one could hypothesize that the rapid signal transduction seen in CD28-based CAR T cells may be hampering T cells survival and proliferation.

Another possible explanation for the observed differences in longevity of CD28 and 4-1BB based CAR-T cells could relate to differences in the CARs ability to mobilize Ca^{2+} . Although Ca^{2+} plays an important role in T cell activation and proliferation, increased Ca^{2+} signaling may lead to increased apoptosis among activated T cells. Indeed, Orai1-dependent Ca^{2+} entry and NFAT activation have been shown to be crucial for activation of downstream cell death programs in effector T cells (Kim et al., 2011). Although we were unable to demonstrate differences in NFAT activation, the observed differences in Ca^{2+} signaling patterns may ultimately lead to changes in the balance of pro- and anti-apoptotic gene transcription as a result of excessive NFAT activation over multiple CAR-T cell and effector cell interactions. Furthermore, Ca^{2+} is also able to influence the activation of other transcription factors that play an important role in T cell apoptosis including canonical NF- κ B and MEF2 signaling (unpublished data, (Byrum et al., 2013)).

Although much investigation remains to be conducted before a CAR signaling model can be sketched out, these initial findings pave the way to postulate a model. Considering a CAR is constructed using native stimulatory molecules of a T cell, TCR-associated proteins will likely participate in CAR signaling. For example, CD28 contains several intracellular motifs that are critical for its effective signaling (June et al., 1990). Two dominant signaling cascades are initiated during CD28-mediated activation. One involves the phosphorylation of tyrosine residue within the membrane proximal YMNM motif that leads to the recruitment SH2-domain containing proteins, especially PI3K, Grb2 and GADS (August et al., 1994; Schneider et al., 1995). Activation of PI3K initiates the mTOR pathway resulting production of survival elements such as Bcl-xL, Bcl2 as well as the production of IL2 and other proliferative signals (Vanhaesebroeck and Alessi, 2000). The other pathway is initiated by two distal motifs that are proline-rich and bind to Itk and Lck via SH3-domain. Activation of these domains is responsible for bringing Lck and lipid rafts into the immune synapse via filamin-A (Hayashi and Altman, 2006). PI3K-dependent signaling is likely the major pathway initiated by CD28 signaling and given our findings, one could speculate that a CD28-containing CAR signals through this pathway well. Experiments done in conjunction with these studies also tested the activation of the second pathway mentioned above, but further investigation is currently underway.

Native 4-1BB, on the other hand, signals through a different set of signaling proteins, although there is some overlap with the CD28-mediated activation pathways. As a prominent member of the TNFR-family, 4-1BB mediates T cell activation through

pathways involving NF- κ B, JNK, p42/p44 mitogen activated protein kinase (MAPK) and p38 MAPK(Aggarwal, 2003). It primarily recruits specific TNFR-associated factors (TRAFs), a family of signaling adapter that link TNFRs to NF- κ B and stress kinase signaling pathways(Arch and Thompson, 1998). Two members of the TRAF family, TRAF1 and TRAF2, are critical for interaction with and the proper downstream signaling of 4-1BB, since their absence severely impairs ERK activation. Some preliminary co-immunoprecipitation experiments done in parallel to this thesis project have revealed direct interaction of the 4-1BB containing CAR with TRAF2, suggesting a possible mechanism similar to that of endogenous 4-1BB, and this needs to be evaluated further.

A major consideration in drawing out possible signaling models of CAR costimulatory domains is the very nature and conformation of these coreceptors in their native environment. Endogenous CD28 for example exists and signals as a disulfide-linked homodimer(Greene et al., 1996). Although this oligomerization state is not a prerequisite for CD28 signaling, interruption of the sulfide bond leads to suboptimal costimulation of the immununological synapse(Lazar-Molnar et al., 2006). Similarly, 4-1BB exists as a trimeric molecule in its native form in order to initiate a complete signaling cascade in T cells(Won et al., 2010). Whether all CARs containing these domains also interact with each other in a similar manner on the T cell surface is not confirmed, although there are some studies hinting towards possible dimerization of CARs(Bridgeman et al., 2010).

In summary, our results confirm that the signal transduction pathways CAR ICDs propagate are comparable to those pathways initiated by their endogenous homologues in

primary human T cells. The key findings uncovered here are the differences in activation status and sustenance of these activation signals by different class of CAR costimulatory domains. By extension, these initial bursts of signals can have profound effects on the overall activation and function of these cells, especially when used as a therapeutic tool. These findings should be taken into account when designing such synthetic molecules to target desired tumor antigens for clinical investigations.

Experimental Procedures

CAR constructs

A schematic of the CAR constructs used in the study is shown in Fig 4.1A. This study was conducted using CARs specific to a liquid tumor model (CD19) as well as a solid tumor model (SS1). The CD19 CARs were generated at St Jude Children's Research Hospital (Imai et al., 2004) and as previously described (Milone et al., 2009; Nicholson et al., 1997). The SS1 CAR contained the scFv that recognizes human mesothelin. Maintaining the scFv region constant, we varied the intracellular signaling domains ranging from a first generation CAR (ζ) to second generation CARs (28 ζ and BB ζ), as described earlier (Carpenito et al., 2009; Milone et al., 2009; Zhao et al., 2010). The cDNA for these CAR constructs were subcloned into pGEM.64A based vector by replacing GFP of pGEM-GFP.64A to produce pGEM.64A based CD19 or SS1 vectors. The vectors were confirmed by sequencing.

RNA manufacture and expression

For *in vitro* transcribed (IVT) RNA, the T7 mScriptTM RNA system (Cellscript, Madison WI) was used as per the manufacturer's instructions (and as described previously (Zhao

et al., 2010)). The IVT products were purified with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and the purified RNA was eluted in RNase-free water at 1 µg/µL.

Primary human CD4 T cells were obtained from anonymous healthy donor at the University of Pennsylvania Apheresis Unit. These cells were cultured in R-10 media (RPMI 1640 supplemented with 10% FCS, 100U/mL penicillin, 100µg/mL streptomycin sulfate) in the presence of anti-CD3/28 beads (Life Technologies) over a period of 10-12 days, until the cells rested down to a size of 350fL as described (Levine et al., 1997). To achieve a high efficiency of CAR expression on the cell surface, we resorted to using an electroporation-based system of gene transfer. RNA was electroporated into the pre-expanded human T cells using the BTX CM380 (Harvard Apparatus BTX, Holliston, MA USA) machine as previously described (Zhao et al., 2010).

CAR expression was examined by incubating cells with biotin-labeled polyclonal goat anti-mouse F(ab)₂ antibodies (Jackson ImmunoResearch, West Grove, PA) at 4°C for 30 minutes, followed by two washes with wash buffer (PBS with 3% BSA) and staining with phycoerythrin-labeled streptavidin (BD Pharmingen, San Diego, CA). The expression was then analyzed by flow cytometry on a LSRFortessa (BD Biosciences).

***In vitro* CAR T cell stimulation**

For *in vitro* stimulation of CAR T cells, recombinant mesothelin-Fc fusion protein or anti-CD29 idiotype was coupled with Dynabeads M-450 Tosylactivated (Life Technologies) according to manufacturer's instructions. The coated beads were washed three times in R-10 media before use for *in vitro* stimulation. For stimulation, the CAR T cells were co-cultured with beads at a bead:cells=3:1 ratio at 37°C.

Western blotting

For western blotting, the cell pellets were boiled with 1% SDS lysis buffer (1% SDS, 50mM Tris pH 8.0, 10mM EDTA) for 10 minutes. For normalization of gel loading, the protein lysates were assayed by the Lowry method (Bio-Rad Dc protein assay). These lysates were mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer with NuPAGE Sample Reducing Agent (Life Technologies), boiled for 10 min, and loaded on a 4–12% Bis-Tris polyacrylamide gel (Life Technologies). Separated proteins were transferred to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA) membranes. Antibodies for pZAP70, ZAP70, pErk, Erk, pAkt, Akt and β -actin were purchased from Cell Signaling Technology. Identified proteins were detected with secondary antibodies, anti-mouse IgG and anti-rabbit IgG conjugated with IRDye 800 and IRDye 680 on the Odyssey Imaging system (Li-Cor).

Calcium flux

Flow cytometry-based measurement of cytoplasmic calcium concentration was performed according to a previously published method (Posey et al., 2015). For each sample, 3×10^6 cells were resuspended in 1 ml of culture medium containing Indo-1-AM (1mM; Life Technologies, San Diego, CA) and incubated at 37°C for 30 min. The cells were then washed and stimulated with soluble mesothelin-Fc fusion protein, as well as biotinylated anti-CD3 (OKT3 clone) and streptavidin. The ratio of the two emission wavelengths (405/20 and 530/30 with a 450-nm long-pass filter) was measured over a period of 15 minutes with an LSR II flow cytometer (BD Biosciences, MD) equipped

with a 325-nm laser. Ionomycin (15 $\mu\text{g/ml}$; Sigma-Aldrich, MO) was used as a positive control to confirm proper Indo-1 loading and the functional potential of cells.

To measure intracellular calcium concentration by microscopy, T cells were loaded with Fura-2 AM (1mM, Life Technologies) for 10 minutes at room temperature. Cells were loaded on poly-L lysine coated coverslips and allowed 10 minutes to adhere. While perfused in 0mM or 2mM Ca^{2+} buffer, cells were stimulated with antigen-coated magnetic beads and ratio of Fura-2 Ca^{2+} free fluorescence (380nm Ex; 510nm Em) to Fura-2 Ca^{2+} bound fluorescence (340nm Ex; 510nm Em) was measured over several minutes of observation.

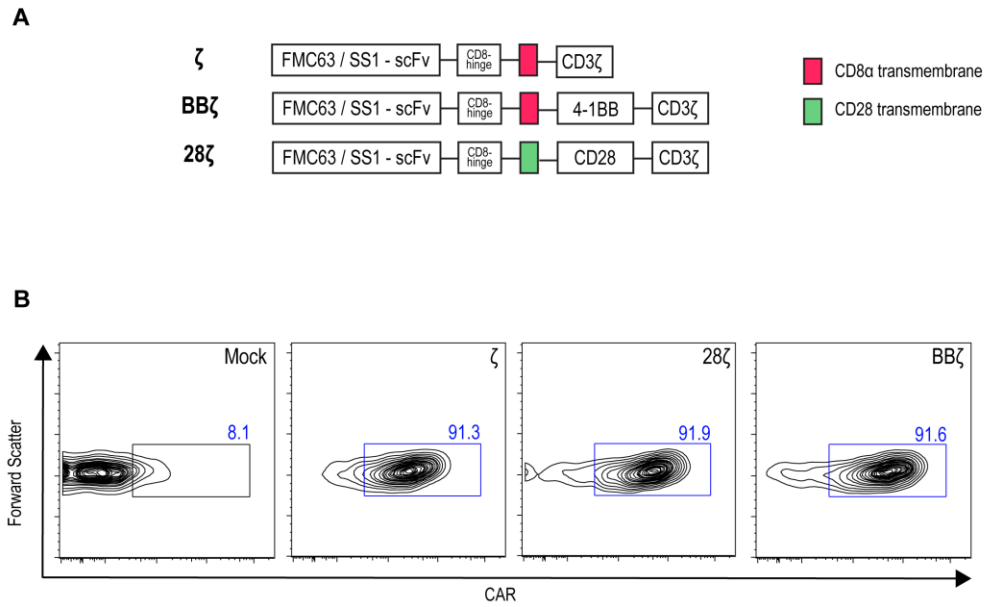


Figure 4.1 Electroporation of in vitro transcribed RNA to generate CAR-positive human CD4⁺ T cells.

(A) Schematic representation of the α -mesothelin or anti-CD19 CAR constructs used in the study.

(B) CAR expression on primary human CD4⁺ T cells. CAR expression was measured 24hours post RNA electroporation by flow cytometry.

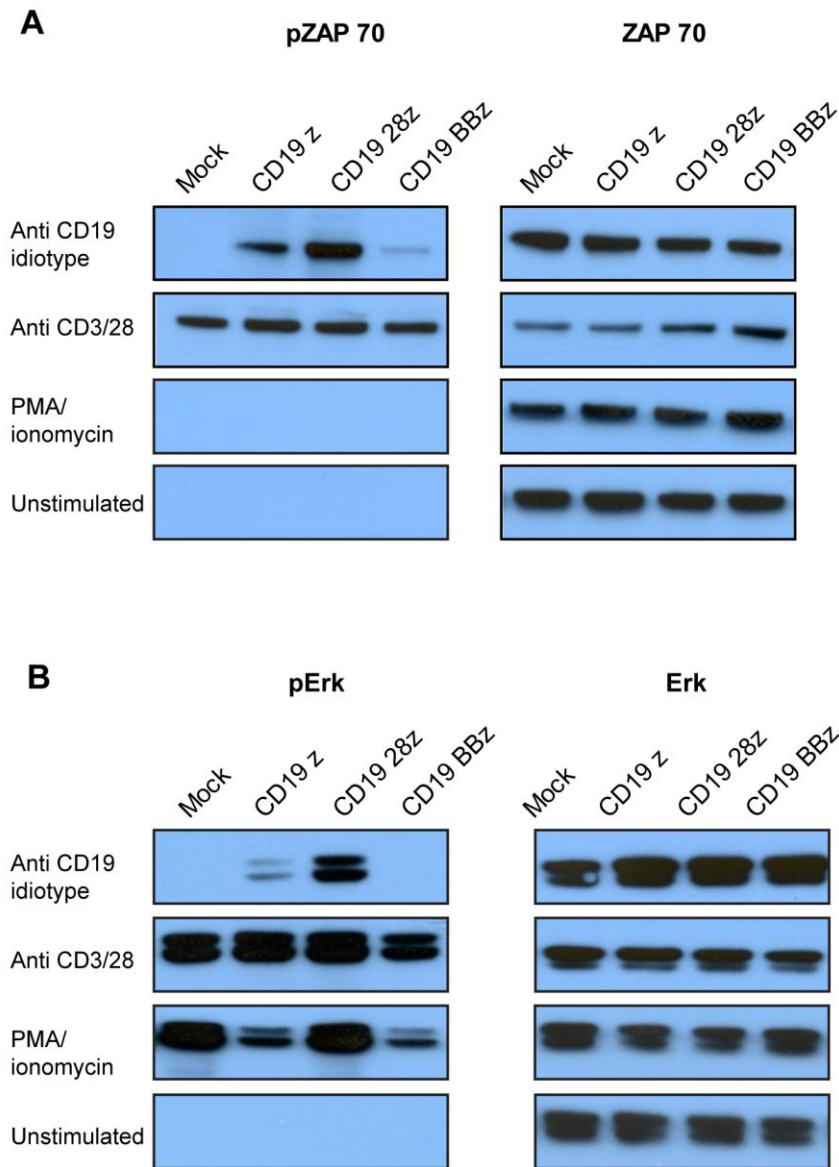


Figure 4.2 Strong induction of phosphorylated proximal and distal signaling proteins in 28 ζ CARs.

CAR T cells were either stimulated with mesothelin-coated beads or α CD3/28 beads for 5 minutes, PMA/ionomycin for 30 seconds, or were left unstimulated. Cell lysates were analyzed for phosphorylated forms of the proximal CD3 ζ -associated protein ZAP70 (**A**) and distal MAPK signaling protein Erk (**B**) by Western blot. Stimulation using mesothelin showed cognate antigen-specific stimulation of CAR T cells, as measured by phosphorylation of proximal and distal signaling proteins.

ZAP 70 phosphorylation

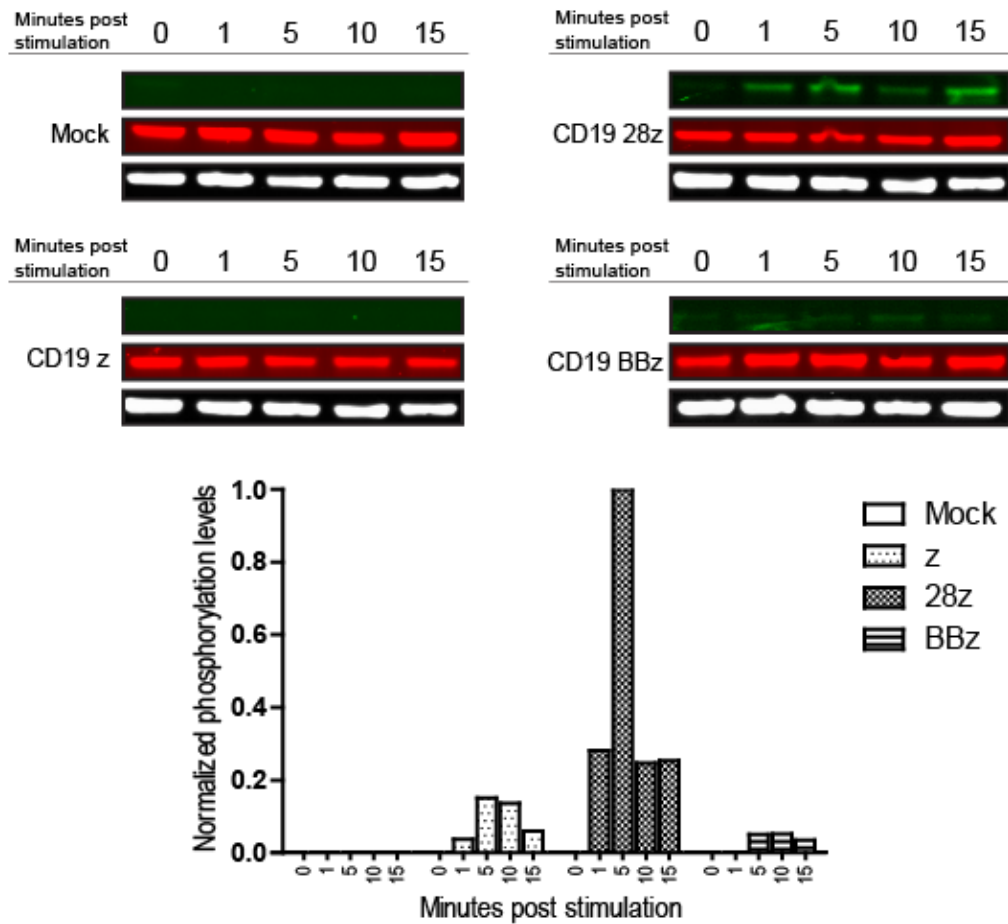


Figure 4.3 Time kinetics of phosphorylation of ZAP70 reveals rapid signal transduction in CAR T cells with the 28 ζ endodomain.

Induction of ZAP70 phosphorylation in CAR T cells after stimulation with cognate anti-idiotype against the CD19 CAR. The phosphorylation levels are quantified in the bottom panel

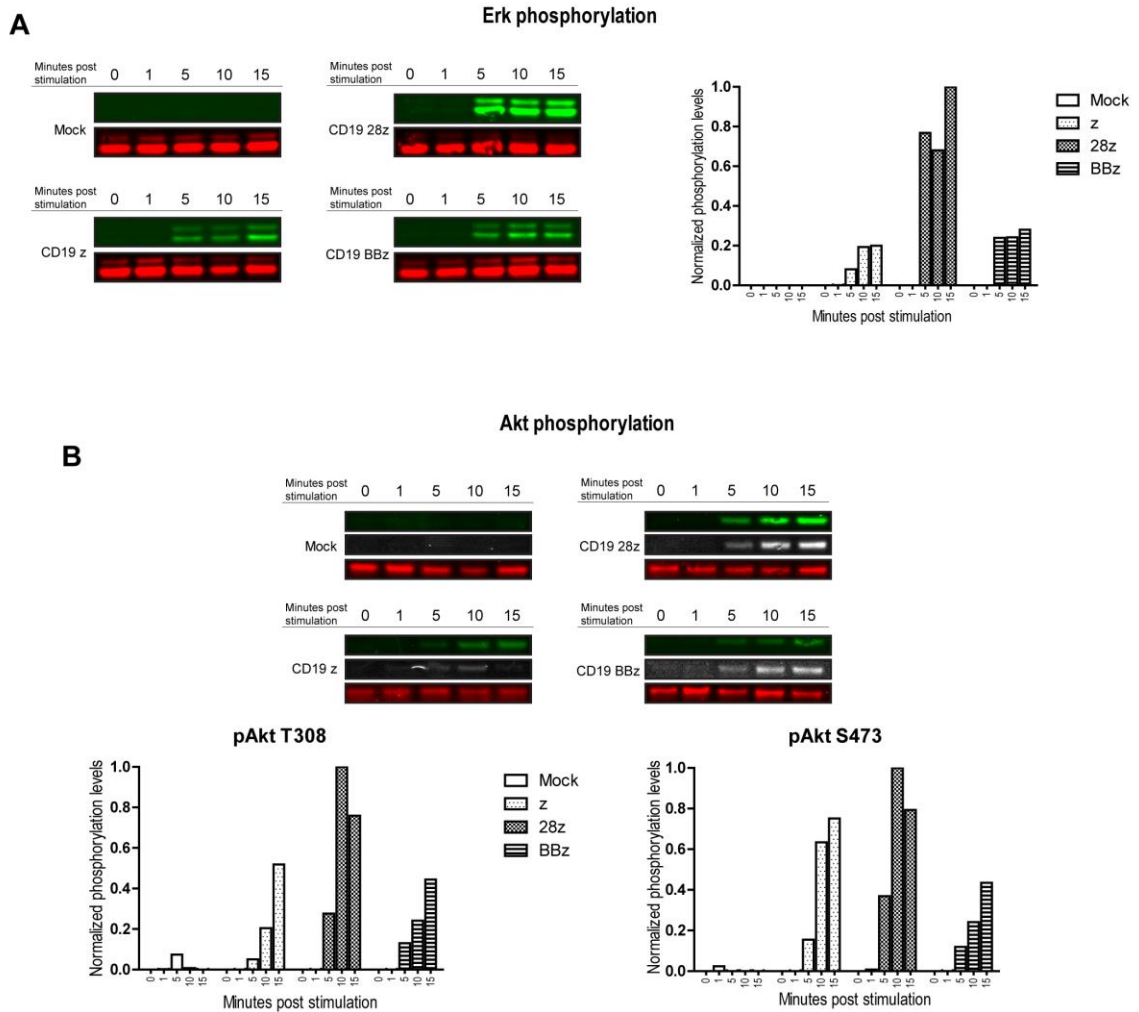


Figure 4.4 Rapid signal induction in 28 ζ containing CAR T cells as measured by phosphorylation of distal signaling proteins.

CAR T cells stimulated at different time-points as indicated were lysed and analyzed for phosphorylated forms of distal MAPK signaling protein Erk (**A**) and both the phosphorylated versions of Akt (**B**). The bar graphs correspond to fold increase in phosphorylation at each specified time point.

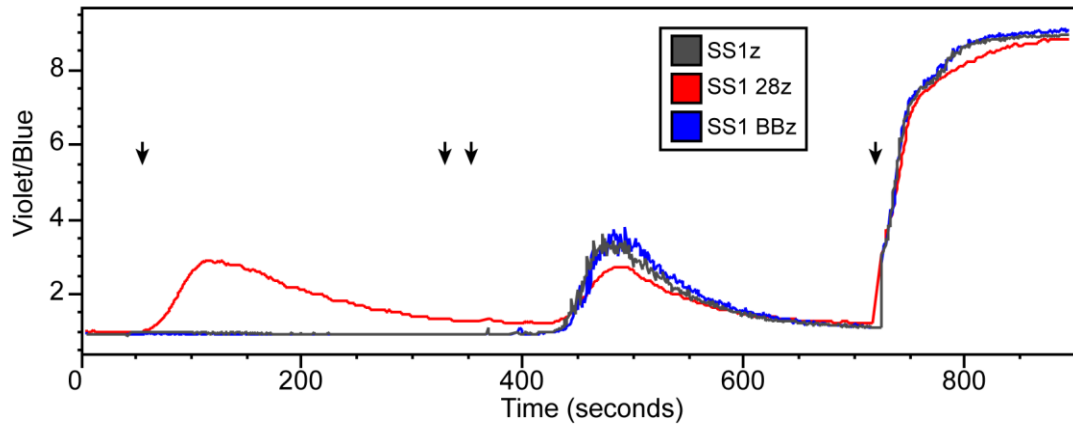


Figure 4.5 28 ζ CAR T cells show intense calcium flux in response to cognate antigenic stimulation

Calcium flux response of CAR T cells after stimulation with soluble antigen-Fc fusion protein depicts a detectable calcium flux only in SS1-28 ζ CARs. All T cell groups demonstrate a calcium flux in response to anti-CD3 ϵ stimulation (OKT3) and the calcium ionophore, ionomycin.

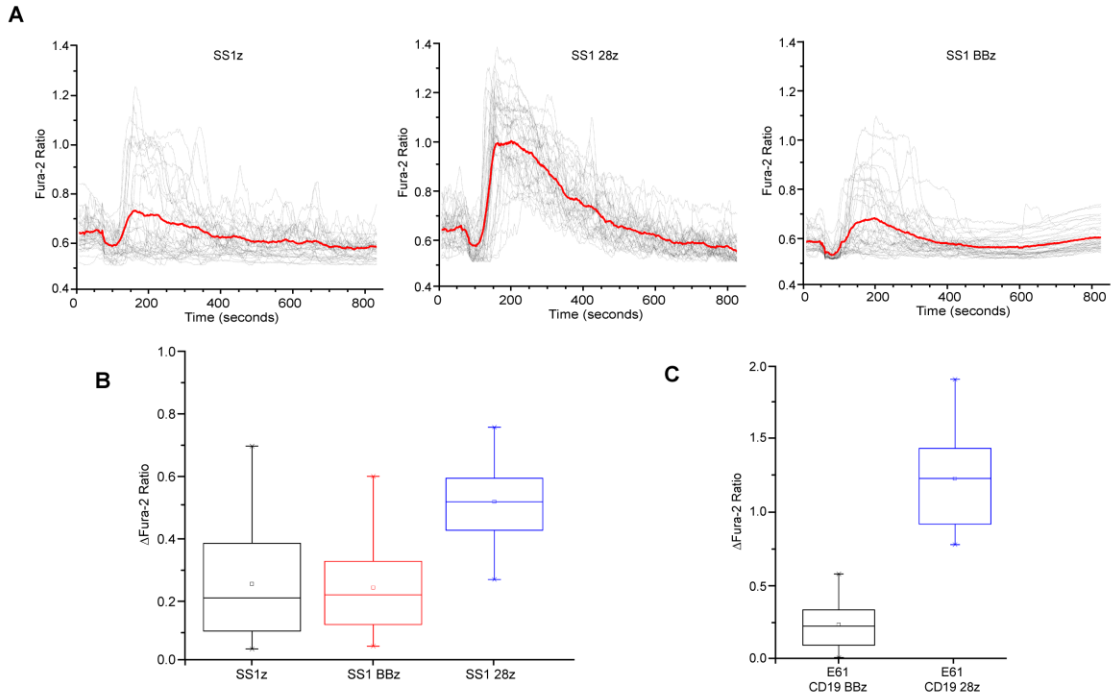


Figure 4.6 Ca^{2+} flux levels induced by CARs with 28 ζ signaling domain are ~2 fold higher

(A) Calcium flux of CAR T cells after stimulation with mesothelin-coated beads using Fura2 ratiometric dye. $[\text{Ca}^{2+}]_i$ is ~2 fold higher in SS128 ζ CAR-T cells in comparison to SS1BB ζ CAR-T cells. The ratio of the increase in fura-2 relative to baseline is plotted for SS1 CAR (B) and CD19 CAR (C).

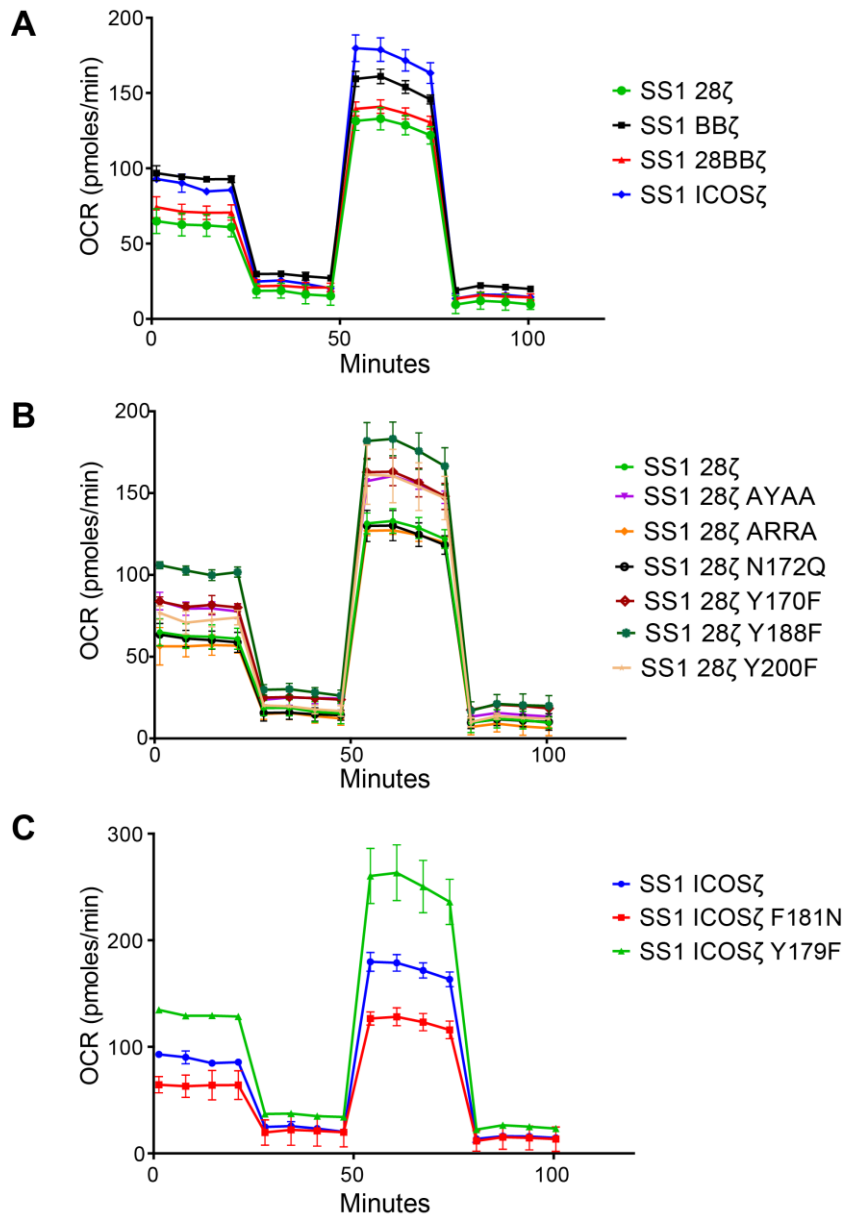


Figure 4.7 Point mutations in CAR signaling domains reveal distinct metabolic profiles.

Oxygen consumption profile measured for (A) three 2nd generation and a 3rd generation CAR, (B) 28ζ CARs and (C) ICOSζ CARs with point mutations as specified in the figure. The location of the amino acids specified in the mutants represent their position in the native forms of these receptors.

Chapter 5

DISCUSSION

Adoptive cell therapy using T cells armed with CARs has come of age with encouraging success in recent clinical trials. The surge of interest in this field can be well explained merely by the number of CAR clinical trials currently being conducted globally. A quick search for the terms “chimeric antigen receptors” on clinicaltrials.gov reveals that there were a total of just five clinical study protocols submitted to the registry between the years 2005 and 2010, the time before this thesis work began. Those same search terms displayed a total of 53 such studies registered between 2010 and the beginning of 2015: a five-fold increase in the last half a decade. As of December 2015, this number has risen to 83 registered clinical investigations at a global scale. These staggering statistics imply the growing interest in this space of cancer therapeutics and emphasize the need for greater understanding of the mechanisms underlying these engineered biological tools.

The premise of adoptive T cell therapy has been to use the patient’s own immune cells and reinvigorate the cell population to detect cancer and destroy it efficiently, with minimal to no damage to normal tissues. The key aspect here being the patient’s *own cells*, i.e, primary human T cells. A major hurdle in investigating the mechanisms of signaling has been the lack of an appropriate model system. The use of immortalized cell lines engineered to express CARs does not replicate the phenomena that occur within primary human cells. Pre-activation of T cells to introduce the CAR transgene using

virus-based system requires stimulating the cells through the endogenous TCR or other mitogens to induce mitosis. Following this, analysis of any CAR-mediated stimulation signal can be misconstrued as being a consequence of the primary activating stimulant. Therefore, the signaling events that specifically occur downstream of CARs cannot be accurately dissected

This thesis addressed these hurdles through the elaboration of a novel method to study the mechanisms of CAR-mediated signaling and activation in primary human T cells. By transient expression of CAR molecules on the cell surface, this patented protocol provides an effective method to study short-term as well as long-term signaling effects of CARs on primary immune cells. The method described in Chapter 2 is a powerful tool to test new CARs at a rapid pace as they are being developed, obviating the need for the lengthy viral-mediated gene transfer protocols. Not only can this method be applied to potentiating the success of ex vivo immune cell expansion, but also permits accurate titration of CAR densities on the cell surface. This could prove invaluable in the context of evaluating receptor affinity and avidity against targets expressing different levels of antigen. Another key advantage of this model is that it obviates the potential risk of chromosomal instability and possible cancerous transformation, which is possible when using integrating viruses for CAR transgenesis (McCormack and Rabbitts, 2004). Overall, this technique provides an excellent model for interrogating the metabolic characteristics and signaling processes of CAR-redirection T cells.

CAR ICDs dictate metabolic fate of human T cells

Thus far, over a 100 patients with CD19+ hematological malignancies have been treated with the anti-CD19 CAR T cells. The impressive clinical results have demonstrated that adoptive cell therapy with CAR-modified T cells hold great promise as a treatment for a number of different cancers. As previously described, the majority of these trials conducted at multiple centers focused on the use of either the 28 ζ or the BB ζ CAR constructs. Chapter 3 demonstrates how a single stimulation, although transient in nature is able to “instruct” proliferative burst and a notable phenotype in primary human T cells. Particularly, this initial signaling event imparts the long-term signaling impact of these CAR ICDs on the expansion kinetics, memory differentiation and metabolic reprogramming of T cells. This dissertation work provides insight into the mechanisms underlying the functional properties of BB ζ CAR-T cells. We provide evidence throughout this dissertation that BB ζ endows CAR-T cells with oxidative features that are permissive for memory differentiation, mitochondrial function, and long-lasting persistence. These findings underscore the importance of optimizing the molecular designs of CARs and the need for in-depth investigation of how CAR ICDs function.

Several clinical studies have demonstrated superior persistence and in vivo expansion of BB ζ CAR T cells (Porter et al., 2015). Studies done with 28 ζ CAR T cells have demonstrated suboptimal in vivo proliferation in leukemic patients (Brentjens et al., 2013; Lee et al., 2015). Our model has recapitulated these clinical observations. One explanation for the differential proliferative rates and prolonged survival can be the distinction in memory differentiation of T cells between BB ζ and 28 ζ CAR T cells. The

finding that BB ζ skews the cell population to a long-lived central memory phenotype whereas 28 ζ promotes an effector phenotype highlights a likely mechanism contributing to differences in survival and anti-tumor efficacy described above (Brentjens et al., 2010; Porter et al., 2011a). These results also have broader implications in predicting the efficacy of ACT treatment. Numerous studies using tumor-specific T cells have reported recurrence or outgrowth of tumor cells. One contributing factor to this observation could be the insufficiency in production of a continuous source of effector progeny. Therefore, if cytolytic T cells persisted longer, they would have a higher chance of mediating durable tumor clearance. However, it is difficult to generalize this for all tumor types. Although resistance to such therapies due to the loss of T cell effector pool has been a significant barrier in solid malignancies, modifications of the CAR architecture have shown great tumor clearance despite suboptimal persistence. For example, Wang et al designed a modified version of the CAR where they fused an scFv to the transmembrane and cytoplasmic domains of KIR2DS2, a stimulatory killer immunoglobulin-like receptor (KIR) (Wang et al., 2015). When expressed on T cells with DAP12, an immunotyrosine-based activation motif-c, these cells inflicted superior antigen-specific effector functions as compared to the conventional second generation CARs despite slightly poorer persistence in vivo. This would imply that although survival of CAR positive T cells contributes to tumor eradication, T cell persistence alone is not a determinant of efficacy. Hence, this complex web warrants further investigation.

The ability to have distinct effects on memory differentiation has implications on whether short-lived or long last immunity is beneficial in certain cancers. These features

can be capitalized upon, if carefully applied in the correct tumor setting. For example, one could speculate that in the case of tumors over-expressing antigens that are also present in low densities on normal tissue, CAR T cells with short-term cytolytic effects may be better tolerated due to potential on-target off-tumor toxicity. On the other hand, in the case of tumors with a high probability of relapse, it would be clinically advantageous to have long-lasting memory CAR T cells.

With the advent of synergistic and combinatorial strategies, our results imply that a mixed population of T cells with different ICDs could maximize benefits of the therapy. Future studies are required to identify the optimal ratio of 28 ζ (supporting rapid effector function) and BB ζ (supporting persistence) CAR-T cells in accordance with the duration and amplitude of cytolytic effects desired. The close association of BB ζ with memory cell differentiation, persistence, and oxidative function is intriguing. Although speculative, an enhanced spare respiratory capacity may provide an energy reserve in BB ζ cells ensuring they can overcome the energy cost of re-exposure to an antigen. These features may contribute to the efficacy of BB ζ CAR-T cells in the treatment of hematological malignancies seen thus far.

A major contribution of this thesis expands on the growing body of work showing how the metabolic aspects of these genetically modified T cells are influenced by the CAR design and how metabolic reprogramming impacts therapeutic efficacy. It is well established that cytoplasmic kinases activated by CD28 and 4-1BB regulate cellular metabolism. CD28 promotes glucose uptake, aerobic glycolysis, and protein translation through activation of PI3K, AKT, and mTOR (Frauwirth et al., 2002). On the other hand,

4-1BB associates with the adaptor proteins TRAF1/2, which drive NF- κ B signaling along with PI3K-AKT-mTOR(Watts, 2005). A recent study demonstrated that 4-1BBL promoted the differentiation of mesenchymal stem cells into thermogenic beige adipocytes(Wu et al., 2012). Additionally, agonist antibodies to 4-1BB improve glucose tolerance and enhanced fatty acid oxidation in obese mice suggesting that this costimulatory pathway may have some unique effects on fatty acid metabolism(Kim et al., 2010). As such, both these pathways can affect metabolism in complex ways. This chapter describes some of the salient features of metabolism and the nutrient requirements of cells driven by 4-1BB compared with CD28 signals in context of an anti-CD19 and anti-mesothelin CARs.

Finally, findings from these studies can be translated towards the optimization of CAR therapy in specific tumor settings. The tumor microenvironment is characterized by limited nutrient and oxygen availability. There is a constant competition for glucose, amino acids, fatty acids and other metabolites between tumor cells and immune cells. More often than not, the tumor outcompetes T cells thereby dampening their cytolytic ability(Sukumar et al., 2015). To overcome this, understanding the preferential metabolic reprogramming of CAR T cells mediated by the intracellular signaling domains can be exploited when designing CAR therapies. For example, studies using antibodies against CTLA-4 have shown restoration of glucose levels in the tumor microenvironment of a murine sarcoma model(Chang et al., 2015). In such a case, one could hypothesize that infiltrating this reconditioned environment with CAR T cells that preferentially rely on glycolysis could be most effective. Similarly, combining other checkpoint blockade

therapies such as anti-PD-1 with CAR T cells strategically designed with the correct signaling domain could potentially improve therapeutic outcomes of CAR T cell therapy.

CAR signaling in T cells – tonic or vital?

The 4th Chapter demonstrates how some CAR ICDs can lead to tonic and persistent signaling in T cells. Although the antigen density used as a stimulant may be vastly different based on the tumor type in the physiological setting, the model here used to activate T cells through the CAR is consistent with the ex vivo configurations applied in the clinical protocols. We observe that 28 ζ CAR T cells have a much more rapid response to antigenic stimulation relative to BB ζ or the ζ chain alone. This lends well to the notion that CD28 plays an early role in T cell activation and carrying out clonal expansion, while 4-1BB plays a role in later stages to sustain immune responses (Cannons et al., 2001; Vallejo, 2005; Watts, 2005). Here, we show that stimulation of CAR T cells with cognate antigen phosphorylates both proximal and distal signaling protein, much like one would see with endogenous TCR stimulation. However, this phosphorylation occurs at earlier time-points with 28 ζ CAR T cells, starting as early as 1 minute post stimulation and peaking at about 5-10 minutes. Interestingly, the phosphorylation event is not sustained in the BB ζ or ζ CAR T cells as it is with the 28 ζ CARs.

The implications of these rapid signal transductions can be multifold.

First, these findings point out a vital role of Akt activation in CAR T cells. Akt kinases are designated as critical mediators of cellular proliferation and survival (Fruman, 2004). These are activated by both costimulatory receptors, CD28 (Parry et al., 1997) and 4-1BB (Starck et al., 2005). Such costimulatory signals act to lower the threshold of

activation of T cells by almost 5 fold, making the cell highly sensitive to antigenic stimulation(Viola and Lanzavecchia, 1996). However, if not tuned appropriately, “over stimulation” or tonic signaling can instead be detrimental to the cell, as has been seen with endogenous CD28(Collette et al., 1998). Whether the relatively quick and prolonged induction in 28 ζ CAR implies a negative influence on the system is a question that cannot be clearly addressed at this point, but warrants further investigation. However, it could explain the relatively inferior persistence of 28 ζ CAR T cells reported in Chapter 3 as well as in other published studies(Brentjens et al., 2013; Lee et al., 2015).

Elevation in intracellular Ca²⁺ is a key mediator of T-cell signaling and activation. In endogenous TCR mediated antigen engagement, the intracellular components of the TCR complex recruit a series of tyrosine kinases, ultimately leading to the phosphorylation and activation of PLC γ (Qian and Weiss, 1997). PLC γ proceeds to cleave PIP₂ in the plasma membrane to generate diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP₃). IP₃ is responsible for release of Ca²⁺ in the cytoplasm either from the endoplasmic reticulum or the extracellular space. In most cells, including lymphocytes, a sustained maintenance of Ca²⁺ keeps the Nuclear Factor of Activated T cells (NFAT) transcriptionally active. This transcription factor is responsible for the regulation of key cytokines, including IL2(Rao et al., 1997) and differential levels of Ca²⁺ induction can have consequences in the later activation of T cells. Ca²⁺ signaling is also sensitized during CD28-costimulation because of the activation of MAP kinase Erk(Byrum et al., 2013). This sensitization leads to formation of immunological synapses, which is CD28 and Lck-dependent. Correlating this with our data suggests a possible mechanism of why

we observe rapid influx of Ca^{2+} in 28 ζ CAR T cells, as compared to ζ or BB ζ CAR T cells.

In addition to having an impact on NFAT-mediated transcriptional regulation of critical survival factors, calcium signaling is critical in maintaining the homeostasis of the mitochondria (Babcock and Hille, 1998). Mitochondria act as Ca^{2+} buffers, taking up Ca^{2+} during periods of activity and gradually releasing it later, thus reducing the size of Ca^{2+} peaks (Hoth et al., 1997). An important consequence of Ca^{2+} uptake by mitochondria is the activation of mitochondrial dehydrogenase to boost the production of NADH and ATP (Jouaville et al., 1999). If we were to combine our findings from Chapter 3 with the Ca^{2+} flux differences we have uncovered in Chapter 4, it seems logical to put forth the hypothesis that these observations could be related. Although it was out of the scope of these studies, this effect of Ca^{2+} regulation in the mitochondria may be an important step in preparing the T cells for the impending increase in biosynthetic and bioenergetic demands that accompany cell cycle entry and activation.

Follow-up Studies

This thesis has uncovered significant findings about the impacts of CAR ICDs on T cell fate, but at the same time, it has opened the doors to several more questions that need to be addressed. Most observations presented in this thesis as well as those reported by other groups have revealed that costimulatory domains within the chimeric receptor function as expected based on knowledge of endogenous costimulatory molecules. However to be at a position where one can enhance the efficacy of this promising anti-

cancer therapeutic tool, further investigation needs to be done. Listed below are some suggested follow up studies.

Role of TFAM. To further investigate the importance of mitochondrial biogenesis on the persistence of CAR T cells, it would be wise to manipulate this phenomenon by genetic regulation of TFAM. Preliminary findings described in Chapter 3 show a marked decrease in the mitochondrial oxygen burning rate in BB ζ CAR T cells upon genetic knockdown of TFAM. Given the role of TFAM in mitochondrial biogenesis, it would seem logical to test if this is in fact due to decreased mitochondrial biogenesis and whether it has any impact on SRC. But would this abrogate the phenotype we observed with BB ζ CAR T cells above? Alternatively, it would be interesting to find out if over-expressing TFAM in 28 ζ CAR T cells would make them more oxidative. On a related note, studies have shown that HMG box 1 phosphorylation of TFAM impairs its ability to bind DNA and to activate transcription(Lu et al., 2013). The question that begs to be address is to see if this phenomenon occurs preferentially in 28 ζ CAR T cells.

Reliance on FAO. The preferential upregulation of FAO in BB ζ CAR T cells lends way to investigate whether inhibiting this metabolic pathway would alter their memory development and persistence. Specifically restricting availability of long chain fatty acids in culture or inhibiting/down-regulating CPT1a should be done. Similarly, loss of cytochrome oxidase C has been shown to cause a shift to glycolysis and impairment of the mitochondrial electron transport chain(Srinivasan et al., 2015). Because we observed a relative increase in mRNA levels of MTCO1 in BB ζ , studies to disrupt its expression in these cells would signify its importance in promoting the above phenotype.

In vivo studies. RNA CARs have now been translated into the clinics with encouraging success(Beatty et al., 2014). Studies done in mouse models have revealed superior persistence of lentivirally-transduced BB ζ CAR T cells(Carpenito et al., 2009; Milone et al., 2009). It would be very relevant to examine the performance of this RNA-CAR based system in vivo. If our observations hold, one would expect to see results similar to those reported by Pearce and colleagues(Pearce et al., 2009; van der Windt et al., 2013).

CAR structure. Structural characteristics of the CAR ecto and endo domains still need to be clearly understood. Furthermore, unveiling the immunological synapse formation at the sites of CAR-antigen engagement will provide mechanistic insights on CAR aggregation on the surface, antigen-density threshold, cytoskeletal rearrangement and requirement or exclusion of certain surface molecules such as LFA. These studies could be performed using classic immunoprecipitation methods or with state-of-the-art microscopy techniques(Dustin and Depoil, 2011).

Signaling motifs of CAR ICDs. Recently, we have initiated site-directed mutagenesis to examine how discrete regions of the CD28 cytoplasmic domain impact distinct molecular pathways. For example, regulation of T cell proliferation versus upregulation of Bcl-X_L, a cell survival factor, are both attributed to different domains of the endogenous CD28 cytoplasmic tail(Burr et al., 2001). One would hypothesize that corresponding functional properties of 28 ζ CAR T cells could be mapped out to these sites on the 28 ζ ICD as well, and thus manipulation of these domains could alter CAR T functions. Similar studies must be performed with BB ζ and other CAR constructs. This

can be tied in with examining how Ca^{2+} helps in stabilizing the synapse through changes in cytoskeletal rearrangement. Additionally, current studies in our group are directed towards understanding the signaling mediated by the 3rd generation CAR, i.e. 28BB ζ .

Lessons for clinical protocol. Current clinical protocols for culture of the ACT product involves the use of defined nutrient-rich culture media. Its nutrient composition is designed to closely resemble physiological conditions in a healthy body. However, once these T cells are infused into a patient, they encounter a nutrient-shock. As discussed earlier, nutrient supply in the tumor micro-environment can be limiting and the competition for nutrients that these T cells have to face is fierce. Additionally, our findings suggest that based on the components of the CAR design, T cells can alter their metabolic needs. Therefore, from a metabolism point of view, different CAR designs could tolerate the new environment in the host differently. So the question that arises is – what if the culture systems could be configured up front based on the CARs used. For example, certain CARs with 28 ζ domain would require more glucose but their BB ζ counterparts may not. Alternatively BB ζ CAR T cells may require more fatty acids that would instruct long term memory formation and enhanced proliferation. Since the phenomenon observed in this report is instructive, is it possible to pharmacologically instruct T cells to preferentially choose one metabolic pathway over the other and thereby favor a particular memory subset formation? Such parameters could prove critical in designing future clinical protocols and in predicting the fate of the infused products when encountering tumor targets.

Future Directions

This thesis is among the first few reported studies detailing the mechanistic impacts of CAR signaling domains. Here, we have provided an accurate and easy-to-use tool that can be employed by investigators to study CAR-mediated effects on primary human T cells. Additionally, this tool can be used as a stand-alone protocol for in vitro cell expansion. Our results describing the influence of CAR signaling domains on the metabolic parameters and biochemical signaling events in T cells, combined with our model system could significantly influence current clinical processes.. It can also be used as a surrogate model to assay potential in vivo performance of CAR T cells.

Much remains to be accomplished to enhance the effectiveness and ensure the safety of cell therapies. However, CAR T therapy has now become a new paradigm in cancer therapeutics. Our hope is that this thesis has contributed to the growing knowledge revolving around this exciting space, and has laid the groundwork for furthering studies in this arm of immunotherapy. With the rapid developments that this field has observed in the past few years, one can surmise that this approach will soon be far from being just an investigational therapy and will be licensed as a standard cancer therapy.

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