UNIVERSITY OF CALIFORNIA

Los Angeles

Developing Inhibitory Chimeric Antigen Receptors for Mitigating CAR T Cell On-Target, Off-Tumor Toxicity

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular and Medical Pharmacology

by

Nathanael Joshua Lui Bangayan

2023

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2023

Professor Owen N. Witte, Chair

Chimeric antigen receptor T cell (CAR T) therapy has shown extraordinary success in the treatment of hematological malignancies. However, its application to solid tumors has been largely limited by the immunosuppressive tumor microenvironment and toxicities. Because most CAR targets are tumor associated antigens, they are also expressed on normal tissues that are killed by CAR T cells in a process known as on-target, off-tumor toxicity. To mitigate this toxicity and increase specificity, Boolean logic gates have been applied to CAR T cells to regulate activity based on multiple antigenic signals. The AND-NOT-gate utilizes an inhibitory chimeric antigen receptor (iCAR) to downregulate T cell activation after normal tissue antigen recognition. Our study found that an iCAR with a single PD-1 domain shows a kinetic delay in inhibition. This delay can be ameliorated by increasing the avidity but not the affinity of the iCAR. Alternatively, the PD-1 domain can be replaced with alternative inhibitory domains from BTLA, LAIR-1, and SIGLEC-9. To further enhance inhibition, we developed a dual-signaling inhibitory CAR (DiCAR) that combines the PD-1 domain with one of these alternative domains. These DiCARs are more efficient at inhibiting cytotoxicity than an iCAR with a single PD-1 domain. Furthermore, preliminary studies have shown that these DiCARs can inhibit cytotoxicity *in vivo*.

The dissertation of Nathanael Joshua Lui Bangayan is approved.

Yvonne Y. Chen

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DEDICATION

One common belief held in this world is that science and faith cannot be intermixed. However, throughout these last eight years as a graduate student, I have come to find that science has been an important component to strengthening my faith. By learning about the intricacies of T cell signaling and even taking part in the difficult part of engineering new cellular therapies, my faith in a God who can create and manage all of these details has only been bolstered. So I would like to dedicate my work to my God for whom I live. To God be the Glory.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

Over the past eight years of graduate school, I have struggled to become a scientist worthy of this doctorate, but with the help of many people and institutions the day has finally come to pass. I would like to spend some moments here acknowledging those who have supported me throughout this endeavor.

First, I would like to thank my advisor, mentor, and teacher – Dr. Owen Witte. Although I suspect I was not the student that he thought I was at the beginning, he took the time and effort to shape me into the scientist I am today. I came into the lab with some technical experience and some grand ideas, but he taught me how to refine these ideas into hypotheses that can and should be answered. By training me in his lab, I have learned to think independently, to read the literature critically, to collaborate with others, and to write grants. I would be dishonest if I were to say there were never tough times, but the lessons I learned will surely help me in my future scientific endeavors. Thank you for teaching me that the best experiment is the one that convinces you to stop working on a project, that science is a team endeavor and to not be afraid to ask for help, and that lab meeting is not a dog and pony show but an opportunity for us to learn from each other. Although I was often out of my comfort zone, I am grateful for the opportunity to be a trainee from your lab. And to fulfill the wish that you mentioned in lab meeting a long time before, rather than saying Dr. Witte, I would like to say, “Thank you, Owen.”

Second, I would like to thank all the lab members past and present who have supported me and my work. I would like to acknowledge the TCR group for giving me an opportunity to take the lead in some of our projects (splicing project) and to contribute to others. Whether it be Jami advising me on cloning, Donghui teaching me how to become the secondary flow expert in the lab, Zhiyuan and Pavlo sdiscussing experiments with me, Xiao-hua inspiring me to keep working hard and critically plan experiments, or Christie, Giselle, and Miyako assisting me with running the many T cell cytotoxicity experiments, you were all important in getting this work done. I would like to acknowledge the many post-docs who also taught and advised me

throughout my time here (Jung-wook, John Lee, John Philipps, Liang, Greg, Evan, Bryan, and Janai). Specifically, I would like to thank John Lee, who took me in on his project and passed the CEACAM5 CAR work onto me. Without his prior work and his ideas, this project would have never gotten off the ground. Although intimidating, John Lee is one of the best physician scientists I know. I would also like to acknowledge Liang Wang, who grew as a post-doc in the lab at the same time I grew as a graduate student. He helped me troubleshoot and set up many experiments, especially the in vivo ones, of which if they didn't happen, the in vivo work in this project would never have been done. I would also like to acknowledge Mireille, a project scientist that was previously in the lab. When I first rotated, she took me under her wing and taught me how things get done in the Witte lab. I am certain that if she was not supervising me during that time, I wouldn't have even had the opportunity to join the lab. At the same time, I want to acknowledge my first Witte lab friend, Tim Chai. He was the first person to reach out to me in the lab and make me feel welcome; he encouraged me to join the lab even when I was fearful; and he constantly supported me these eight years even while away getting his own Ph.D. Thanks for being a good friend. There were many undergraduates that I would also like to thank who helped in the cloning process and my side projects which include (Leo, Brian, Elizabeth, Tiffany, and Kasra).

Finally, in the lab I would like to thank all members of the Squid Squad past and present in all our iterations (Miyako, Lisa, Gmao, Giselle, Wendy, and Minna). You were all the support system I needed to persevere even when my project was constantly failing. Whether you listened to me rant about the difficulties of science, calmed my anxiety by playing board games on afternoons, or saw the good in me that I couldn't see myself, you all kept me going. If somebody were to ask how could I be so resilient in such a tough project, I would have to say that it was in part because of your encouragement. Specifically, I would like to thank Lisa and Gmao, who I first saw as rivals for Dr. Witte's attention, but I now consider two of my best friends.

Outside of the lab, I must acknowledge and thank the many institutions that helped fund this project, which include the UCLA Prostate SPORE, the Parker Institute for Cancer Immunotherapy, the Prostate Cancer Foundation, the Broad Stem Cell Research Center, and the UCLA Tumor Immunology and Virology/Gene Therapy Training Grants. Thank you for your financial support. I want to acknowledge other professors and teachers who have helped me during this process from Dr. Sam Chow, Dr. David Nathanson, Dr. Ting-ting Wu, and most importantly, my committee members – Dr. Antoni Ribas, Dr. Stephen Forman, Dr. Donald Kohn, and Dr. Yvonne Chen. Specific thanks must got to Dr. Chen, who was critical in guiding this project from start to finish, even though I was not a member of her lab. I also want to acknowledge some of the previous scientific mentors I have had who brough me to this point – Dr. Yung-Ya Lin, Dr. Joseph Nabhan, Dr. Quan Lu, Dr. Yasuhiro Katagiri, Dr. Herbert Geller, and my undergraduate advisor and person who encouraged me to move forward in science, Dr. Huiying Li.

Finally, I would like to thank the three most important people in my life. I would like to thank my loving girlfriend, Suwen Li. Being a fellow graduate student from the same cohort, she struggled with her projects just as I did mine, yet she willingly listened to me complain, encouraged me to continue, and supported me even when I wanted to quit. She constantly saw the best in me and saw me as good and worthwhile even when I didn't feel it. So thank you, my love and my best friend. The other two people I would like to thank are my parents. Mom and Dad, I know that I did not become the type of doctor that you wanted me to be but thank you for supporting me in pursuing this career choice. You have always encouraged me to pursue my dreams, to stay true to my faith, and to live the way God wants me to. You have prayed for me in my struggles, made sure that all other things were taken care of so I could focus on my studies and work, and loved me unconditionally. This work could not be done without your help.

Most importantly, I must acknowledge the role that my faith has played throughout this journey. Without my belief in God and Jesus Christ, I surely would have given up before this even began. So all thanks and praise must go to Him.

Curriculum Vitae

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EDUCATION

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Bachelor of Science, Biochemistry and MIMG (Microbio, Immuno, and Mol Genetics) 2011

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University of California – Los Angeles (Molecular and Medical Pharmacology)

Ph.D. Graduate Student (Under direction of Dr. Owen Witte) Sept 2015 - Present

- Developing chimeric antigen receptor T cell (CAR T) therapy for the treatment of neuroendocrine prostate cancer
- Engineering new chimeric antigen inhibitory receptors to mitigate toxicity
- Assisting in the development of TCR therapies targeting prostate cancer

University of California – Los Angeles (Molecular and Medical Pharmacology)

Lab Assistant (Under direction of Dr. Huiying Li) Oct 2012 - July 2015

- Determined the relationship between the skin microbiota and atopic dermatitis using next-generation sequencing
- Characterized the *Propionibacterium acnes* (*P. acnes*) species and phage communities
- Studied the gene expression of *P. acnes*
- Lab safety officer

Undergraduate Researcher (Under direction of Dr. Huiying Li) Oct 2009 – June 2011

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Post-Baccalaureate Fellow (Under direction of Dr. Herbert Geller) Oct 2011 - Sept 2012

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AWARDS

UCLA Virology and Gene Therapy Training Grant (2018-2019); UCLA BSCRC Training Program (2017-2018); UCLA Tumor Immunology Training Grant (2016-2017); UCLA Molecular Pharmacology Genesis Scholarship (2015); UCLA Dean's Honor List (2007-2011); UCLA Summa Cum Laude (2011); UCLA Dunn Award for Excellence in Biochemistry (June 2011); UCLA Dean's Prize Award for Undergraduate Science Poster Day (May 2011); UCLA Undergraduate Research Scholars Scholarship (Sept 2010-June 2011); UCLA Undergraduate Research Fellowship (Dec 2009-June 2010); Robert C. Byrd Scholarship (for Mathematics) (June 2007-June 2011)

Chapter 1: Introduction

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With its recent success and adoption as a treatment for hematological malignancies, chimeric antigen receptor (CAR) T cells have gained traction as a therapy for solid tumors. CARs are engineered receptors that combine a targeting extracellular domain with intracellular T cell signaling domains to direct immune cells to specifically kill tumor cells. However, due to the lack of bona fide tumor specific antigens, most CAR T cell therapies have been shown to also target normal cells that express those antigens. This "on-target, off-tumor" toxicity has hindered its broad use for all cancers and exposed the need for the development of new methods that increase the specificity and regulate the activity of CAR T cells. This thesis project is focused on the development of inhibitory chimeric antigen receptors (iCARs) to mitigate this on-target, off-tumor toxicity. To provide a basic understanding of this topic, this introductory chapter will begin with a brief history and review of T cell signaling, followed by the development of CAR T cells and the current knowledge of its signaling pathways, and lastly, cover the existing methods for regulating CAR T cell activity.

1.1 – T cells – Their History from Vestigial to Critical

1 Corinthians 12:14, 21-22 ESV – For the body does. Not consist of one member but of many…The eye cannot say to the hand, "I have no need of you," nor again the head to the feet, "I have no need of you." On the contrary, the parts of the body that seem to be weaker are indispensable…

In the early 1900s, many organs of importance today were simply considered vestigial. Robert Wiedersheim published a list of them in his book *The Structure of Man an Index to His Past History*. Some of these organs included the pituitary gland, the appendix, the thyroid, and the thymus.[1] The thymus continued to be considered a vestigial organ until the 1960s when Jacques Miller discovered its immunological function. When he thymecotmised mice, he found that mice without a thymus had lower levels of lymphocytes, were more susceptible to infection, and tolerated skin

grafts from different strains of mice.[2] It was after this key experiment that people began to consider that the thymus might have a role in immunology, but what was it?

A few years later with his first graduate student Graham Mitchell, Miller discovered that the thymus was the source of "antigen-reactive cells" that could initiate the differentiation of bone-marrow derived "antibody-forming cells."[3–5] These "antigen-reactive cells" would eventually be recognized as thymic-dependent "T-lymphocytes," while the "antibody-forming cells" would be called thymic-independent (bursa-equivalent) "B-lymphocytes."[6] This is where the terms T cells and B cells originated.

H. Cantor and E. A. Boyse would then discover that these mouse T cells could be further separated into functional groups based on their Ly antigens. T cells that were Ly-1$^+$ helped B cells produce antibodies, while T cells that were Ly-23$^+$ had cytotoxic activity.[7] There human counterparts would then be defined by Reinherz *et al.* using the OKT-4 antibody that bound helper cells and the OKT-5 and OKT-8 antibody that bound cytotoxic cells.[8–13] These populations would later be known as CD4$^+$ and CD8$^+$ T cells respectively.[14] However, it was still unknown as to how these T cells recognized foreign antigens.

Major discoveries in the 1980s answered this question. These discoveries included the concept of MHC-restriction,[15] the presentation of viral antigens by MHC,[16] the cloning and characterization of the T cell receptor (TCR),[17–20] and the crystallization of MHC.[21,22] By uniting these findings, it became clear that each T cell has a unique TCR that specifically recognizes a foreign antigen presented by a MHC molecule. Although important, this was just the tip of the iceberg.

The following decades led to the discovery of co-stimulatory and inhibitory receptors important in T cell regulation, key adaptors and transcription factors in T cell signaling, and T cell byproducts like cytokines and granzymes. By the early 2000s, T cells were no longer seen as vestigial cells but critical players in adaptive immunity that could be engineered to not only stop infection but also eliminate tumors.

1.2 – Triggering T cell activity – The Basics of T cell Signaling

After a T cell recognizes a foreign antigen, it triggers a complex set of signals that rapidly transforms a quiescent cell into a killing machine. But how does ligand binding lead to proliferation, cytokine production, and cytotoxicity? This section will summarize the current understanding of how T cells convert this external signal into immune-related activities in a process called T cell activation.

Simplified Models of T cell Activation

There currently exists three major models for T cell activation. The first model was proposed by Lafferty and Cunningham and was an extension of Bretscher and Cohn's B cell two signal model.[23–25] This model proposed that a T cell needed two signals to be activated – 1) a signal based on antibody-antigen recognition and 2) a species-specific costimulatory signal provided by the stimulating cell.[24,26] Lafferty further refined the model after the discovery of MHC stating that Signal 1 is the TCR-pMHC interaction which gives specificity and Signal 2 is a costimulatory signal provided by the antigen presenting cell.[23,27] This two signal model has now become the accepted dogma for initiation of T cell activation.

The second model is a derivation of the two signal dogma and is known as the tide model. In this model, the first signal is the TCR-pMHC interaction, which primes the cell for activation or tolerance, making it an initiator. The second signal is a summation of both costimulatory (i.e. CD28, 41BB, OX40) and coinhibitory receptors (i.e. PD-1, CTLA-4) that drive the cell toward "Response" or "Un-Response," making it a modulator of the initiating signal. By integrating signals from multiple receptors at once, the immune response rises and falls like a tide based on the signals given.[28] Rather than thinking of signaling as a simple "on-off" switch, this model takes into account the gradient of activity that can occur from multiple modulators.

The third model accounts for certain T cell subsets that require cytokine signaling and is aptly termed the three-signal model.[29] Unlike memory T cell subsets which need only two signals to induce full effector function, naïve $CD4^+$ and $CD8^+$ T cells also require a third signal provided by the presence of IL-1 and IL-12 respectively.[30,31] Without the presence of IL-12, naïve $CD8^+$ T cells

become tolerant rather than fully activated.[30] This three signal model explains the need for cytokine signaling but is not applicable to all T cells.

Regardless of the model, T cell activation begins with the TCR binding peptide-MHC (p-MHC) and initiating a signal that is further driven by interactions of secondary molecules on the T cell surface. The following subsections will briefly describe these signal transduction pathways.

The Formation of the Immunological Synapse

When T cells interact with APCs/target cells that present their cognate antigen, an immunological synapse is formed between both cells. This synapse is composed of a central supramolecular activation cluster that contains multiple TCRs (c-SMAC), a peripheral supramolecular activation cluster (p-SMAC) composed of the proteins LFA-1 and TALIN, and a distal SMAC (d-SMAC) that contains proteins like CD45 and CD43.[32,33] The formation of this synapse was thought to be a critical step in the activation and signaling of a T cell;[34] however it was later shown that signaling could occur even before the formation of the synapse at TCR microclusters in the periphery.[35–38] This synapse was actually a consequence of these TCR-CD3 complexes migrating and forming a c-SMAC.[38] Rather than acting as an initiator of signaling, this synapse is now considered an important regulator that balances TCR signaling and degradation.[36,39] So what happens at the microclusters?

The Signal Cascade Following TCR-pMHC Engagement

The microclusters are the sites where TCR signaling is first initiated. When a single TCR binds p-MHC, the interaction triggers biochemical changes that lead to downstream signaling that activates the T cell.[40] Many theories, such as kinetic proofreading, serial engagement, kinetic segregation, and mechanosensing, try to explain how ligand binding triggers this first signal, but the process is still unclear.[41] However, the signaling pathway following initiation is more defined. The TCR is composed of a disulfide linked alpha and beta chain that is stabilized on the cell surface by six CD3 molecules (CD3γ, CD3δ, two CD3ε, and two CD3ζ). This heterodimeric complex contains unique motifs known as ITAMs (immunotyrosine based activation motifs), where

the initial signal takes place. When ligand binding occurs, CD4/CD8 molecules recruit the kinase Lck to the TCR/CD3 complex.[42] Activated Lck phosphorylates ITAMs found on the CD3 chains,[43] like those on CD3ζ.[44] Another kinase ZAP-70 then binds the ITAMs of the CD3ζ chain via its SH2 domain and further phosphorylates adaptors important in T cell signaling like LAT and SLP-76.[43] These adaptors recruit other key signaling molecules like PLC-γ, Nck, and Vav to the membrane.[45,46] In addition to Zap-70 activation, PI-3 kinase (PI3K) is also recruited to the cell membrane and begins to convert PIP_2 to PIP_3, an important metabolite for downstream events.[47] In total, four key events occur after the initial signal: 1) transcription factor (i.e. NFAT, AP-1, and NF-κB) activation, 2) metabolic transformation, 3) enhanced integrin adhesion, and 4) actin cytoskeletal rearrangement. The following details are summarized from Janeway's I*mmunobiology*.[45]

The first key event is transcription factor activation of NFAT, AP-1, and NF-κB. The activity of PLC-γ plays a critical role in initiating these signal cascades. PLC-γ is an enzyme that is recruited to the membrane by the LAT-SLP76-Gads adaptor complex. It breaks down PIP_3 at the cell membrane (generated by PI3K) to the metabolites DAG (diacylglycerol) and IP3 (inositol 1,4,5-trisphosphate). DAG is membrane-bound and recruits PKC-θ and RasGRP, which drive the activation of NF-κB and AP-1. IP3, on the other hand, diffuses into the cytosol and binds receptors in the ER that trigger calcium influx and the activation of NFAT. These three transcription factors change the transcriptional landscape following T cell activation.

The second key event is metabolically related. These changes are mostly driven by Akt, which is recruited to the membrane by PIP_3. When activated, Akt promotes cell survival by the downstream activation of Bcl-2 and induces lipid production, ribosome biosynthesis, mRNA biosynthesis, and protein translation through the mTOR pathway. This anabolism helps the activated T cells to rapidly proliferate.

The last two events involve changing the structure of a T cell to enhance its interaction with the target cell. The first event is the aggregation of the integrin LFA-1 and increases cell adhesion.

With the help of adaptors like ADAP, WASp, and Nck, LFA-1 forms the p-SMAC and stabilizes the immunological synapse. The second event involves actin polymerization and cytoskeletal reorganization by WASp, Nck, and Cdc42. All together these structural changes modify how the T cell interacts with the target cell.

Costimulatory and Inhibitory Receptors Involved in T cell Signaling

In addition to the primary signal provided by the TCR-pMHC interaction, a number of costimulatory and inhibitory receptors contribute to enhancing or diminishing T cell activation. Two examples of well-studied costimulatory receptors are CD28 and 41BB. CD28 is widely accepted as "the" costimulatory Signal 2 required for T cell activation. It is constitutively expressed in almost 100% of $CD4^+$ T cells and 50% of $CD8^+$ T cells[48] and binds CD80 and/or CD86.[45] Once phosphorylated, CD28 augments TCR signaling by lowering the threshold of activation, by recruiting PI3K, PKC-θ, Lck, and Ras to the cell membrane, by remodeling chromatin, and by switching metabolism towards glycolysis.[49] 4-1BB, on the other hand, is a costimulatory receptor whose expression is induced by TCR and CD28 signaling. It performs its costimulatory function by recruiting and activating Lck, CD3ε, CD3ζ, and SLP-76 to lipid rafts,[50] and enhancing NF-κB signaling through TRAFs.[49] 4-1BB can enhance cytokine production and proliferation, as well as, rescue T cells from anergy and exhaustion.[49] Additional costimulatory receptors that help in activation, memory cell generation, cytokine production, and survival are ICOS and OX-40.[51,52] These are only four of the many costimulatory molecules that exist to augment T cell activation.

T cells are also equipped with many inhibitory receptors whose function is to titer down the immune response. One class of inhibitory receptors contain domains known as immunoreceptor tyrosine-based inhibitory motifs or immuoreceptor tyrosine-based switch motifs (ITIM/ITSM). Like ITAMs, when phosphorylated on their tyrosine residue, these motifs can bind the SH2 domains of phosphatases like SHP-1, SHP-2, and SHIP.[53] To inhibit T cell signaling, SHP-1 and SHP-2 dephosphorylate key kinases and adapters like Zap-70, SLP-76, CD3ζ, LAT, and PI3K,[54] while SHIP hydrolyzes molecules like PIP_3.[53,55] Examples of these are PD-1, BTLA, and LAIR-1.[54]

PD-1 is one of the most well-studied inhibitory receptors and is a successful target for a class of drugs known as checkpoint inhibitors (i.e. Pembrolizumab and Nivolumab).[56] It is known to mostly bind and enact its inhibitory function through SHP-2, but can also downregulate activity without this phosphatase present.[57,58] BTLA, on the other hand, preferentially binds and acts through SHP-1.[58] Interestingly, BTLA may also play a role in T cell activation since it contains a Grb-2 binding motif that may recruit PI3K to the cell membrane.[59,60] Unlike BTLA and PD-1 which only contain one ITAM, LAIR-1 contains two.[61] LAIR-1 is constitutively expressed in naïve T cells and is associated with SHP-1, suggesting that it may function to not only downregulate activation but also to prevent initiation.[62,63] Many other ITIM-containing receptors known for T cell inhibition include members of the SIGLEC family (i.e. SIGLEC-7 and SIGLEC-9) and TIGIT.[54,64,65]

In addition to the ITIM-containing receptors, there are many other T cell inhibitory receptors that are known checkpoint inhibitors like CTLA-4, TIM-3, and LAG-3. CTLA-4 is a receptor that has homology to CD28 and has higher avidity for its target antigen B7.[66] It is a negative regulator that is expressed about 48 hours after T cell activation and inhibits T cell proliferation and activation.[66–68] It is thought to work by competitively binding B7.1 and B7.2 preventing CD28 binding and costimulation as well as interacting with the phosphatases SHP-2 and PP2A.[54] CTLA-4 is the target for the clinically successful checkpoint inhibitor Ipilimumab.[69] TIM-3 and LAG-3 are also targets for checkpoint inhibitors in the clinic.[70,71] Although it is not fully known how both of these receptors inhibit, they contain unique properties in their intracellular domains that are important for their function. LAG-3 contains a KIEELE motif and an FxxL motif that have been shown to be necessary for inhibition,[72,73] while TIM-3 contains two tyrosine residues that when phosphorylated lead to reduced Lck recruitment and activity.[71] Additional receptors without ITIM domains known to be involved in T cell inhibition are CD5[74] and VISTA.[75]

Mechanisms of T Cell Cytotoxicity

Once T cells are activated, they can then begin to kill target cells that present their cognate antigen. T cells are known to kill using three different methods. The first and primary way of killing is to

release perforin, which creates pores in the target cell membrane. The T cell then delivers granzymes like Granzyme B which activate caspases and trigger apoptosis.[76] The second method of killing utilizes the FAS-FASL pathway. When activated, $CD4^+$ and $CD8^+$ T cells express FASL, which binds to the FAS receptor on target cells. When engaged, a death-inducing signaling complex (DISC) that contains Caspase-8 is formed in the target cell, leading to a cascade of signals that causes apoptosis.[77] The third method that T cells use is the release of cytokines. Cytokines can not only recruit and promote the activation of immune cells like macrophages, NK cells, and other T cells, but some like IFN-γ can bind receptors on tumor cells that also trigger apoptosis.[78]

Overall, T cell activation begins with antigen recognition by the TCR within the context of MHC and ends with the proliferation of T cells, the production of cytokines, and the killing of the target cell. Several signaling pathways like the PI3K/Akt/mTOR pathway, the Ras/Raf/MAPK pathway, and the PLC-γ pathway contribute to transforming the T cell through transcription factor activation, cytoskeletal rearrangement, integrin aggregation, and metabolic switching. Specific ligand binding coupled with complex signaling transforms a non-activated T cell into a killing machine that can eliminate infections and even tumors.

1.3 – Tinkering with a T Cell – Part 1: Adopting T cells for Immunotherapy

William Coley earned the title the Father of Immunotherapy, because of his work treating bone and soft tissue sarcomas with Coley's Toxins, a cocktail of bacteria and/or bacterial products. He believed that these toxins could trigger infection and stimulate the immune system to kill cancer, making it one of the first formal uses of immunotherapy. Although it sounded strange, he surprisingly was able to demonstrate tumor shrinkage in some patients. However, due to the high risk of infection, the variability of preparation and delivery, and the lack of reproducibility, it was not widely accepted.[79] Nonetheless, it set the stage for others to consider how the immune system could play a role in cancer therapy.

Although Coley had already been trying to treat cancer with his crude form of immunotherapy,

Paul Ehrlich was the first person to propose that the immune system surveilled the body for cancer cells and eradicated them.[80,81] About 50 years later, Sir MacFarlane Burnet and Lewis Thomas would refine this hypothesis into the concept of immune surveillance, which states that the immune system is responsible for removing dangerous cells that acquire mutations that can become neoantigens that provoke an immunological reaction.[82–84] It was likely this concept that inspired others like Steven Rosenberg to pioneer work in adoptive cell therapy. Rosenberg showed that lymphokine-activated killer cells (LAKs) could be obtained from a patient's blood, expanded in IL-2, and kill fresh tumor cells. These LAKs were sensitive to antibodies that targeted CD3 and CD8, suggesting that some of the population might be T cells.[85] Based on this work, he began to perform studies in which he would obtain tumor infiltrating lymphocytes (TILs) from tumors, grow them up *ex vivo*, and transfer them back into patients to treat melanoma.[86] In one of these seminal clinical trials, 11 out of 20 patients partially responded to the therapy, demonstrating that adoptive cell therapies could work.[87]

Unfortunately, one major drawback of using TILs is that not all patients have them. Some tumor tissues have few antigen-specific infiltrating T cells. In those cases, an alternative strategy is necessary. One strategy is to engineer specificity into T cells by introducing a specific TCR into T cells using a retro- or lentiviral vector. Early proof-of-concept experiments were performed with TCRs that targeted MART-1, MDM2, and gp-100.[88–90] Eventually, these TCR-engineered T cells would be tested in clinical trials with varying degrees of success. One example was with the MART-1 directed TCR that was used to treat patients with progressive metastatic melanoma. Although only two out of the fifteen patients responded, the study showed that it was possible to engineer T cells to treat tumors in patients.[91] Another example with more success was the NY-ESO-1 directed TCR. In different clinical trials, patients with myelomas, synovial cell sarcomas, or melanomas treated with a NY-ESO-1 directed TCR therapy responded with a 70%, 61%, and 55% objective clinical response rate respecitively.[92,93] With that type of success, it is not a surprise that as of October 2021, there have been a total of 119 clinical trials that involve adoptive transfer

of TCR T cells that target 118 antigens.[94] Although no TCR-directed adoptive cell therapy has yet to be approved by the FDA, the future looks promising for T cell engineered therapies.

1.4 – Tinkering with a T cell – Part 2: Building a CAR (Chimeric Antigen Receptor)

First-generation CARs – Breaking Free from MHC

Although the future looks bright with engineered TCR T cells, one major limitation is MHC restriction. Because TCRs must recognize antigens in the context of MHC, only patients with HLA alleles that match the corresponding TCR can receive the therapy. This significantly reduces the number of patients that can be treated. To overcome this barrier, Yoshihisa Kuwana and Zelig Eshhar independently found a way to provide specificity without MHC restriction. In both of their papers, they combined the VL or VH chain of an antibody with the TCR alpha or TCR beta chain. When both these chimeric receptors were introduced into T cells they triggered specific activation independent of MHC.[95,96] These initial constructs were prototypes of what would eventually become the CAR.

In 1993, Zelig Eshhar improved upon his previous design by combining the scFv of an antibody that recognized 2,4,6-trinitrophenyl (TNP) with the CD3ζ or FcRγ chain. Unlike his previous design that required two receptors, this engineered receptor was a single construct. When introduced into T cells, it specifically stimulated the cells to produce cytokines and kill TNP-modified target cells.[97] Although weak in activity, this structure became the blueprint for the modern day chimeric antigen receptor (CAR).

A CAR is an engineered receptor that combines the specificity of an antibody with the potent activation of T cell signaling domains. It is normally composed of an antigen binding domain, an extracellular spacer/hinge, a transmembrane domain, and T cell costimulatory and activation domains. These components are like modular building blocks, that to an extent, can be swapped one for another. The antigen binding domain is usually a scFv of an antibody that specifically binds the desired cell surface antigen; however, alternatives that use natural receptors and ligands have been utilized.[98] The extracellular spacer/hinge region provides flexibility for the CAR

to bind its target and have from come CD4, CD8, CD28, and the IgG Fc region.[99] The transmembrane domain anchors the receptor to the membrane and can be derived from CD3ζ, CD4, CD8, and CD28.[99] The business end of the receptor is found on the C-terminus, where the costimulatory and activation domains lie. Although not obligatory, the costimulatory domains (i.e. the intracellular components of 41BB, CD28, ICOS, OX-40) tend to enhance CAR T cell function. The activation domain, which initiates signal transduction, is often the CD3ζ chain, but CD3ε and FcRγ have been used as well.[99]

Second-generation CARs – Adding Costimulation to Improve Activity

As described earlier, Eshhar's first CAR had weak activity and was only composed of a scFv chain linked to the CD3ζ chain.[97] This initial construct is now termed a first-generation CAR. In his paper, he described the potential to use additional costimulatory domains that could enhance its activity.[97] CARs that contain this additional costimulatory domain are now known as second-generation CARs. Many different domains have been incorporated to improve CAR T cell function. The two most common domains are CD28 and 4-1BB, which have been shown to enhance cytokine production, proliferation, resistance to exhaustion, cytotoxicity, and tumor killing *in vivo*.[100–103] CARs with these domains are currently being used in the clinic.[104] Additional domains that have been shown to improve CAR T cell function compared to their first-generation counterparts are OX-40, ICOS, and CD27.[105–107] Recently, a screening strategy known as CARPOOL was used to find new domains that can be incorporated into second-generation CARs. This method revealed that even domains normally associated with the B cell lineage like BAFF-R and TACI could improve CAR T cell activation and reduce exhaustion.[108] As more domains are found, this list of second-generation CARs will continue to grow.

Third-generation CARs – Building One upon Another

If one costimulatory domain can improve function, could multiple costimulatory domains create a synergistic effect? This is the question that many groups have tried to answer by generating third-generation CARs that combine multiple costimulatory domains into one construct. The most

common combination is CD28 and 4-1BB. CARs that contain both these domains have shown mixed results, with some reporting that it improves cytotoxicity and cytokine production,[109,110] while others see little to no further enhancement compared to their second-generation counterparts.[111,112] In a clinical trial where this combination was directly compared to a second-generation CAR that only contained CD28 in the same patient, more expansion and persistence was seen with the third-generation CAR, suggesting that it might be better.[113] Other combinations that have been tired are CD28 and OX-40,[105] 4-1BB and ICOS,[114] and even CD28 and the STAT-binding domains IL-2Rβ and a YXXQ motif.[115] A CARPOOL screening method has even been used to find new third-generation CARs in a high throughput manner, yielding combinations like CD40, CD3ε ITAM, and DAP12 or FCεR1γ, OX40, and CD3ζ ITAM3.[116] More work will need to be performed before a verdict can be made as to whether third-generation CARs are truly superior.

Fourth-generation CARs – Equipping CAR T cells with ARMOR

Rather than modifying the CAR construct itself to enhance function, a new class of CAR T cells known as ARMORED CARs or TRUCKs are being engineered to resist the tumor microenvironment through the secretion of cytokines and checkpoint inhibitors.[117,118] TRUCKs deliver IL-12, IL-18, IL-7 and CCL19 to the tumor microenvironment, making it less immunosuppressive and even recruiting endogenous immune effector cells to the tumor bed.[117,119–123] In this way, a CAR T cell is not only being used as a direct killer but also a recruiter.

A New generation of CARs – Genetically Modifying the CAR T Cell

Currently, CARs are not the only things being modified, but the T cells themselves are also being engineered to become more resistant to the tumor microenvironment. Using CRISPR mediated strategies, genes like PD-1, RASA2, MED12, CCNC, and NR4A have been knocked out to improve CAR T cell function.[124–128] One study, where PD-1 was knocked out through the direct integration of the CAR into its locus, has even reported that 7/8 B cell non-Hodgkin's lymphoma patients that received these CAR T cells went into complete remission in the 12 month median observation period.[125]

In contrast to deletion, genes have also been overexpressed or added into CAR T cells to enhance their function. One example is with c-Jun, which has been shown to increase proliferation, prevent exhaustion, and improve the potency of CAR T cells, allowing them to detect cells with low levels of antigen density.[129] Ligands for costimulatory receptors like CD80 and 4-1BBL have also been overexpressed to provide costimulation apart from the CAR.[130] One group has even introduced a dominant negative form of the TGF-β receptor that can bind the immunosuppressive cytokine TGF-β but not signal.[131] Whether by adding or removing genes, T cells are becoming more resistant to the tumor microenvironment.

Over the span of thirty years, the simple first-generation CAR that gave T cells MHC-independent specific activation has become a complex fourth-generation receptor that can stimulate a patient's own immune cells to battle cancer. Through iterative improvements like the addition of costimulatory domains, this initially weak receptor has now become one of the most important tools in the cancer immunotherapy toolbox. It will be interesting to see what further advances will be made in CAR T cell design in the next thirty years.

1.5 – CAR signaling – Key Differences between CARs and TCRs

Because CARs utilize the CD3ζ domain of the TCR complex, it was initially thought that they signal in the same manner as TCRs. Although similar in some respects, research into the mechanisms behind CAR signaling have revealed major differences between the two. One major difference between CARs and TCRs is the antigen threshold that needs to be reached for T cell proliferation, cytokine production, and cytotoxicity. It is suggested that a single TCR-pMHC interaction is enough to trigger T cell activation,[40,132] but hundreds to thousands of molecules are necessary for CARs.[133] Watanabe *et al* demonstrated that there are actually two different antigen thresholds for CARs – one for cytotoxicity which only needed several hundred molecules and one for cytokine production and proliferation which required several thousand.[133] These results explain why in two studies where TCRs and CARs were compared, the TCR was found to be more sensitive than the CAR.[134,135]

One reason for this threshold difference may be the difference in the immunological synapse. Unlike TCRs which form a "bull's eye" like structure composed of a c-, p-, and d-SMAC, CARs are less organized. Most CAR T cells do not form a stable c-SMAC, instead they gather together in microclusters that are interspersed with CD45 molecules.[136] These microclusters are already associated with Lck, making signaling rapid but short lived.[137] However, when CAR T cells do form a c-SMAC, CD45 is segregated around the d-SMAC as expected.[136] How this immunological synapse relates to effectiveness is still unknown; however, it has been proposed that the stability of it may determine CAR effectiveness in long-term killing and *in vivo* assays.[138]

The second reason for this threshold difference may be differences in signaling pathways. In general, CARs utilize the same pathways that TCRs do. Like TCRs, the primary signal is likely to be triggered by a combination of receptor oligomerization, mechanosensing, receptor deformation, and kinetic segregation of CD45 phosphatases outside the CAR microclusters after binding.[99,139] The binding then triggers the phosphorylation of ITAMs in the CD3ζ domain that can recruit and activate kinases and adapters like Zap-70, LAT, SLP-76, and PLC-γ.[136,140,141] Transcriptomic analysis of CAR T cells even without antigen recognition have shown gene expression patterns that are indicative of T cell activation, suggesting that they utilize similar pathways.[142] Phosphoproteomic analysis by Salter *et al* showed that there was a near one-to-one concordance between TCR or CAR activated samples 45 minutes after activation.[143] However, there were a few key differences. First, certain proteins like CD3δ, CD3ε, CD3γ, and LAT were either not phosphorylated or less phosphorylated when activated by the CAR compared to the TCR.[143] It is not surprising that the other CD3 chains are not phosphorylated by CARs, since CARs primarily use CD3ζ as their activation domain; however, it was unexpected that LAT was less phosphorylated.[143] Interestingly, another study supported this result by showing that although LAT could enhance activation and cytokine production it was dispensable for CAR signaling.[136] The second difference was in the kinetics and intensity of signals produced by the CAR and TCR tested, but this may simply be due to the difference in targets.[143] The result is not surprising

though if one considers the position of costimulation. With CARs, costimulation occurs in-line with CD3ζ activation, but with TCRs, costimulation happens on separate molecules that might be upregulated over time.[144] Additionally, in another study, Salter *et al* showed that even though second-generation CARs activated similar pathways regardless of their costimulatory domain (CD28 vs. 41BB), the intensity and kinetics of phosphorylation differed.[141] If two CARs had differences in kinetics, what more TCRs and CARs?

Although antigen density thresholds differ, CARs still kill target cells in a similar manner to TCRs. They kill through granzymes and perforins, the Fas-FasL axis, and cytokines.[145] Due to the complexity of obtaining TCRs and CARs with the same affinity towards the same antigen, it can be difficult to compare the signaling pathways of these two receptors concurrently. Also, there is the added complication of which CAR is being used for comparison, since costimulation can alter signaling. Additional research will need to be performed to further elucidate the mechanisms behind CAR signaling, so that CARs can be designed with these pathways in mind.

1.6 – CAR Keys – Important Factors that Control CAR T Activity

All components of a CAR from the antibody scFv chain to the internal signaling domains contribute to CAR T cell activation and signaling. For example, the length of the hinge/spacer region has been shown to be important based on the epitope being targeted. Short spacers work better for epitopes that are more distal from the membrane while long spacers are optimal for epitopes that are more proximal.[146,147] Even, the origin of the spacer and transmembrane can lead to better activity. In one instance, a first-generation CAR's activity was improved to the levels of a second-generation CAR by simply replacing the hinge and transmembrane domains with those derived from CD28.[148] A small modification to the CAR can lead to drastic changes in activity. But if every domain of a CAR is important, what are the key factors that should be focused on to control CAR activity? It seems that there are three factors that are most critical – 1) the affinity of the CAR, 2) the avidity of the CAR, and 3) the internal signaling domains of the CAR.

Affinity

The affinity of a CAR describes how strongly the CAR binds its target epitope. In general, the affinity is mostly determined by the scFv chain of the antibody and correlates with CAR strength and activity. CARs with higher affinity more strongly activate T cells than CARs with lower affinity.[146,149] The affinity of a CAR can even be modulated to provide selectivity against target cells. Multiple groups have shown that the affinity of a CAR can be tuned to provide specificity. Higher affinity CARs kill target cells regardless of the level of antigen expressed, but lower affinity CARs can distinguish between cells with high and low antigen density.[150–152] By reducing affinity, the CAR becomes more dependent on avidity, the second key factor in CAR activation.

Avidity

Avidity considers both the affinity of a CAR as well as the number of CARs that bind. It can be regulated by the amount of antigen found on target cells as well as the number of CAR receptors expressed on T cells. In a study conducted in 2017, Walker *et al* demonstrated that both antigen density of anaplastic lymphoma kinase (ALK) on target cells and CAR density on T cells highly affected cytotoxicity.[153] The contribution of antigen density on CAR activation has also been seen with CARs targeting CD20, GPC2, EGFR, and CD22.[133,150,154,155] Ramakrishna *et al* has even suggested that antigen density may affect the conversion of CAR T cells toward a memory phenotype, contributing to persistence and effectiveness *in vivo*.[155] The threshold of antigen density differs based on the CAR being used, but it is clear that it contributes to cytotoxicity, proliferation, and cytokine production.[148] The importance of avidity in CAR T cell killing is so important that even a reduction in tumor antigen density can lead to resistance against CAR T cells.[156,157] By modulating the avidity through increased expression of antigen on target cells or CAR on T cells, CAR activity can be enhanced.

Internal Signaling Domains

As mentioned above, depending on the CAR being used, the antigen density threshold differs. The major driving factor in that study was the costimulatory domain incorporated into the CAR. The CAR equipped with a CD28 costimulatory domain responded to lower amounts of antigen

compared to the CAR equipped with a 41BB domain.[148] This response to low antigen density targets is only one difference between these two domains. Differences in metabolism,[158] signaling kinetics,[141] constitutive tonic signaling,[159–161] memory formation,[158,160] and exhaustion[160] have all been reported when comparing CD28 and 41BB-containing CARs. Overall, the CD28 domain is preferred for low antigen density targets, where rapid killing and activity is needed, while the 41BB domain is preferred for those of higher densities, where persistence is desired.[49,162] Alternative domains like OX-40 and ICOS can also contribute to activity changing the amount of cytokines released as well as the phenotype of the T cells respectively.[101,106] Even the location of these domains proximal or distal to the transmembrane affect their function.[102,114,163] The importance of signaling components is not limited to the costimulatory domain. The number and location of ITAMs in the CD3ζ domain have also been optimized to enhance activity. CD28-3ζ second-generation CARs containing only one functional ITAM more proximal to the membrane outperformed CARs that contain all three ITAMs in *in vivo* "stress test" studies, where CAR T dosage is purposedly lowered to fail.[164] The selection of domains, especially, costimulatory domains is critical in generating CARs; therefore, one should consider persistence, antigen density on normal cells, and even which cytokines are desired to be produced when building a CAR.[163]

Affinity, avidity, and the signaling domains used in a CAR are all key factors in determining CAR T cell activation and signaling. Careful decisions must be made about the antibodies being used for recognition and the signaling domains included into the construct based on antigen density on tumor cells and normal tissues. Building the right components into a CAR can be the deciding factor between clinical success or dangerous toxicity.

1.7 – Driving CARs into the Clinic

CAR T trials against hematological malignancies

CAR T cell therapy has rapidly become one of the primary immunotherapeutic treatments for cancer. This is largely due to its success targeting leukemias and lymphomas. One of the first

successful treatments of patients with refractory chronic lymphocytic leukemia (CLL) was reported in 2011 with a CD19-targeting second-generation 41BB-3ζ CAR. Previous clinical trials had shown modest responses, but this study showed ongoing remission for at least 10 months after treatment.[165] Following studies utilizing a CD19-targeting CAR with either a CD28 or 41BB costimulatory domain showed similar successes for relapsed or refractory acute lymphoblastic leukemia.[166–170] One study even reported that up to 90% of patients underwent complete remission one month after treatment, and 67% had event-free survival after six months.[166] Because the number of patients, the time of assessments, the lymphodepleting regiment, and the constructs being tested in each trial differ, it is hard to compare them, but it is clear that CD19-targeting CARs are effective treatment options for patients with hematological malignancies. Success has also been seen with the treatment of multiple myeloma with CARs targeting BCMA.[171,172] For this reason, the US FDA has now approved six CAR T cell products (Tisagenlecleucel, axicabtagene ciloleucel, brexucabtagene autoleucel, ciltacabtagene autoleucel, idecabtagene vicleucel, and lisocabtagene maralecucel) for twelve different indications, including B-ALL (B-cell acute lymphoblastic leukemia), LBCL (large B cell lymphoma), mantle cell lymphoma, and follicular lymphoma.[104] CARs targeting other antigens found on hematological malignancies like CD22, CD30, CD7, and CD20 have also seen success and are reviewed by Labanieh and Mackall.[104]

CAR T clinical trials against solid tumors

Many CAR T clinical trials have attempted to treat patients with solid tumors. Antigens like GD2, HER2, IL-13Rα2, EGFR, Mesothelin, Claudin-18.2, PSMA, CAIX, and CEACAM5 have all shown varying degrees of success and toxicity.[104] In one patient treated with a IL-13Rα2-targeting CAR, a transient but complete response was seen against recurrent multifocal glioblastoma.[173] In another trial, a mesothelin-targeting CAR in combination with PD-1 blockade showed glimpses of a response in patients that had malignant pleural mesothelioma.[174] However, success against solid tumors has been limited due to difficulties like antigen specificity, tumor heterogeneity, CAR

T trafficking, and the immunosuppressive micronvironment.[175] The strength of CAR T cells will need to be enhanced to overcome these barriers, but it will have to be balanced with ways to mitigate the concerning dose-limiting toxicities that are also seen with this therapy.

Toxicities associated with CAR T therapy

There are three major toxicities associated with CAR T cell therapy: cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS/neurotoxicity), and on-target, off-tumor toxicity. The first two are more general systemic toxicities, while the latter is mediated by the expression of antigen on normal tissues. These toxicities have ranged from manageable to death and should be considered and planned for before embarking on CAR T trials.

Cytokine release syndrome (CRS) is a toxicity associated with T cell activation and secretion of cytokines in the blood. These cytokines include but are not limited to IL-6, IFN-γ, IL-5, and IL-10.[168] The most common symptom is fever, but it can easily progress to hypoxia, hypotension, and edema.[168,176] It can also damage renal, musculoskeletal, hepatic, gastrointestinal, and neuronal tissues.[176] A meta-analysis of CAR T cell clinical trials have even associated it with cardiotoxicity.[177] CRS has been seen with CD22, BCMA, and CD19-directed CARs, but the degree of toxicity has varied.[157,178] Most often it has been associated with high tumor burden, suggesting that it is in response to the treatment.[166,167] Currently, steroids and tocilizumab (anti-IL6 antibody) are administered to reduce CRS, but it seems to compromise treatment efficacy.[166,168–170]

The presence and severity of CRS has been correlated with neurotoxicity, although one occurring does not necessarily mean the other will as well.[179] Neurotoxicity or ICANS is a toxicity that exhibits in some patients treated with CD19 or BCMA-targeting CAR T therapy, although the cause is unknown.[178] Patients have experienced symptoms that range from headaches, confusion, and delirium to hallucinations, encephalopathy, and seizures.[166,168,170,179] In one clinical trial run by Juno Therapeutics, five patients died from cerebral edema.[180] Although in most cases

reversible, concern should be given to this debilitating toxicity. Interestingly, recent reports have found that CD19 is expressed at the RNA level in brain mural cells[181] and BCMA in the neurons and astrocytes of basal ganglia.[182] This expression may trigger on-target, off-tumor toxicities that may explain this phenomenon.

On-target, off-tumor toxicity has not been implicated as the cause of neurotoxicity as of yet, but this toxicity is still concerning. There are only a few tumor-specific antigens that exist. Most antigens are tumor associated, in that, they are highly expressed in tumors but are still expressed at low levels in normal healthy cells. CAR T cells cannot easily distinguish normal versus tumor cells; therefore, it will kill any cell that expresses its target antigen. This specific killing of normal tissues is known as on-target, off-tumor toxicity. This is commonly seen with the CD19 CAR eliminating healthy B cells and causing aplasia.[166,183] But it has also been seen with CARs targeting solid tumor antigens like CEACAM5, ERBB2, and CAIX. In a clinical trial where a patient was injected with a ERBB2-targeting CAR, the patient experienced respiratory distress and eventually died due to low ERBB2 expression in the lungs.[184] Some believe that this may be due to CRS, but whether on-target, off-tumor toxicity played a role is still debatable. Acute respiratory toxicities were also seen with a CEACAM5-directed CAR T cell therapy in patients being treated for metastatic CEACAM5^{+} tumors, due to expression of CEACAM5 in the lung. This toxicity posed enough of a risk that the clinical trial was closed.[185] CAIX is another target that has been associated with on-target, off-tumor toxicity in the liver due to expression in bile duct epithelial cells.[186,187] It is critical that accurate surveys of normal tissue be performed before selecting a CAR antigen to reduce this toxicity.

Although CAR T cell therapy has shown great promise in the clinic, its success is accompanied with caveats. CAR T cells have shown little to no success in the treatment of solid tumors. The immunosuppressive tumor environment will require enhancements in CAR T cell strength and trafficking, but this augmentation will need to be balanced with regulation to avoid debilitating side effects like CRS, neurotoxicity, and on-target normal tissue damage. By developing clever

strategies that regulate CAR T cell activity, this dream to apply CAR T cell therapy to solid tumors may become a reality.

1.8 – Tuning the CAR T Cell Engine to Regulate Its Activity

Due to the major toxicities associated with CAR T cell therapy, multiple strategies have been developed to regulate its activity. They break down into four major categories: elimination, drug regulation, affinity tuning, and Boolean logic gates. Each strategy will be discussed in the sections below.

Elimination of CAR T cells

One method to stop activity is to remove the CAR T cells completely. RNA transient transfection is a way CAR expression can be restricted to a limited amount of time.[188] Unlike viral transduction and CRISPR gene editing, which integrate the transgene into the genome, transfection is temporary. As CAR T cells proliferate, the transgene dilutes and is lost after multiple cycles. If short-term activity was enough for tumor killing, then this method would be ideal. However, since CAR T cell persistence is a major factor in successful treatments, repeated injections of fresh newly transfected CAR T cells would be necessary to achieve long-term tumor remission. Another method to eliminate CAR T cells is to incorporate suicide genes (i.e. HSV-TK,[189–191] inducible Caspase-9,[192–194] truncated EGFR,[195] CD20[196]) that target CAR T cells for destruction. By introducing drugs associated with these genes, CAR T cells can be rapidly eliminated from the body. Unfortunately, this process is irreversible, so deciding when to administer the drug is critical, since efficacy will be compromised if CAR T cells are removed before tumors are eliminated.

Drug Regulation of CARs

Rather than eliminating the T cells completely, strategies have been developed to control the expression, the dimerization, the signaling, and even the degradation of CARs using drugs. For example, by placing the CAR transgene behind an inducible tetracycline (Tet)-On promoter, CAR expression can be controlled with the addition of Doxycycline.[197] To control dimerization, groups split the antigen binding domain and the signaling domains of a CAR into two membrane bound

components that contained drug-dependent dimerization domains. By adding a specific drug, they could permit or inhibit dimerization and thereby control CAR activation.[198–200] At the signaling level, Dasatanib, a FDA approved tyrosine kinase inhibitor, has been used to inhibit LCK phosphorylation and block CAR downstream signaling.[201,202] A more complex way to control signaling is to use SNIP CARs (Signal neutralization by an inhibitable protease)[203] or VIPER CARs (Versatile protease regulatable CARs),[204] which both use proteases to cleave the connection between the binding and signaling domains of a CAR. Only by adding a protease inhibitor can the domains remain attached and signal after ligand binding. Even CAR degradation can be controlled by drugs using the CAR-LID (CAR-Ligand induced degradation) system. Here CARs contain a cryptic degron (degradation signal) that is exposed after the addition of a drug, labeling it for degradation. It has been shown to be reversible and tunable but the modification leads to reduced surface expression and activity.[205] These clever strategies for controlling CAR expression are promising, but many have problems with leakiness, due to ligand-independent expression or degradation.

Tuning/Controlling Affinity

Because affinity is a key factor in determining CAR activity, Chmielewski *et al* suggested that tuning the affinity of the scFv domains could help a CAR T cell distinguish between cells that express high and low levels of antigen.[149] This concept was then applied to multiple CARs targeting ErbB2, EGFR, and CD19 to show that by simply controlling the affinity of a CAR, one could control which target cells that express the antigen are killed.[150–152] By tuning a CAR's affinity, T cells can be made to kill tumor cells that express high levels of antigen while sparing normal cells that express low levels. However, this strategy also increases the chance of tumor escape, because tumors merely need to reduce antigen expression rather than completely lose it.

Instead of tuning the affinity of a single scFv chain in a CAR, switchable CARs introduce temporary antigen recognition through a different module. In this strategy, a CAR is not designed to recognize the tumor antigen directly but an epitope or domain that is linked to an scFv chain

that recognizes the antigen. In this manner, a CAR T cell can circulate throughout the body inert until the introduction of the antigen binding module. This was first introduced in 2012 by having a CAR recognize FITC, which was linked to antibodies that recognize tumor associated antigens. By introducing different FITC-conjugated antibodies, the CAR T cell could recognize and kill multiple tumor types as well as be switched off once activity was undesirable.[206] Variations of this strategy include Uni-CARs, which have CARs target an epitope like the 5B9 tag,[207,208] and SUPRA CARs, which utilize leucine zippers as domains that associate the CAR and antigen binding domain.[209] These switchable CARs allow for the removal of CAR activity through metabolism of the antigen binding module but also allow them to be reintroduced if necessary. Additionally, they provide a way for CARs to switch targets if an antigen is lost during treatment tumor evolution.

Boolean-Logic Gates

The last strategy that has been utilized to regulate CAR T cell activation is to use Boolean logic gates. There are three major logic gates that have been utilized OR-gates, AND-gates, and AND-NOT-gates. The first gate is mostly utilized to prevent tumor antigen escape, while the latter two are used to prevent on-target, off-tumor toxicity. Unlike suicide genes and drug regulation, which require a doctor to decide when to administer a drug, Boolean logic gates allow the CAR T cell to regulate itself based on the environment, making it a powerful tool that theoretically can rapidly control activity as necessary.

Antigen loss is one of the main ways that tumors escape from being killed by CAR T cells. In two clinical trials using a CD19-targeting CAR, patients relapsed due to the outgrowth of a $CD19^-$ population.[169,170] To avoid this from happening, CAR T cells have been engineered to recognize two antigens rather a single one, so that they can kill a tumor cell whether it sees one antigen **OR** the other. One method of achieving this is to inject two CAR T cell populations that recognize two different antigens. This was performed by both Hamieh *et al*[156] and Ruella *et al*[210] by targeting CD19 and CD22 or CD19 and CD123 respectively. Ruella *et al* also showed that the process could be simplified by placing both the CD19-targeting CAR and the CD123-targeting CAR into

the same T cell.[210] Because transducing two CARs into a T cell is difficult due to vector constraints, tandem CARs were developed that link both scFv domains in tandem to the signaling domains of one construct. Various combinations, which include CD19 and CD22,[157] CD19 and HER2,[211] CD19 and CD20,[212,213] and HER2 and IL13Rα-2[214] have all been successfully targeted with this strategy. The applicability of these tandem CARs has even been extended to three antigens.[215] With the discovery that antigen binding can be performed using 33 amino acid designed ankyrin repeat proteins (DARPins) rather than full scFv's, it might be possible to make OR-gates that recognize even more.

The second Boolean logic gate is known as an AND-gate and is utilized to limit on-target, off-tumor toxicity. In general, the AND-gate requires a CAR T cell to recognize two tumor associated antigens before activation occurs. It must receive a signal from one antigen **AND** the other. Kloss *et al* created an AND-gate by introducing two receptors with different specificities in a T cell.[216] The first receptor was a first-generation CAR which required co-stimulation to activate. The second receptor was a chimeric costimulatory receptor that provided the costimulation. Only by recognizing both antigens would the CAR T cell be able to fully activate and function. This strategy has been applied to breast cancer by targeting ERBB2 and MUC1.[217] A similar method that utilizes two receptors is the Synthetic Notch (SynNotch) system, which is more of an IF-THEN gate. In this system, **IF** a synthetic Notch receptor recognizes a tumor antigen using its scFv chain, **THEN** it will cleave and release a transcription factor that drives the expression of a CAR that recognizes a second antigen.[218,219] It has been used to target combinations like EpCAM and ROR1,[220] CD19 and ROR1,[220] and ALPPL2 and MSLN or HER2.[221] One drawback is that when human components of the Notch receptor were used instead of mouse, the system was found to be leaky and weak, but modifications have been made to improve its performance.[222] Alternative AND-gate strategies include a masked CAR, which use a tumor-associated protease as a second antigen,[223] and bispecific T cell engagers (BITEs), which are only released after the first antigen is recognized.[224] AND-gates can be powerful tools to reduce on-target, off-tumor toxicity, but they

also increase the chances of tumor escape, because a tumor only needs to downregulate one of the antigens to avoid being killed.

The last gating strategy, the AND-NOT-gate, is an alternative way to mitigate on-target, off-tumor toxicity. This method was first developed by Fedorov *et al* in 2013 and utilized two receptors to distinguish tumor and normal tissues.[225] The first receptor is a standard CAR that recognizes a tumor associated antigen and fully activates when engaged. The second receptor is an inhibitory CAR that links an scFv that recognizes a normal tissue antigen to a T cell inhibitory signaling domain like PD-1 or CTLA-4. When the T cell interacts with normal tissues where the CAR **AND** iCAR bind their respective ligands, then the T cell is told **NOT** to activate. The proof-of-concept model combined a CD19-targeting CAR and a PSMA-targeting iCAR, which are not co-expressed on normal tissues.[225] The iCAR was then developed to target HLA-alleles which are often downregulated by tumors to make it clinically applicable.[226–231] Further modifications have been made to incorporate alternative inhibitory signaling domains outside of PD-1 and CTLA-4 to improve its function.[232,233] This strategy has shown major promise in inhibiting on-target, off-tumor toxicity and is the focus of this dissertation.

1.9 – Conclusion

The thymus was thought to be a vestigial organ unnecessary for survival, but research has shown that it is anything but expendable. Not only are T cells important in protecting us from external infections but also from cancerous tumor cells. By equipping these T cells with CARs, they have been engineered to recognize and to kill tumors specifically. However, a major limitation is on-target, off-tumor toxicity. Although many strategies have been developed to alleviate this problem, like affinity tuning and Boolean logic gates, they have had limitations that have prevented them from being clinically applicable. Specifically, the AND-NOT-gate has been shown to be inefficient at inhibiting CAR T cell activation, requiring high levels of iCAR and iCAR target antigen to function. The focus of this dissertation has been to better understand the inhibitory CAR and its limitations, in order to develop a better iCAR that inhibits CAR T cell

activation more efficiently. The hope is that by doing so, CAR T cells can be safely and effectively used to treat both liquid and solid tumors.

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Chapter 2: Dual inhibitory domain iCARs improve efficiency of the AND-NOT gate CAR-T strategy

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*The following chapter is a modified version of a manuscript that is currently in press with the Proceedings of the National Academy of Sciences (PNAS).

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Competing Interest Statement: O.N.W. currently has consulting, equity, and/or board relationships with Trethera Corporation, Kronos Biosciences, Sofie Biosciences, Breakthrough Properties, Vida Ventures, Nammi Therapeutics, Two River, Iconovir, Appia BioSciences, Neogene Therapeutics, 76Bio, and Allogene Therapeutics. T.S. currently has consulting relationships with Dren Bio. A.M.W. currently has consulting, equity, and/or board relationships with ImaginAb and Novartis Institute for Biomedical Research. None of these companies contributed to or directed any of the research reported in this article. None of these companies contributed to or directed any of the research reported in this article. A provisional patent listing N.J.B. and O.N.W. has been submitted based on this work through the UCLA Technology Development Group.

Keywords: Chimeric antigen receptor; Inhibitory CAR (iCAR); on-target, off-tumor toxicity; immunotherapy; AND-NOT logic gate

Abstract

CAR (chimeric antigen receptor) T cell therapy has shown clinical success in treating hematological malignancies, but its treatment of solid tumors has been limited. One major challenge is on-target, off-tumor toxicity, where CAR T cells also damage normal tissues that express the targeted antigen. To reduce this detrimental side-effect, Boolean-logic gates like AND-NOT gates have utilized an inhibitory CAR (iCAR) to specifically curb CAR T cell activity at selected nonmalignant tissue sites. However, the strategy seems inefficient, requiring high levels of iCAR and its target antigen for inhibition. Using a TROP2-targeting iCAR with a single PD1 inhibitory domain to inhibit a CEACAM5-targeting CAR (CEACAR), we observed that the inefficiency was due to a kinetic delay in iCAR inhibition of cytotoxicity. To improve iCAR efficiency, we modified three features of the iCAR – the avidity, the affinity, and the intracellular signaling domains. Increasing the avidity but not the affinity of the iCAR led to significant reductions in the delay. iCARs containing twelve different inhibitory signaling domains were screened for improved inhibition, and three domains (BTLA, LAIR-1, SIGLEC-9) each suppressed CAR T function but did not enhance inhibitory kinetics. When inhibitory domains of LAIR-1 or SIGLEC-9 were combined with PD-1 into a single dual-inhibitory domain iCAR (DiCARs) and tested with the CEACAR, inhibition efficiency improved as evidenced by a significant reduction in the inhibitory delay. These data indicate that a delicate balance between CAR and iCAR signaling strength and kinetics must be achieved to regulate AND-NOT gate CAR T cell selectivity.

Significance Statement

On-target, off-tumor toxicity is a major barrier to the application of CAR (chimeric antigen receptor) T therapy to solid tumors. Boolean logic gates like the AND-NOT gate have utilized an inhibitory CAR (iCAR) to reduce this toxicity. We investigated the role of avidity, affinity, and internal signaling domain composition on the kinetics of iCAR inhibition. With this knowledge, we designed a new dual-inhibitory domain CAR (DiCAR) that combines two immune cell inhibitory signaling domains to specifically regulate CAR T cell cytotoxicity and improve inhibition efficiency compared to an iCAR with a single PD1 domain.

Introduction

Genetically engineered adoptive cell therapies that target tumor-associated antigens have recently shown success in the clinic. One such therapy is chimeric antigen receptor (CAR) T cell therapy which introduces an engineered receptor that combines an extracellular antigen binding domain and T cell signaling domains into T cells to specifically kill tumor cells. CAR T cells targeting CD19 and BCMA (B-cell maturation antigen) have successfully treated hematological malignancies such as relapsed or refractory acute lymphoblastic leukemia, large B-cell lymphoma, and multiple myeloma (1, 2). Although application of CAR T cell treatment for solid tumors has rapidly grown in number of clinical trials (3), its success has been limited due to two major obstacles: the immune restrictive tumor microenvironment (4) and on-target, off-tumor toxicity (5–7). To overcome this inhibitory environment, CAR T cells have been generated to be more potent, but these improvements are still accompanied with neurotoxicity, cytokine release syndrome, and/or on-target, off-tumor toxicity (8). Improvements must be made to balance the strength and efficacy of CAR T therapy with the potential toxicities associated with it.

On-target, off-tumor toxicity occurs when CAR T cells recognize normal tissues that express the targeted tumor associated antigen. These toxicities have ranged from manageable with the CD19 CAR and B cell aplasia (8) to lethal with the ERBB2 (Erb-B2 Receptor Tyrosine Kinase 2) CAR and respiratory distress (9). Additional CARs targeting CEACAM5 (Carcinoembryonic antigen-related cell adhesion molecule 5) in the lung and CAIX (Carbonic anhydrase IX) in the liver have also shown toxicities that were considered too debilitating to advance clinically (5–7).

Various strategies have been developed to reduce CAR T cell toxicity. Elimination of CAR T cells through drug-induced suicide genes and secondary markers (10–13), affinity tuning of the antigen binding domain (14, 15), and control of CAR T cell recognition through small molecules and targeting modules (16–19) have all been tested. Each of these strategies has been capable of reducing toxicity but at the cost of efficacy due to the loss of persistence or increased tumor

escape.

Rather than compromising the efficacy of CAR T treatment, Boolean logic gates have been applied to CAR T cells as safety switches. By integrating signals from multiple receptors at once, these CAR T cells can better regulate their activity based on their environment. For example, AND-gate strategies utilize two receptors that recognize different tumor antigens to trigger CAR T cell activation. Variations of this strategy have combined a masked CAR and proteases (20), a chimeric co-stimulatory receptor and a first-generation CAR (21), a Synthetic Notch receptor and a CAR (22), and a logic-gated intracellular network (LINK) CAR (23). Although promising, the strategy suffers from two limitations: 1) tumor escape can occur due to loss of a single antigen and 2) leakiness of either one of the receptors can lead to toxicity (23).

An alternative logic gate, that can provide more protection, is the AND-NOT gate, which utilizes two receptors – an activating CAR that contains T cell co-stimulatory and activation domains and an inhibitory CAR (iCAR) that contains a T cell inhibitory signaling domain. The CAR recognizes a tumor antigen and activates a T cell, while the iCAR recognizes a normal tissue antigen and inhibits T cell activity. In this manner, the CAR T cell can distinguish a tumor cell and normal cell that express the same CAR target. Over a decade ago, Fedorov *et al.* published the proof-of-concept of this strategy by linking a scFv chain that recognized PSMA (Prostate-Specific Membrane Antigen) to the PD-1 or CTLA-4 inhibitory signaling domains. This iCAR was capable of inhibiting T cell proliferation, cytokine production, and cytotoxicity when combined with a TCR or CD19-targeting CAR. However, its ability to efficiently inhibit T cell activity was limited to when the iCAR specific antigen was highly expressed (24).

Improvements to iCAR design have focused on targeting relevant normal tissue antigens and increasing the potency of iCAR signaling. HLA-C1, HLA-A2, and HLA-A3 have all been described as iCAR targets that limit killing to tumor cells with loss of HLA alleles (25–28), but this subjects CAR T therapy to HLA-restriction. LIR-1 (Leukocyte Immunoglobulin Like Receptor B1) and TIGIT (T Cell Immunoreceptor With Ig And ITIM Domains) have been reported as replacements to PD-

1 (26, 29, 30), but how they enhance iCAR inhibition is unknown.

The AND-NOT gate strategy is compelling, but a deeper understanding of the mechanisms and key drivers of specific iCAR inhibition is necessary to achieve a tighter regulation of CAR T cell activity. Unlike CARs, the role that affinity and avidity play in iCAR function and kinetics has not been well-studied. To better understand how to enhance specific iCAR inhibition of CAR T cell activity, we studied the role of dosage, affinity, and internal signaling components in iCAR inhibitory kinetics. This knowledge led us to develop a class of iCARs that combine two different inhibitory signaling domains into a single construct termed the dual-inhibitory domain iCAR (DiCAR). DiCARs more efficiently inhibit CAR T cell activity than an iCAR with a single PD1 domain.

Results

The TROP2 PD1 iCAR displays a kinetic delay in inhibition of CAR T cytotoxicity.

To develop a model for the AND-NOT gating strategy, we selected two epithelial cell targets as antigens for the CAR and iCAR. CEACAM5 (**CEA**) was chosen as a CAR target because of its high expression in neuroendocrine prostate cancer (31), colorectal cancer (32), gastric cancers (33), and small cell cancers of the lung (34). Due to its normal tissue expression in the colon, bladder, kidney, and lung, some adoptive cell therapies targeting this antigen have displayed dose-limiting on-target, off-tumor toxicities that could be reduced with an AND-NOT Boolean logic gate (5, 33, 35).

As an iCAR target, we selected TROP2 (*TACSTD2 (Tumor Associated Calcium Signal Transducer 2)* or **TROP2**), which is widely expressed in epithelial cells of the lungs, skin, esophagus, kidney, liver, and pancreas (36). Antibody-drug conjugates targeting TROP2 have been used in the treatment of metastatic triple-negative breast cancer, making it an amenable target for immunotherapies (37). Our previous work with TROP2 made it a useful surrogate epithelial cell marker for studying the AND-NOT gating strategy (38, 39)

As an activating module, we enhanced a previously published CEACAM5-Long-CD28-3z CAR by

replacing its extracellular spacer and co-stimulatory domain (SI Appendix, Fig. S1A) to increase *in vivo* functionality (31, 40–42). This CEACAM5-42NQ-41BB-3z targeting CAR (**CEACAR**) elicited the same levels of IFN-γ production and cytotoxicity against a CEACAM5$^+$ engineered cell line as our previously published CAR (SI Appendix, Fig. S1B and S1C) (31). This CEACAR was also able to eliminate CEACAM5$^+$ tumors *in vivo* compared to an untransduced T cell control (SI Appendix, Fig. S1D).

To develop the iCAR, antibodies were identified through phage display. Recombinant TROP2 was used as an antigen for panning a single fold scFv (single-chain fragment variable) phage display library as further described in the Methods section (43). Using one of the antibodies (H11) with the highest binding affinity, we generated an iCAR construct as described by Fedorov *et al*. (24). The TROP2 scFv chain was linked to an extracellular spacer (Long Spacer - IgG4 hinge, CH2, and CH3 domain), a CD28 transmembrane domain (TM), and a PD1 intracellular signaling domain to form the TROP2-Long-PD1 iCAR (**TROP2-PD1 iCAR**) (Fig. 1A).

To establish whether the TROP2-PD1 iCAR could inhibit CEACAR T cell activity, T cells transduced with both the CAR and iCAR were co-cultured with engineered DU145 prostate cancer cell lines. A DU145 cell line in which the TROP2 gene was deleted using CRISPR/Cas9n (44) (**CEA$^-$/TROP2$^-$**) was engineered to express GFP and either CEACAM5 alone (**CEA$^+$/TROP2$^-$**) or both CEACAM5 and TROP2 (**CEA$^+$/TROP2$^+$**) by lentiviral transduction. T cell activity was expected when CEACAM5 alone was expressed; but inhibition was expected when TROP2 was present as well (Fig. 1A). Because high levels of iCAR were reported to be necessary to inhibit allogenic T cell cytotoxicity by Fedorov *et al.* (24), the multiplicity of infection (MOI) of the iCAR was 10-fold higher than the CEACAR. T cells enriched to be at least 80% CAR$^+$/iCAR$^+$ were then co-cultured with these engineered DU145 cell lines.

Two hallmarks of T cell activation that were inhibited by CAR$^+$/iCAR$^+$ T cells after co-culture with CEA$^+$/TROP2$^+$ target cells were cytokine production and cytotoxicity. Approximately 90% less IFN-γ was produced by the CAR$^+$/iCAR$^+$ T cells compared to the CAR$^+$ only control (Fig. 1B). This

difference was not seen when the target cells expressed CEACAM5 alone. There was also a 30% reduction in the death of $CEA^+/TROP2^+$ target cells by the $CAR^+/iCAR^+$ T cells compared to the CAR^+ only control 48 h after co-culture (Fig. 1C) – a difference not observed with the $CEA^+/TROP2^-$ control. However, 50% of the population was still killed compared to the untransduced negative control, suggesting that the TROP2-PD1 iCAR could inhibit CEACAR cytotoxicity but not completely.

T cells rapidly integrate both positive and negative signals to determine how they will interact with a target cell. Since CAR T signaling and activity is dynamic (45), we hypothesized that this incomplete inhibition might be due to a delay in the TROP2-PD1 iCAR's inhibitory function. To test this hypothesis, $CAR^+/iCAR^+$ T cells were co-cultured with $CEA^+/TROP2^+$ target cells and observed by Incucyte live cell image analysis over 150 h (Fig. 1D and SI Appendix, Fig. S2A). Forty-eight hours after co-culture, target cells were killed by the $CAR^+/iCAR^+$ T cells. However, at 72 h, the adherent target cells appeared to be replicating compared to those cultured with the CAR^+ only control based on relative confluence over time. By 150 h, the target cells were confluent (SI Appendix, Fig. S2A), suggesting that inhibition had occurred. Flow cytometry analysis of target cells recovered after co-culture confirmed the continued expression of CEA and TROP2, removing the possibility that the target cells survived due to CAR target antigen loss (SI Appendix, Fig. S2B). Regardless of the three independent donors used to generate the $CAR^+/iCAR^+$ T cells in replicate experiments, the TROP2-PD1 iCAR was able to inhibit CAR T cell activity, but this inhibition was delayed (Fig. 1D).

Increasing iCAR avidity reduces the kinetic delay in inhibition.

Both affinity and avidity contribute to the efficacy of antibody-based tumor targeting therapies (46). Avidity was recently shown to contribute to an iCAR's ability to inhibit CAR NK cell activity (28). This prompted us to ask whether the delay in iCAR inhibition of T cells was also affected by its avidity. Since avidity is based on the number of receptors and antigens interacting, we investigated these variables by modulating the surface level expression of the antigens on target

cells and the receptors on T cells.

To control the level of CAR and iCAR target antigen, the $CEA^{-}/TROP2^{-}$ target cell line was transduced with lentiviruses that contained CEA (CAR antigen) and TROP2 (iCAR antigen). These cells were single cell cloned and screened for target cell lines that had high CEA and low TROP2 expression (**$CEA^{HI}/TROP2^{LO}$**) and low CEA and high TROP2 expression (**$CEA^{LO}/TROP2^{HI}$**) (Fig. 2A; SI Appendix, Fig. S3A). $CAR^{+}/iCAR^{+}$ T cells were then cocultured with both these cell lines, and the delay in inhibition was compared over time. To compare the delays between groups and experiments, the area under each cytotoxicity curve (AUC) was calculated and normalized to an untransduced T cell control. The closer the normalized AUC was to 1 the more the cytotoxicity curve matched the untransduced control, suggesting a shorter delay. When $CAR^{+}/iCAR^{+}$ T cells were cocultured with target cells that expressed higher levels of the iCAR antigen TROP2 and lower levels of the CAR antigen CEA, the delay in inhibition was reduced ($CEA^{LO}/TROP2^{HI}$ vs $CEA^{HI}/TROP2^{LO}$, Fig. 2B and C). The data showed that as target cells became more sensitive to the iCAR and less sensitive to the CAR, inhibition efficiency improved. Increasing the avidity by higher surface expression of the iCAR target antigen TROP2 reduced the delay in inhibition.

Another way to adjust the avidity was to alter the levels of CAR and iCAR on the surface of T cells. To increase the surface expression of the iCAR, primary T cells were transduced with increasing MOIs for the TROP2-PD1 iCAR lentivirus (MOI: 1, 3, 10), while holding the MOI of the CEACAR lentivirus constant (MOI: 1). Flow cytometry confirmed that as the MOI increased, the surface expression as measured by mean fluorescence intensity (MFI) of the iCAR increased. Concurrently, the MFI of the CAR decreased (Fig. 2D; SI Appendix, Fig. S3B). Overall, the iCAR:CAR ratio gradually increased as we raised the MOI of the iCAR, leading to a higher potential avidity for the iCAR.

These $CAR^{+}/iCAR^{+}$ T cells were then co-cultured with the $CEA^{LO}/TROP2^{HI}$ target cell line and observed for approximately 170 h. As seen in Fig. 2E and F, as the MOI of the iCAR increased,

the efficiency of inhibition increased as measured by the cytotoxicity curves and AUCs. The CAR T cells with an iCAR at an MOI of 10 had a curve that largely overlapped with the untransduced control (Fig. 2E). Although we could not determine the exact iCAR:CAR ratio necessary for complete inhibition, the data suggests that efficiency of inhibition can be controlled through the avidity of the iCAR.

Increasing the affinity of the TROP2-PD1 iCAR does not improve efficiency of AND-NOT gate inhibition.

Affinities of CARs have been tuned to improve the activity and specificity of CAR T cells (14, 15, 47). Likewise, we hypothesized that we could improve the efficiency of iCAR inhibition and reduce the delay observed by increasing the affinity of the iCAR to TROP2.

Using Bio-Layer Interferometry (BLI), the C3 and B11 scFv chains in our phage display library were found to have a 4.6X and 2.6X higher binding affinity compared to the H11 clone in our original iCAR construct respectively (C3 K_d = 0.78nM; B11 K_d = 1.37nM; H11 K_d = 3.55nM) (Fig. 3A). The C3 and B11 scFv chains were incorporated into iCARs by replacing the H11 scFv chain in our original TROP2-PD1 iCAR (H11-TROP2-PD1 iCAR). These three iCARs (C3-TROP2-PD1 iCAR, B11-TROP2-PD1 iCAR, and H11-TROP2-PD1 iCAR) were introduced into primary T cells with the CEACAR at an MOI of 1 for both the CAR and iCAR to ensure that avidity would not confound the results. All three iCARs were confirmed to have similar surface expression levels by flow cytometry (Fig. 3B). Enriched $CAR^+/iCAR^+$ T cells were co-cultured with target cells and cytotoxicity observed over time.

Approximately a week after coculture, no improvement was seen in the inhibition efficiency of both higher affinity iCARs (C3-TROP2-PD1 iCAR and B11-TROP2-PD1 iCAR) (Fig. 3C). When calculating for normalized AUC, they were even found to be significantly worse (Fig. 3D). When TROP2 levels were reduced ($CEA^{HI}/TROP2^{LO}$), these iCARs showed no significant difference between each other regardless of affinity. Since the epitopes of these scFv chains were not mapped and the long spacer used may not be ideal for C3 and B11, variations of these iCARs

were generated that had a short spacer (IgG4 hinge). These iCARs were less expressed on the surface of the cell compared to their long spacer counterparts and also showed worse inhibition (SI Appendix, Fig. S4). These results demonstrated that further increasing the affinity of our iCAR did not improve iCAR efficiency.

iCARs with immunoreceptor tyrosine-based inhibition or switch motifs (ITIM/ITSM) can inhibit CAR T cell activation and cytotoxicity.

CARs have been enhanced by replacing their co-stimulatory and activation domains with alternative domains (i.e. 41BB, ICOS, JAK/STAT, OX-40) that improve proliferation, cytokine production, and *in vivo* persistence (41, 48–50). It was recently shown that alternative inhibitory domains, such as CTLA-4, LIR-1, and TIGIT, could also replace the function of PD1 in an iCAR in T cells (24, 26, 29). Domains including KIR2DL1, LIR-1, CD300A, NKG2a, and LAIR-1 were also tested in an iCAR construct in NK cells (51).

We selected a series of inhibitory receptor signaling domains as potential modules that could inhibit CAR T cell activity. Some domains were derived from receptors that have been targeted as checkpoint inhibitors like TIM-3 (T-Cell Immunoglobulin And Mucin Domain-Containing Protein 3), CTLA-4, and LAG-3 Lymphocyte Activating 3) (52–54). Other domains like CD5, PCDH18 (Protocadherin 18), and VISTA (V-Set Immunoregulatory Receptor) were selected due to their previous roles in T cell inhibition in mouse knock-out models (55–59). Domains from BTLA (B And T Lymphocyte Associated), LAIR-1 (Leukocyte Associated Immunoglobulin Like Receptor 1), TIGIT, SIGLEC-7 (Sialic Acid Binding Ig Like Lectin 7), and SIGLEC-9 (Sialic Acid Binding Ig Like Lectin 9) were all chosen for their inclusion of ITIM/ITSMs, which both inhibit signaling through the recruitment of phosphatases (60).

To test these domains for inhibitory function, twenty-two iCARs were constructed by linking the H11 TROP2 scFv chain, an extracellular spacer of variable length (Short or Long as described in Methods), a CD28 TM, and the intracellular domain of the inhibitory receptor as designated by Uniprot (61) (SI Appendix, Fig. S5B and Table S1). All constructs were confirmed to be expressed

on the cell surface by flow cytometry against an HA-tag on its N-terminus (SI Appendix, Fig. S5C). Short spacer iCARs trafficked less effectively to the surface of the cell compared to those that contained long spacers regardless of the inhibitory signaling domain used (SI Appendix, Fig. S5C). To rapidly screen through these iCARs, a Jurkat-NFAT-ZsGreen reporter cell line was cotransduced with both an iCAR and a CEACAM5-Long-CD28-3z CAR (iCAR MOI: 25, CAR MOI: 1) and tested for activation after coculture. When activated, these Jurkat cells increase the expression of ZsGreen and can be detected by flow cytometry, but if inhibited, they cannot (SI Appendix, Fig. S5A). Sorted $CAR^+/iCAR^+$ Jurkat cells were co-cultured with target cells that expressed CEA and/or TROP2 for 24 h. Specific inhibition mediated by the iCAR was calculated by comparing the percentage of $ZsGreen^+$ Jurkat cells when co-cultured with $CEA^+/TROP2^-$ target cells compared to $CEA^+/TROP2^+$ cells.

Approximately 75% of CAR^+ Jurkat cells were activated when co-cultured with target cells that expressed CEACAM5 regardless of TROP2 expression. However, the TROP2-PD1 iCAR decreased the percentage of activated cells to ~40% when TROP2 was present (SI Appendix, Fig. S5D). In total, eleven additional inhibitory signaling domains were screened for their ability to inhibit CAR T cell activity. Of the twenty-two additional iCAR constructs tested, eight of them specifically inhibited CAR T cell activation when cocultured with the $CEA^+/TROP2^+$ line compared to the $CEACAM5^+$ line. These eight constructs all contained an ITIM/ITSM motif (SI Appendix, Fig. S5D). The TROP2-Long-SIGLEC9 iCAR showed the greatest specific inhibition with a difference of ~40%.

iCARs containing BTLA, LAIR-1, and TIGIT inhibited CAR T cell activation even when TROP2 was not expressed. This ligand-independent inhibition may be due to tonic signaling of the iCAR at this avidity. Some non-ITIM-containing iCARs such as LAG-3 and CD5 also showed ligand-independent inhibition, but because no specific inhibition was observed, they were not further pursued.

A selection of iCARs (BTLA, LAIR-1, SIGLEC-9) that functioned best in the reporter assay were

then tested for their ability to inhibit cytotoxicity in primary T cells equipped with the CEACAR (Fig. 4A). As a negative control, the VISTA iCARs were included. To lower the contribution of avidity and potential tonic signaling seen in the Jurkat reporter assay, the MOI of the iCAR was reduced to an MOI of 10. All iCARs were confirmed to be expressed on the surface of primary T cells (Fig. 4B).

Observing the kinetics of cytotoxicity, we found that the TROP2-Long-BTLA, LAIR-1, and SIGLEC-9 iCARs all inhibited CAR T cell cytotoxicity at a similar rate as the TROP2-PD1 iCAR when cocultured with target cells that expressed high levels of TROP2 (Fig. 4B and C). When the TROP2 level was reduced in target cells ($CEA^{HI}/TROP2^{LO}$), the TROP2-Long-SIGLEC9 iCAR showed a reduced delay in inhibition compared to the TROP2-PD1 iCAR, suggesting that it might be more efficient.

To test whether changing the extracellular spacer length might improve the efficiency of the iCAR, we tested these same constructs with a shorter spacer length. We found that these iCARs had approximately 30 to 70% less surface expression and were less efficient at inhibiting cytotoxicity compared to their long spacer counterparts (Fig. 4B and C). These data indicate that ITIM/ITSM-containing iCARs can inhibit CAR T cell cytotoxicity.

DiCARs improve iCAR inhibitory kinetics and efficiency.

Third-generation CARs, which combine multiple costimulatory domains into one construct, have been reported to increase CAR T cell survival and antitumor efficacy (62–64). This led us to ask whether combining multiple inhibitory signaling domains into a single construct could further enhance inhibition efficiency.

A series of dual-inhibitory domain iCARs (DiCARs) were designed by linking the TROP2-PD1 iCAR with an additional domain from PD-1, BTLA, SIGLEC-9, or LAIR-1 on its C-terminus (Fig. 5A). These domains were chosen since they functioned as a single-domain iCAR. Only the long extracellular spacer was incorporated into the DiCARs because short spacer constructs were consistently shown to be less efficient as single domain iCARs (Fig. 4B and C).

Primary T cells were transduced with the CEACAR at a MOI of 1 and the DiCAR at a MOI of 1 to further reduce the contribution of avidity to inhibition. All DiCARs were confirmed to traverse to the cell surface as detected by flow cytometry (Fig. 5B). DiCAR surface expression was similar between all constructs except for the PD1-BTLA DiCAR, which always had the lowest expression and transduction efficiency (SI Appendix, Fig. S6). Enriched $CAR^+/iCAR^+$ T cells (>94%) were co-cultured with target cells that expressed CEACAM5 and/or TROP2 and monitored for cytotoxicity over a week to observe the delay in inhibition. Three DiCARs (PD1-PD1, PD1-SIGLEC9, PD1-LAIR1) inhibited CAR T cell cytotoxicity more efficiently than the TROP2-PD1 iCAR as indicated by a faster recovery of target cells (Fig. 5C). The delay in inhibition was significantly decreased as calculated by AUC (Fig. 5D) regardless of high or low TROP2 expression. This trend of improved inhibition by DiCARs was found to be reproducible in three independent experiments although the quantitative effect varied (SI Appendix, Fig. S7).

Discussion

Epithelial cell markers like TROP2 can be used as iCAR targets for AND-NOT gating strategies.

By combining a CD-19 targeting CAR and a PSMA targeting iCAR, Federov *et al.* showed that an AND-NOT gating strategy could potentially solve the on-target, off-tumor toxicity problem of CAR T cells (24). To make it clinically applicable, many groups began to target HLA molecules with the iCAR. Because HLA is expressed on most normal tissues but downregulated by tumor cells, this target could provide broad protection (25–28, 30). However, by using HLA-directed iCARs, CAR T therapy becomes subject to HLA-restriction, circumventing a key benefit it provided over TCR-based immunotherapies. Additionally, HLA-directed iCARs can lead to inhibition of proliferation during the production of $CAR^+/iCAR^+$ T cells since HLA is expressed on T cells.

Here we report that a CAR can also be combined with an iCAR targeting a normal epithelial cell marker like TROP2. Although TROP2 is highly expressed in tumors of epithelial cell origin (65) and has been championed as a CAR target (66), in the correct context, it can be used as an iCAR target as well. TROP2 is widely expressed in normal tissues of the kidneys, lung, and skin, and

if matched with a CAR that targets cancers without TROP2 expression can provide protection without HLA-restriction (36). Future work targeting other broadly expressed epithelial cell markers like EpCAM (67), E-Cadherin (68), and Claudin-4 (69) could also be promising.

Balancing the levels of CAR and iCAR signaling is critical to obtaining specific inhibition.

While testing the TROP2-PD1 iCAR for specific inhibition against the CEACAR, we observed a delay in its ability to inhibit cytotoxicity. This delay was found to be avidity dependent and correlated to the iCAR:CAR ratio. This result may explain why in previous studies with both T cells and NK cells, iCAR inhibition was enhanced with its overexpression (24, 28). Interestingly, as the amount of iCAR increased, the level of ligand independent inhibition also increased (Fig. 2E). Our data suggests that a balance between the number of CARs and iCARs signaling is critical to obtain specific inhibition. Accurate quantification of CAR and iCAR expression is necessary to determine a therapeutic window for this strategy.

Increasing the affinity of the iCAR did not enhance inhibition efficiency.

As an alternative to balancing the ratio of CAR and iCAR, we sought to build a more efficient iCAR. We first sought to increase the affinity of the iCAR scFv chain by three- to five-fold (Fig. 3). Although enhancing iCAR affinity was expected to increase function, it did not. Though surprising, this result is not unprecedented in CAR engineering. It has been reported that if CARs reach an affinity threshold further enhancement does not improve activity (47, 70). Because all three scFv chains tested were of “high” affinity, we might have already reached that threshold. Differences in affinity of 10- to 20-fold may be required to see significant changes.

It is unclear as to why the H11-TROP2-PD1 iCAR seemed to function better than the C3 and B11 ones which had higher binding affinities when TROP2 levels were high (Fig. 3). One hypothesis was that the spacer length for both the C3 and B11 antibodies were not optimized for binding their corresponding epitopes. To address this possibility, we changed the length of the spacer in SI Appendix, Fig. S4. However, this modification further reduced the efficiency, suggesting that another variable or combination of variables might be contributing to this difference.

ITSM but not non-ITIM/ITSM inhibitory domains improve iCAR efficiency.

A second change made to potentially increase iCAR inhibition efficiency was to replace the PD-1 domain with a non-ITIM/ITSM containing domain like LAG-3, TIM-3, or CTLA-4. None of the seven domains, including CTLA-4 which was reported by Fedorov *et al.* (24) to function, were capable of specifically inhibiting activity in our Jurkat activation screen (SI Appendix, Fig. S5). Although thirteen constructs were evaluated, the potential combinations of spacer/hinge, transmembrane domain, and signaling domain were not exhausted. Because spacers and transmembranes are known to affect CAR function (71, 72), we cannot exclude the possibility that inhibition could have been seen if another construct was used.

It is unclear as to why intracellular signaling domains from known checkpoint inhibitors like LAG-3 and TIM-3 did not specifically inhibit CAR T cell activation in this assay. Alternative inhibitory mechanisms utilized by these non-ITIM containing inhibitory receptors may be unable to inhibit CAR T cell activation. LAG-3 functions through its KIEELE and FxxL motif, but its mode of inhibition is unknown (73, 74). TIM-3 is thought to function by either destabilizing the immunological synapse through the recruitment of phosphatases or recruiting FYN and CSK to the membrane to inactivate Lck (75, 76). It may be that SHP-1 and/or SHP-2 phosphatases that are recruited via the ITIM motif are necessary for CAR inhibition.

This concept is further strengthened by the fact that the domains that have been shown capable of replacing PD-1 in an iCAR by other groups and ours all contain ITIM/ITSM motifs. The LIR-1 domain described by Hamburger *et al.* contains four ITIM motifs (26), while all the NK receptor domains tested by Li *et al*. (KIR2DL1, LIR-1, CD300a, NKG2A, and LAIR-1) all contain varying numbers of ITIM or ITIM-like motifs (51). Because these motifs are important for the recruitment of the phosphatases SHP-1 and/or SHP-2, which dephosphorylate T cell activation proteins like Zap-70 and LAT (60), the number of ITIMs may correlate to iCAR inhibition efficiency. This may explain why the PD1-LAIR1 DiCAR can perform at similar efficiencies as the PD1-PD1 and PD1-SIGLEC9 DiCARs although it has lower surface expression than the PD1-PD1 and PD1-SIGLEC9

DiCARs (Fig. 5 and SI Appendix, Fig. S6). In total, the PD1-LAIR1 DiCAR would have three ITIMs and one ITSM, while the PD1-BTLA, PD1-PD1, and PD1-SIGLEC9 DiCARs would all have two ITIMs with varying numbers of ITSM or ITIM-like domains (60, 77, 78). By having one additional ITIM, the PD1-LAIR1 DiCAR may recruit more phosphatases to the membrane, increase dephosphorylation, and more rapidly inhibit CAR T cell activation even with lower numbers of receptors on the cell surface. Furthermore, the LAIR-1 domain has been found to be constitutively associated with the phosphatase SHP-1 (79) and could increase the kinetics of inhibition. Alternatively, since these phosphatases bind ITIM domains via a SH2 domain that can affect their activation (80, 81), as well as proximity to CARs, the geometry of ITIMs in these DiCARs may contribute to its inhibition efficiency.

Future work should be focused on two major aspects of enhancing this AND-NOT gate design. First, efforts must be concentrated on determining which combinations of spacers, transmembrane domains, and inhibitory domains can be combined to generate DiCARs with enhanced specificity and reduced ligand-independent inhibition. The combination of domains assessed in DiCARs here were not exhaustive, and additional constructs may further enhance the dynamic range of this strategy.

Second, experiments should be performed to determine the *in vivo* specificity of CAR^+, $DiCAR^+$ T cells. For these *in vivo* studies, a replacement pair of CAR and DiCAR antigens that are clinically relevant should be investigated. These antigens should match the following criteria: 1) the CAR antigen should have low expression in normal tissues, 2) the DiCAR antigen should have high expression in normal tissues that express the CAR antigen, and 3) the DiCAR antigen should be stably expressed on the surface of the cell, ubiquitously expressed in all normal tissues where on-target, off-tumor toxicity would be anticipated, and not be prone to cleavage. The TROP2 antigen selected in this study is suspected to be cleaved *in vivo* by proteases like ADAM17 (39), matriptase (82), and/or ADAM10 (83), which may explain why in preliminary studies we have found reduced expression of this antigen. Optimization of CAR dosage, DiCAR dosage, T cells

injected, and antigen expression in tumors cells will need to be determined and optimized to achieve tumor elimination with reduced toxicity *in vivo* and are currently underway.

Just as second-generation CARs combined a costimulatory domain with the activation domain to enhance CAR T cell function, the DiCARs presented here combine two inhibitory domains to become a second-generation iCAR. The AND-NOT gating strategy can be applied to reduce on-target, off-tumor toxicity by balancing the enhanced strength of CARs with the better regulation of DiCARs.

Materials and Methods

Cell Line Generation

The DU145 prostate cancer target cell line was previously modified to knock-out TROP2 expression ($CEA^-/TROP2^-$) using a CRISPR-Cas9 strategy (44). To generate target lines that express CEA and/or TROP2, CEA and TROP2 were cloned into separate lentiviral constructs and transduced into the $CEA^-/TROP2^-$ cell line. Each cell line was also engineered to express GFP for cytotoxicity assays. Following transduction, cells were single cell sorted for CEA, TROP2, and/or GFP expression. Clones were selected that had the desired surface expression of CEA and/or TROP2. Surface expression of CEA and TROP2 were confirmed by flow cytometry using the antibodies listed in SI Appendix, Table S2. The Jurkat-NFAT-ZsGreen reporter cell line was a gift generated and given by Dr. David Baltimore's lab.

Lentivirus Production

Lentivirus for the various CARs and iCARs were generated using a previously published protocol (84). Briefly, 293T cells were grown in DMEM (Dulbecco's Modified Eagle Medium) + 10% FBS (Fetal Bovine Serum). 293T cells were transfected with Mirus TransIT 293 (Mirus, MIR2705). One day after transfection, cells were treated with 10mM sodium butyrate for 6 to 8 h. Media was replaced with Collection Media (Ultraculture/Pro293-AM + Glutamax + 20mM HEPES). Two days later, viral supernatant was collected, filtered through a 0.45-uM filter, and concentrated using Amicon Ultra-15 (100,000 NMWL) filters (Millipore, UFC910024). Virus was frozen and titered on

293T cells.

Identification of TROP2 binding antibodies using phage display

A human scFv phage display library previously published by Li *et al.* was used to discover antibodies binding TROP2 (43). The phage library was panned with recombinant TROP2 extracellular domain-Fc chimera (R&D Systems, 650-T2-10). Clones that bound TROP2 were found using an anti-M13 antibody that recognizes the phage by ELISA. Complete antibody molecules (scFv-Fc) were generated by linking the scFv to human IgG1 Fc on the C-terminus and cloned into an expression vector. Stable transfectants for antibody production were generated using Zeocin selection. The supernatant from these transfections were collected, filtered, purified, and concentrated to yield a concentration of 0.1 to 1mg/mL. These antibodies were confirmed to specifically bind TROP2 by flow cytometry against an engineered TROP2$^+$ cell line. Binding kinetics of each antibody were determined using Bio-Layer Interferometry (BLI). Recombinant TROP2 extracellular domain protein was bound to the sensor surface and anti-TROP2 antibodies added in concentrations ranging from 400 to 12.5nM. Binding affinity was calculated using FortéBio Data Analysis software. Sequences of the desired scFv's were then utilized as the antigen-binding domain of iCARs.

CAR and iCAR Vector Construction

The CEACAM5 CAR was previously designed and produced by combining the CEACAM5-targeting scFv (Labetuzumab) (85), an IgG4 hinge, the IgG4 CH2 and CH3 constant domains, a CD28 transmembrane domain, a CD28 co-stimulatory domain, and a CD3ζ activation domain (31). Modifications to the CEACAM5 CAR were made to replace the spacer region (IgG4 Hinge + CH2 + CH3) with a spacer developed by Hudecek *et al.*, which we termed the 4/2NQ spacer (40). Additional changes were made to replace the CD28 co-stimulatory domain with the 41BB costimulatory domain to generate the CAR used throughout this paper (CEACAR). iCARs were generated using a similar structure to that previously published (24). Antibodies that react to TROP2 as identified by screening a phage display library were converted into scFv chains. The

scFv chain was linked to various extracellular spacers (Short – IgG4 Hinge; Long – IgG4 Hinge + CH2 + CH3), the CD28 transmembrane domain, and a series of intracellular signaling domains from immune cell inhibitory receptors. The exact amino acids that were used for the intracellular signaling domains are listed in SI Appendix, Table S1. DiCARs are generated by linking an anti-TROP2 scFv chain to an extracellular spacer, a CD28 transmembrane domain, a PD-1 signaling domain as listed in SI Appendix, Table S1, and an additional signaling domain (i.e. PD-1, BTLA, SIGLEC-9, LAIR-1) as listed in SI Appendix, Table S1. Both the CAR and iCAR were cloned into a third-generation lentiviral vector pCCL-c-MNDU3 generously given by Dr. Gay Crooks and Dr. Donald Kohn.

Primary CAR T cell Generation, Enrichment, and Characterization

Peripheral blood mononuclear cells (PBMCs) were purchased from All Cells, LLC from various donors. Unless stated otherwise, in each experiment, a single donor was used for all groups being compared to remove donor variability within the experiment. T cells and PBMCs were grown in TCM (T Cell Media) Base supplemented with the listed cytokines (TCM Base = AIM-V Media (Thermo Fisher, 12055) supplemented with 5% human heat-inactivated AB serum (Omega Scientific, HS-25), Glutamax (Thermo Fisher, 35050-061), and 55uM of Beta-mercaptoethanol. PBMCs were initially thawed and cultured in TCM Base + 50U/mL IL-2 (Peprotech, 200-02). PBMCs were activated with Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher, 11132D) at a 1:1 cell:bead ratio and plated overnight at 37°C at a concentration of 1x10^6 cells/mL. The following day activated cells with beads were collected and resuspended in fresh TCM + 50U/mL IL-2 and diluted to a concentration of 0.5x10^6 cells/mL and plated into a 24-well plate. Cells were transduced with lentivirus containing the iCAR at the appropriate MOI of 1, 3, or 10. Infections were supplemented with Protamine Sulfate at a concentration of 100ug/mL. Six hours after incubation with the iCAR lentivirus, supernatant was removed, and CAR lentivirus was added with fresh Protamine Sulfate. The next day an additional 1mL of media was added to each well. Seven days after activation, Dynabeads were removed, and T cells were transferred to TCM Base

+ 50U/mL IL-2 + 0.5ng/mL IL-15 (Peprotech, 200-15) media at a concentration of 1x10^6 cells/mL. On day 9, T cells were enriched for CAR+, iCAR+ T cells using magnetic bead enrichment. Briefly, CAR^+ T cells were selected after staining with an Anti-FLAG-PE antibody and enriched using the EasySep Release Human PE Positive Selection Kit (Stemcell, 17654) since CARs were linked to a FLAG-tag on their N-terminal end. These cells were then selected for $iCAR^+$ T cells by staining with an Anti-HA-APC antibody and enriched using the EasySep APC Positive Selection Kit (Stemcell, 17681) since iCARs were linked to a HA-tag on their N-terminal end. On day 11, magnetic beads used for enrichment were removed. On day 12, T cells were characterized by flow cytometry and used for various cytotoxicity assays. For ELISAs, 48h after co-culture began, supernatant was harvested from each well. Supernatant was used to measure IFN-γ using the BD OptEIA Human IFN-γ Set (BD, 555142).

Jurkat Activation Reporter Assay

To rapidly screen an iCAR's potential to inhibit CAR T cell activity, a Jurkat reporter assay was utilized. The Jurkat-NFAT-ZsGreen reporter cell line was generously provided by Dr. David Baltimore. These cells were transduced with a lentivirus containing the CEACAM5-Long-CD28-3z CAR previously published by our lab at a MOI of 1 (31). CAR^+ Jurkat cells were also transduced with a lentivirus containing the selected iCAR at a MOI of 25. $CAR^+/iCAR^+$ Jurkat cells were sorted and used in a coculture assay. Jurkat cells were incubated with DU145 target cells for 24 h at an effector:target ratio of 1:1 in RPMI (Roswell Park Memorial Institute media) + 10% FBS + Glutamine (RPMI10+). Jurkat cells were then collected from the culture and the percentage of ZsGreen+ cells were measured by flow cytometry. Gating was performed on CD3+ cells to ensure that GFP+ target cells were not contributing to the measurement.

T Cell Kinetic Cytotoxicity Assay

Plates are coated with 0.001% Poly-L-Lysine for at least 30 min at 37°C. DU145 target cells that are GFP+ are collected from culture and plated in RPMI10+ (RPMI + 10% FBS + 40mM Glutamine) at the desired concentration into the coated plate. Effector CAR T cells are collected from culture

and washed with 1X PBS. CAR T cells are counted and plated at the desired concentration in RPMI10+ into wells that contain target cells. Cocultures are performed at the effector:target ratio descried in the figures. Cocultures are imaged using an Incucyte Zoom Live Cell Analysis System (Sartorius) over a week at approximately 2-h intervals. Masking is performed to calculate the area covered by GFP+ target cells. AUC analysis is performed using GraphPad Prism over time.

Flow Cytometry Analysis

Cells are collected from culture and washed with 1X PBS (Phosphate Buffered Saline). Cells are stained with the selected antibodies in FACS (Fluorescence-Activated Cell Sorting) Buffer (1X PBS + 3% Fetal Bovine Serum + 0.09% Sodium Azide). Antibodies that were used are listed in SI Appendix, Table S2. After staining, cells are washed with 1X PBS and resuspended in FACS Buffer. Cells are run on the BD FACS Canto, the BD FACSAria, or the HT LSR II. Quantification of the amount of CAR and iCAR surface expression was performed using Quantum Simply Cellular anti-Mouse IgG (Bangs Laboratories, 815A) and anti-Rat IgG beads (Bangs Laboratories, 817A) using geometric MFI.

Xenograft model for CEACAR tumor killing

Animal experiments were conducted according to a protocol approved by the Division of Laboratory Medicine at the University of California, Los Angeles. NSG mice were obtained from The Jackson Laboratory at 68 wk of age. Engineered DU145 lines that express CEACAM5 and/or TROP2, GFP, and YFP-Luciferase were mixed with Matrigel Matrix Basement Membrane (Corning 354234) and engrafted into mice subcutaneously on the right flank. T cells were prepared as described in Primary CAR T cell Generation, Enrichment, and Characterization. Approximately, 3 wk after engraftment, when tumors were measurable (10 to 100 mm^3), 2 X 10^6 or 4 X 10^6 T cells were injected into mice via tail vein. Weekly caliper measurements were obtained of the tumors starting the second week after T cell injection.

Data, Materials, and Software Availability

All study data are included in the article and/or SI Appendix.

Acknowledgments

We thank Dr. Yvonne Chen for her advice throughout this project and the basic constructs for developing our CARs. We thank Dr. David Baltimore's lab for the Jurkat-NFAT-ZsGreen reporter system used in these assays. We thank Dr. Gay Crooks and Dr. Donald Kohn for the lentiviral backbone pCCLC-MNDU3 in which the CAR constructs were cloned. We thank Dr. Evan Abt, Dr. Greg Varuzhanyan, and Dr. John Lee for reading and commenting on the manuscript. We also thank Dr. Keyu Li for his assistance in deriving the TROP2 antibodies used in this study. We thank the many undergraduate researchers (Tiffany Pariva, Elizabeth Tran, Leo Hoang) who helped in cloning constructs. We thank the BSCRC flow and microscopy cores for the use of their facilities. N.J.B. was supported by these training grants: the UCLA Tumor Immunology Training Grant (USHHS Ruth L. Kirschstein Institutional National Research Service Award # T32 CA009056) (2016-2017), the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research at UCLA (BSCRC) Training Program (2017-2018), the UCLA Interdisciplinary Training in Virology and Gene Therapy Training Grant (2018-2019). This project was supported by the UCLA Prostate Cancer SPORE (Award Number P50CA092131) and the Parker Institute of Cancer Immunotherapy.

Figures

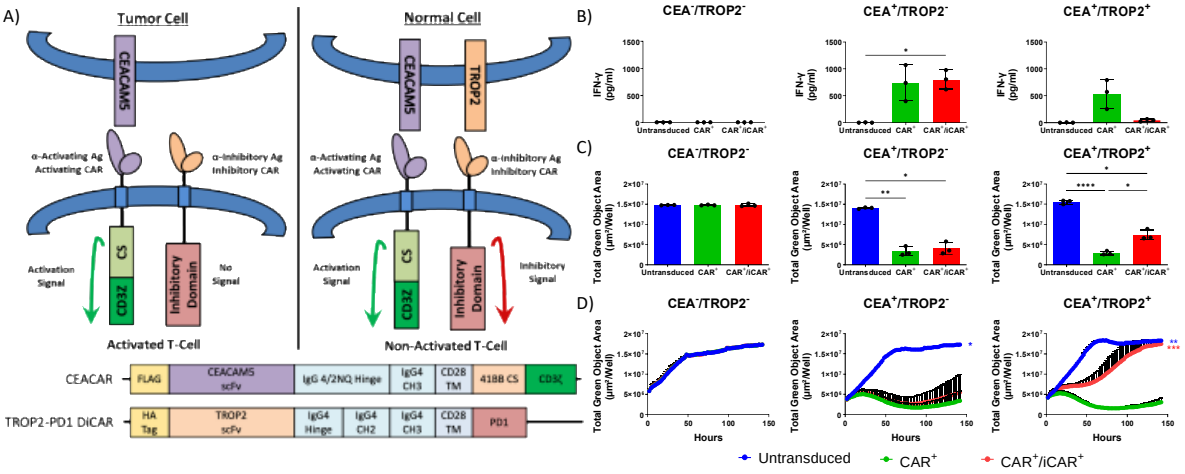


Fig. 1. Inhibition of CAR T cell cytotoxicity by the TROP2-PD1 iCAR is delayed.
A) The model illustrates the "AND-NOT"-gate CAR T strategy for specifically targeting CEA$^+$ tumor cells. The CAR and iCAR target CEA and TROP2, respectively. The CEACAR consists of a FLAG tag, an scFv chain that recognizes CEA, a IgG 4/2NQ hinge, an IgG4 CH3 constant domain, a CD28 transmembrane domain (TM), a 41BB costimulatory (CS) domain, and a CD3ζ activation domain. The TROP2-PD1 iCAR consists of an HA tag, an scFv chain that recognizes TROP2, the IgG4 hinge, CH2, and CH3 constant domains, a CD28 TM, and a PD1 signaling domain.

B) CAR$^+$/iCAR$^+$ T cells can inhibit CAR T cell IFN-γ production as measured by ELISA 48 h after co-culture of T cells with DU145 target cells that express CEA and/or TROP2.

C) CAR$^+$/iCAR$^+$ T cells that can specifically inhibit CAR T cell cytotoxicity after 48 h in co-culture with DU145 target cells that express CEA and TROP2. Target cell presence was measured by total green object area (μm^2/well) of DU145 target cells that express CEA and/or TROP2.

D) Inhibition of cytotoxicity is delayed in CAR$^+$/iCAR$^+$ T cells when co-cultured with DU145 target cells. The cytotoxicity curve shown is a composite of three donors. Measurements of total green object area of GFP+ target cells were measured over ~140 h by Incucyte live cell image analysis at intervals of 2 h. Statistics are calculated based on the total green object area (μm^2/well) at the last time point compared to the CAR$^+$ only control.

The data are reported as a mean ± SE (n = 3 donors). Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value ≤ 0.05, **P value ≤ 0.01, ***P value ≤ 0.001

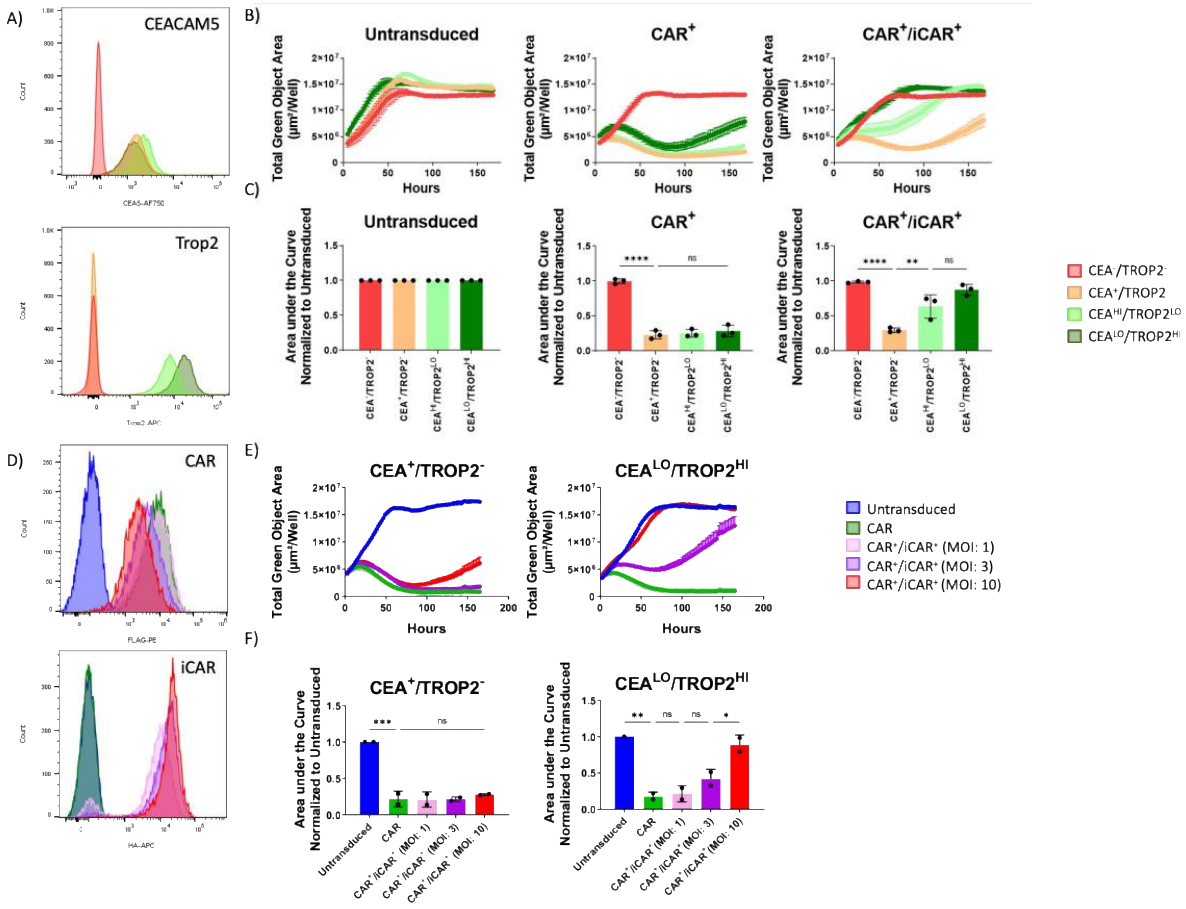


Fig. 2. Controlling the avidity of iCAR interactions reduces the delay in inhibitory signaling kinetics.

A) Engineered target cell lines have different surface level expression of CEA and TROP2 measured by flow cytometry. Histograms are representative images from one of three experiments comparing CEA and TROP2 expression of each target cell line.

B) Increasing the target antigen density reduces the delay in iCAR inhibition as measured by cytotoxicity over time. The cytotoxicity curves are representative images from one experiment measuring the total green object area of the target cells over time (µm^2/well).

C) CAR$^+$/iCAR$^+$ T cells co-cultured with target cells that express high levels of TROP2 have reduced delays in inhibition. The delay in inhibition was measured by calculating the area under each cytotoxicity curve. The AUC was normalized against the AUC calculated for untransduced T cells co-cultured with target cells. The normalized AUC quantified is the mean $\pm$ SD (n=3).

D) Representative histograms measuring the Mean Fluorescence Intensity (MFI) indicate the difference in CAR and iCAR surface expression between CAR T cell groups being tested. Groups have been transduced with CAR lentivirus at a MOI of 1 and iCAR lentivirus at a MOI of 1, 3, and 10, respectively.

E) Increasing the surface level expression of the iCAR in primary T cells reduces the delay in iCAR inhibition as measured by cytotoxicity over time. Representative cytotoxicity curves from

one experiment are displayed comparing the killing ability of CAR T cells with different surface level expression of the iCAR when co-cultured with DU145 target cells that express CEA or CEA and TROP2.

F) $CAR^+/iCAR^+$ T cells with higher iCAR surface expression have reduced delays in inhibition when co-cultured with $CEA^{LO}/TROP2^{HI}$ target cells. The delay in inhibition was measured by calculating the area under each cytotoxicity curve. The AUC was normalized against the AUC calculated for untransduced T cells co-cultured with target cells. The normalized AUC quantified is the mean $\pm$ SD (n=2).

Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value $\leq$ 0.05, **P value $\leq$ 0.01, ***P value $\leq$ 0.001

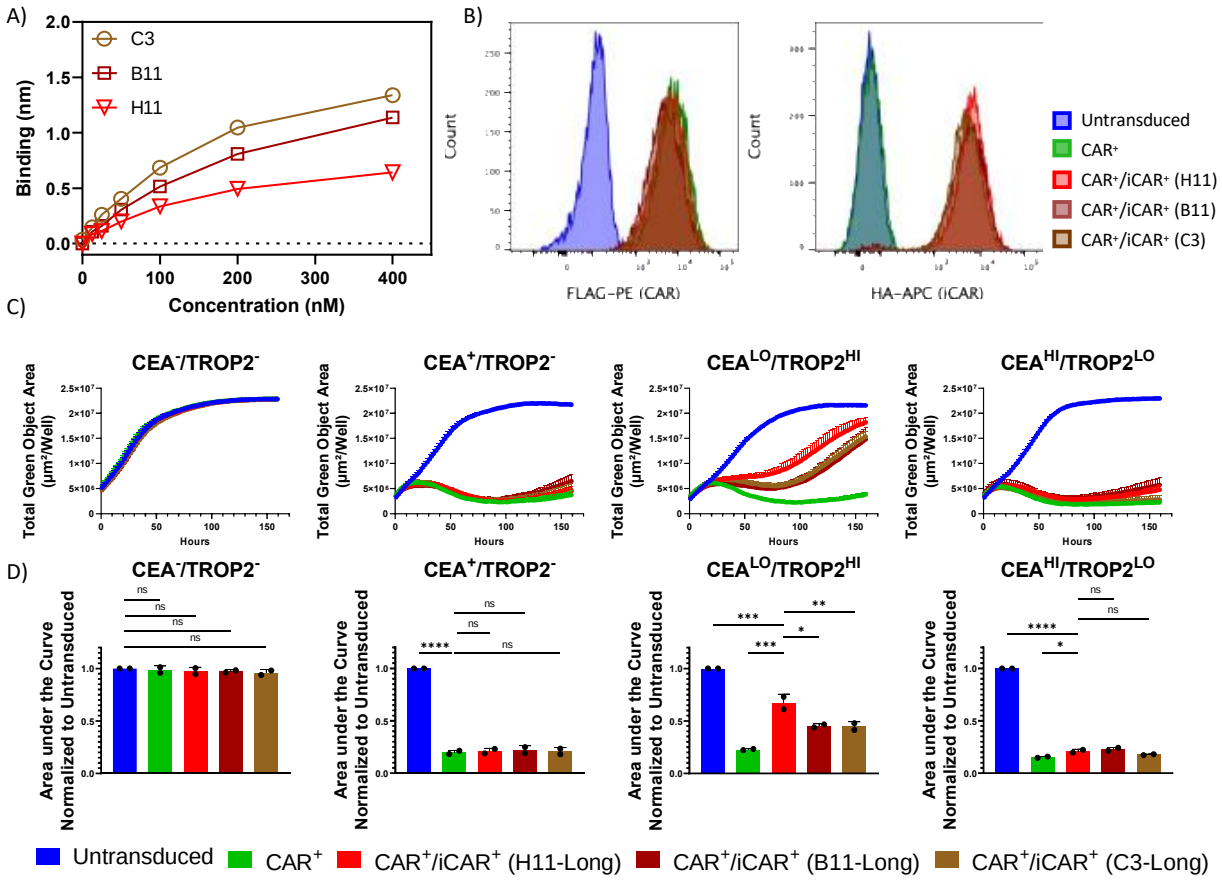


Fig. 3. Increasing the affinity of the TROP2-targeting iCAR does not increase inhibition efficiency.

A) Comparison of binding affinities between three different antibodies targeting Trop2. Binding affinity kinetics were measured by Bio-Layer Interferometry (BLI) using biosensors precoated with recombinant TROP2 protein. Antibodies were serially diluted in concentrations ranging from 400 to 12.5nM. The binding values were obtained and plotted against concentrations of antibody (nM).

B) Representative histograms from one experiment that show CAR and iCAR surface expression is similar between all T cell groups being tested. CAR and iCAR expression are measured using flow cytometry with antibodies against the FLAG- and HA-tags on the engineered receptors respectively.

C) CAR$^+$/iCAR$^+$ T cells with the C3, B11, and H11 scFv show similar levels of cytotoxicity to each other. Representative cytotoxicity curves are displayed from one experiment where the total green object area (µm/well) of GFP$^+$ DU145 target cells that express CEACAM5 and/or TROP2 were measured over approximately 160 h.

D) Area under the curve analysis of cytotoxicity curves. The delay in inhibition was measured by calculating the area under each cytotoxicity curve. The AUC was normalized against the AUC calculated for untransduced T cells cocultured with target cells. The normalized AUC quantified is the mean $\pm$ SD (n=2) from two independent experiments. The significance values shown are comparisons between a control group. For the CEA$^-$/TROP2$^-$ cell line, values are compared to the untransduced control. For the CEA$^+$/TROP2$^-$ cell line, values are compared to the CAR control.

For the $CEA^{LO}/TROP2^{HI}$ or $CEA^{HI}/TROP2^{LO}$ cell lines, values are compared to the $CAR^{+}/iCAR^{+}$ (H11-Long) group.

Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value $\leq$ 0.05, **P value $\leq$ 0.01, ***P value $\leq$ 0.001

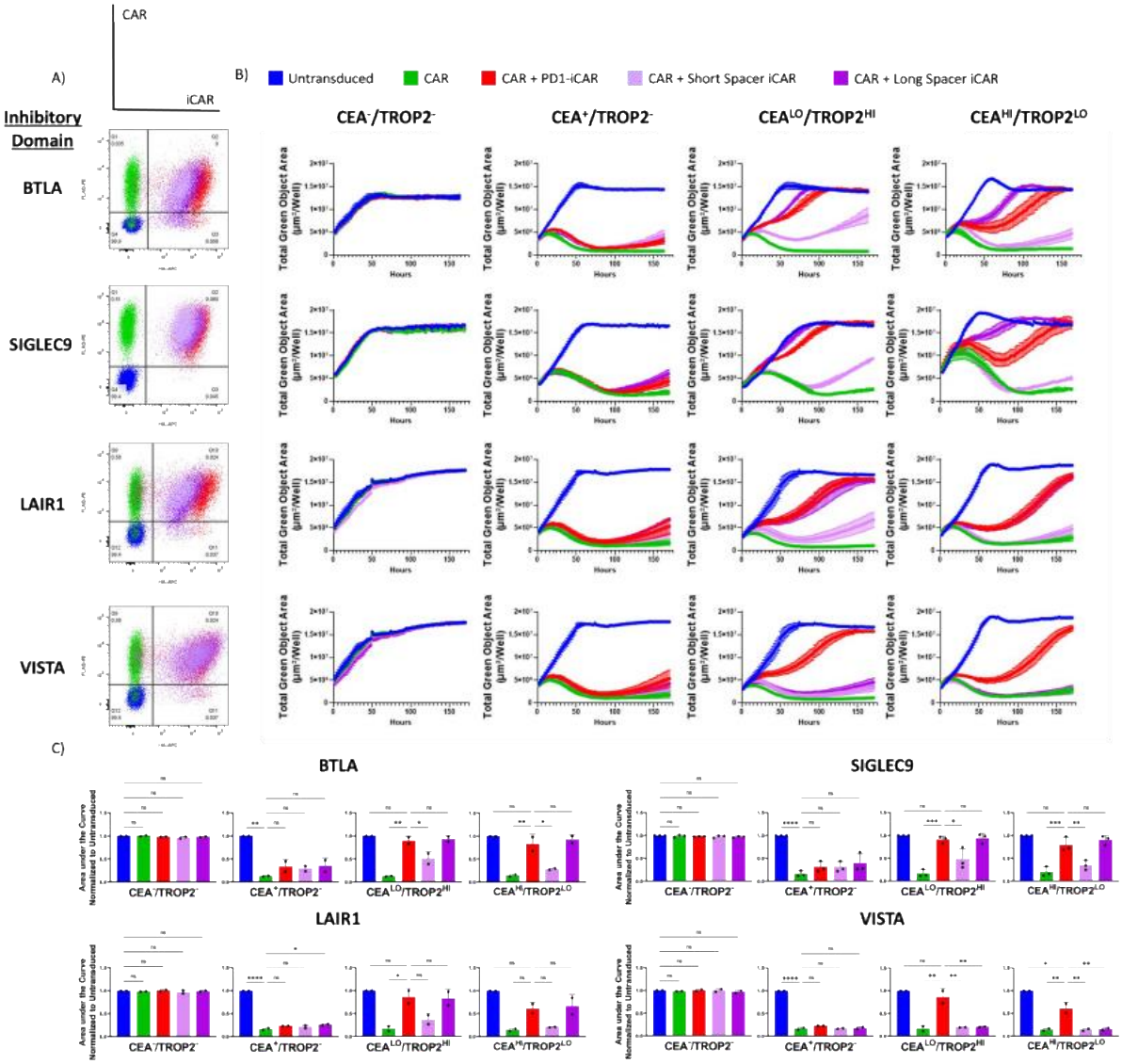


Fig. 4. iCARs containing signaling domains with ITIM motifs can reduce CAR T cell cytotoxicity.

A) The five different populations (untransduced, CAR, CAR + TROP2-PD1 iCAR, CAR + TROP2-Short iCAR, CAR + TROP2-Long iCAR) tested for each inhibitory signaling domain are plotted in a flow cytometry plot that corresponds to each color in the legend. The flow cytometry plot is a representative from one experiment.

B) $CAR^+/iCAR^+$ T cells that were engineered with inhibitory signaling domains with an ITIM motif can inhibit CAR T cell cytotoxicity as measured by total green object area (µm/well) of DU145 target cells that express CEA and/or TROP2 over 150 h. Each curve represents a coculture with the CAR T cell population represented by the color in the legend. These cytotoxicity curves are representative images from one experiment.

C) $CAR^+/iCAR^+$ T cells that were engineered with inhibitory signaling domains with an ITIM motif can inhibit CAR T cell cytotoxicity with a similar efficiency as the TROP2-PD1 iCAR as measured by area under the cytotoxicity curve. AUC was normalized to the untransduced population

cocultured with the target cells. The normalized AUC quantified is the mean ± SD of at least two independent experiments (BTLA – n=2; LAIR1 – n=2, SIGLEC9 – n=3, VISTA – n=2).

The significance values shown are comparisons between a control group. For the $CEA^{-}/TROP2^{-}$ cell line, values are compared to the untransduced control. For the $CEA^{+}/TROP2^{-}$ cell line, values are compared to the CAR control. For the $CEA^{LO}/TROP2^{HI}$ or $CEA^{HI}/TROP2^{LO}$ cell lines, values are compared to the CAR + PD1 iCAR group.

Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value ≤ 0.05, **P value ≤ 0.01, ***P value ≤ 0.001

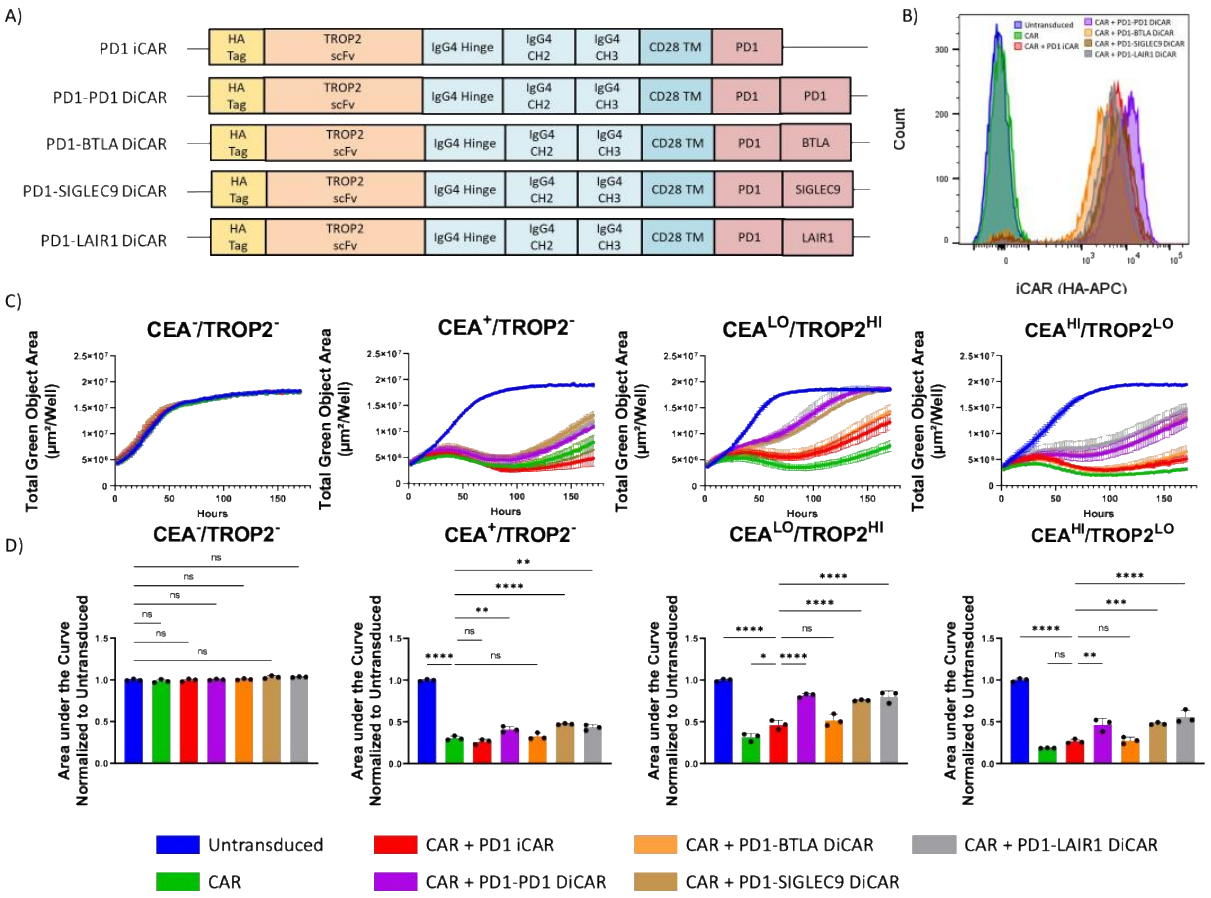


Fig. 5. DiCARs increase the efficiency of inhibition in the AND-NOT-gate CAR T strategy.

A) The models represent the structure of each DiCAR tested. The DiCARs are composed of a TROP2 scFv, the IgG4 Hinge, CH2, and CH3 constant domains, a CD28 TM, the PD-1 inhibitory signaling domain, and the additional inhibitory signaling domains PD-1, BTLA, SIGLEC-9, or LAIR-1.

B) The representative histogram indicates that iCAR/DiCAR surface expression level is similar between the groups of DiCARs being compared. The DiCAR surface expression was determined by flow cytometry for an HA-tag located on the N-terminus of the iCAR/DiCAR.

C) Representative cytotoxicity curves of each CAR T cell population demonstrate that CAR T cell populations with a DiCAR have a reduced delay in inhibition compared to the TROP2-PD1 iCAR. CAR T cells were co-cultured with DU145 target cells that express GFP and CEACAM5 and/or TROP2. Presence of target cells was measured by total green object area (μm^2/well) over time as measured by Incucyte live cell image analysis over 150 h.

D) The delay in inhibition of the iCAR was measured by area under the cytotoxicity curve analysis of each cytotoxicity curve and normalized to the coculture with the untransduced T cell group. This AUC is a representative of one experiment in which triplicate wells were analyzed. Three biological replicates were performed and reported in SI Appendix, Figs. S6 and S7.

The significance values shown are comparisons between a control group. For the CEA$^-$/TROP2$^-$

cell line, values are compared to the untransduced control. For the $CEA^{+}/TROP2^{-}$ cell line, values are compared to the CAR control. For the $CEA^{LO}/TROP2^{HI}$ or $CEA^{HI}/TROP2^{LO}$ cell lines, values are compared to the CAR + PD1 iCAR group.

Statistics performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value $\leq$ 0.05, **P value $\leq$ 0.01, ***P value $\leq$ 0.001

SI Appendix

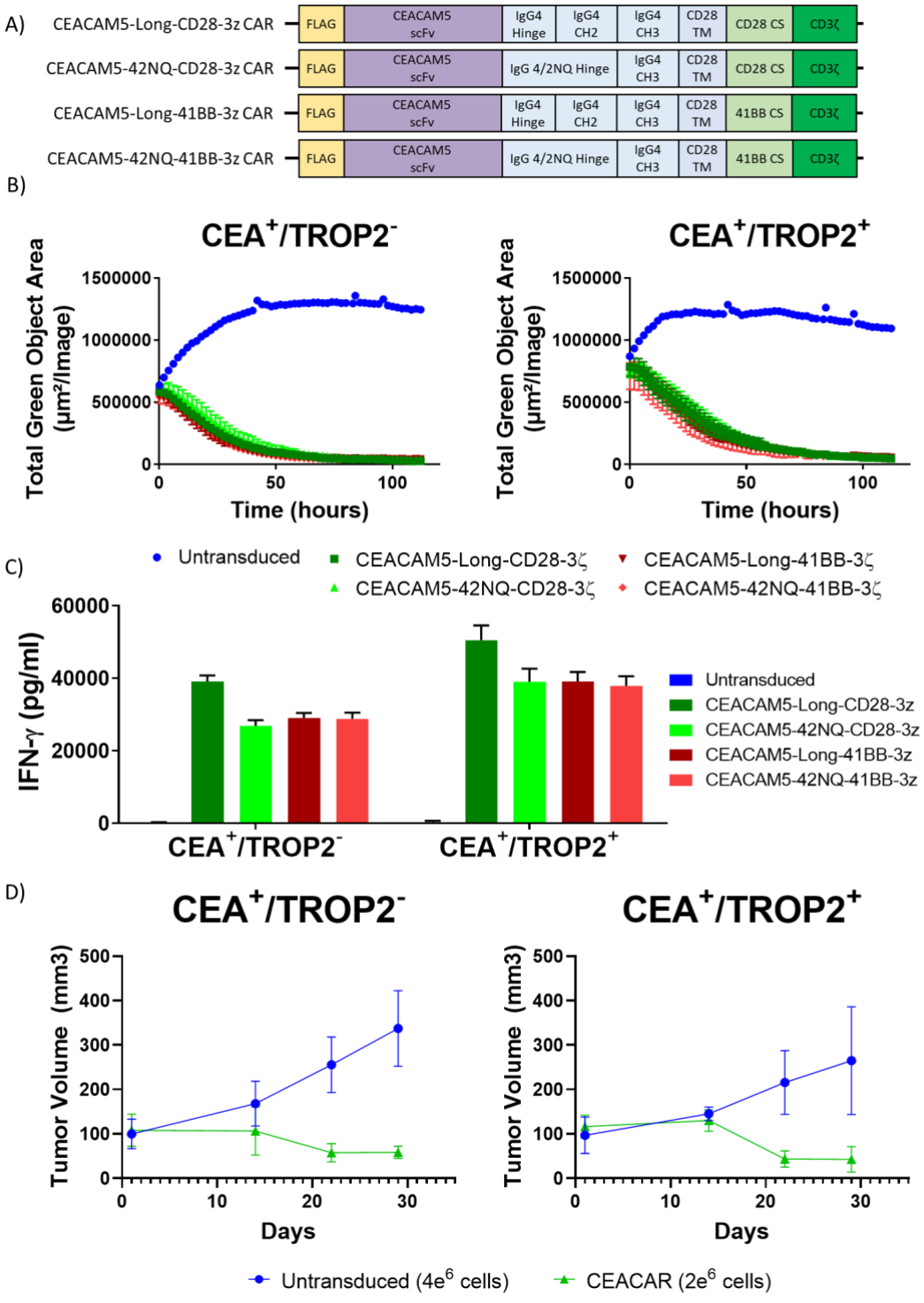


Fig. S1. CEACAM5 CARs with the natural or modified IgG4 hinge and different co-stimulatory domains have comparable cytotoxicity and IFN-γ production.

A) The illustration depicts the different CAR structures being tested. All constructs use the same scFv chain that recognizes CEACAM5. They differ in the hinge that is used. The 4/2NQ modified

hinge is derived from Hudecek *et al.* (1) which combines the hinge of IgG4 and IgG2. The IgG4 CH3 constant domain is used in all constructs. Another difference is the costimulatory domain which is derived from CD28 or 41BB. TM = Transmembrane Domain; CS = Co-stimulatory Domain.

B) All CEACAM5 CARs show comparable cytotoxicity of DU145 target cells that express CEACAM5 as measured by Incucyte live cell image analysis of total green object area of target cells (μm^2/image) over time.

C) All CEACAM5 CARs show comparable production of IFN-γ as measured by ELISA 48 hours after CAR T cells are co-cultured with DU145 target cells that express CEA or CEA and TROP2.

D) CEACAR T cells can kill $CEA5^+$ tumors *in vivo*. Mice were engrafted with either a $CEA5^+/TROP2^-$ OR $CEA5^+/TROP2^+$ tumor. Mice were injected with $4e^6$ untransduced or $2e^6$ CEACAR T cells. Caliper measurements of tumors were measured weekly two weeks after injection of T cells. n = 4 or 5 mice/group.

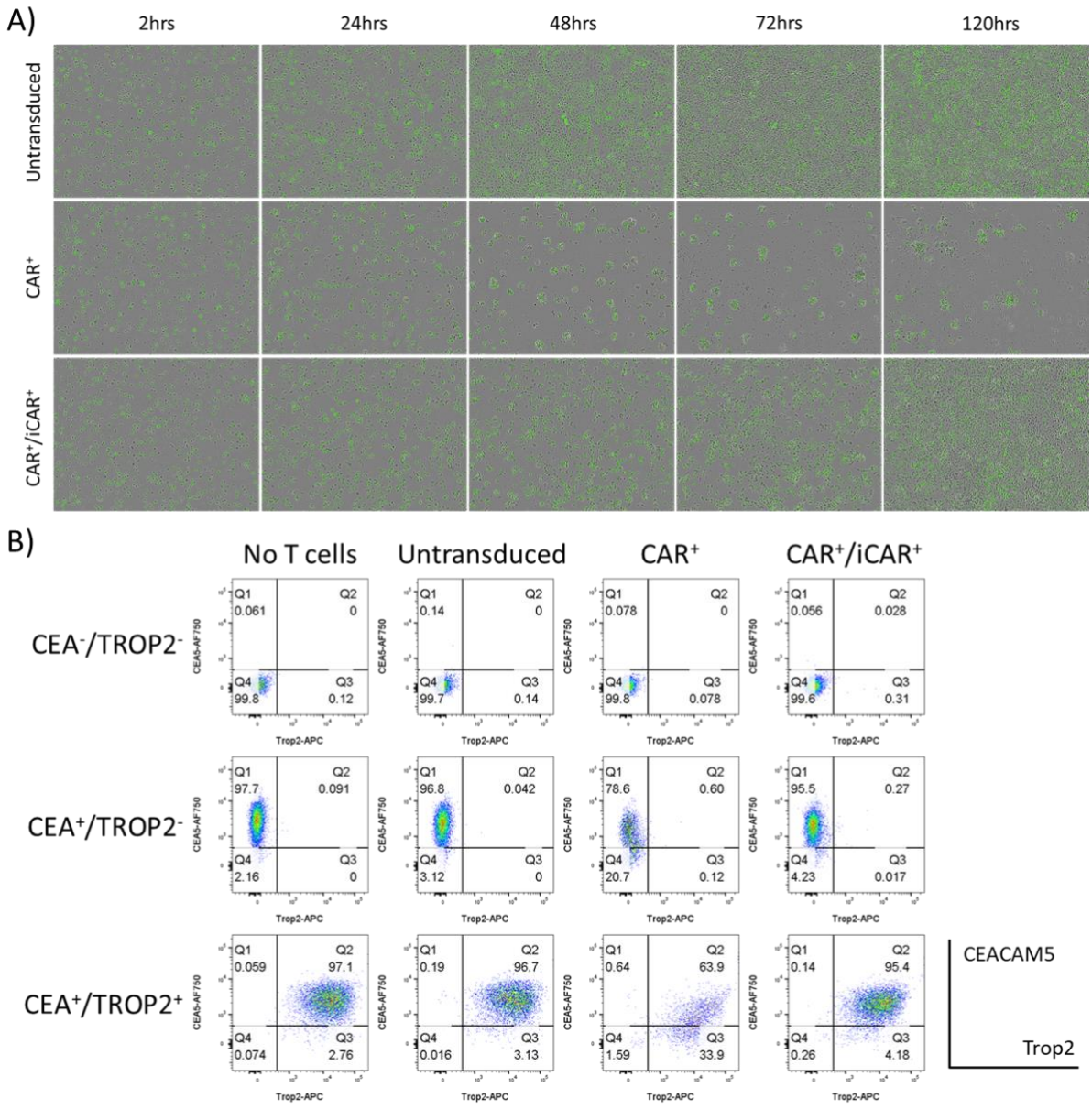
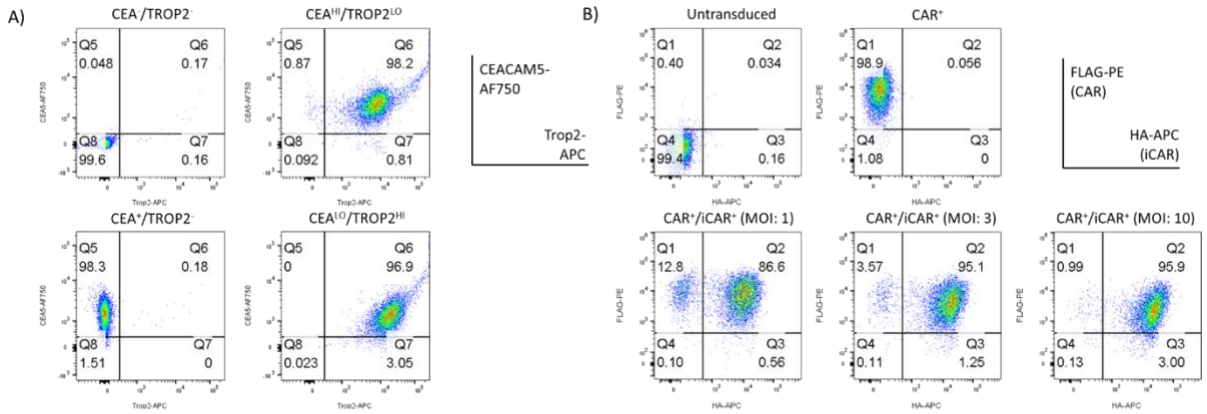


Fig. S2. CEA$^+$/TROP2$^+$ cells regrow after co-culture with CAR$^+$/iCAR$^+$ T cells

A) Images of CEA$^+$/TROP2$^+$ DU145 target cells at 2, 24, 48, 72, and 120 hours after co-culture with untransduced, CAR$^+$, or CAR$^+$/iCAR$^+$ T cells.

B) Target cells continue to express CEACAM5 and TROP2 after co-culture with CAR$^+$/iCAR$^+$ T cells. In a separate experiment, nine days after co-culture, target cells were harvested from wells where DU145 target cells that expressed CEACAM5 and/or TROP2 were co-cultured with untransduced, CAR$^+$, or CAR$^+$/iCAR$^+$ T cells. Expression of CEACAM5 and TROP2 after co-culture was measured by flow cytometry using Anti-CEACAM5 and Anti-TROP2 antibodies.



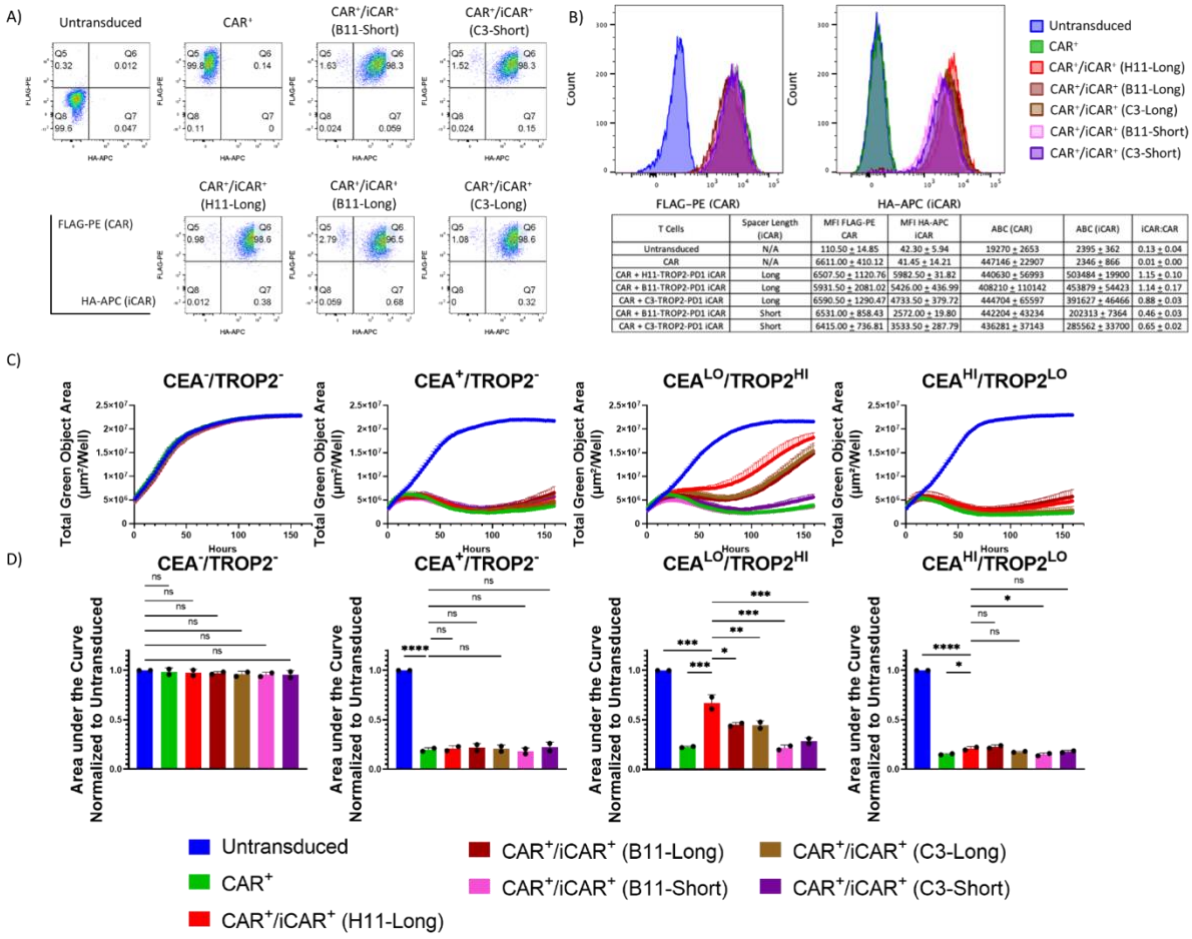
Target Cell Line	Average Geometric Mean Fluorescenc Intensity CEACAM5-AF750	Average Geometric Mean Fluorescenc Intensity Trop2-APC
$CEA^-/TROP2^-$	43 ± 16	0 ± 26
$CEA^+/TROP2^-$	1485 ± 196	11 ± 21
$CEA^{HI}/TROP2^{LO}$	2010 ± 494	7368 ± 3440
$CEA^{LO}/TROP2^{HI}$	1152 ± 136	17810 ± 3534

T Cells	Average Geometric Mean Fluorescenc Intensity FLAG-PE	Average Geometric Mean Flourescence Intensity HA-APC
Untransduced	101 ± 0	76 ± 23
CAR	6429 ± 676	72 ± 22
$CAR^+/iCAR^+$ (MOI: 1)	5766 ± 1030	8947 ± 346
$CAR^+/iCAR^+$ (MOI: 3)	4056 ± 405	14898 ± 1002
$CAR^+/iCAR^+$ (MOI: 10)	3033 ± 971	19099 ± 161

Fig. S3. Quantitative analysis of antigen expression in target cells and receptor expression in T cells

A) Engineered DU145 target cell lines have different surface level expression of CEA and TROP2. The flow cytometry plots are representative images from one of three experiments comparing the CEA and TROP2 expression of each target cell line. The average mean fluorescence intensity and standard deviation from three experiments are reported in the table below.

B) Engineered CAR T cells express both CAR and iCAR on the surface of the cells. Representative flow cytometry plots depict surface level expression of CAR and iCAR in the populations. Primary T cells were transduced with lentivirus containing the CAR at a MOI of 1 and lentivirus containing the iCAR at a MOI of 1, 3, and 10. The average mean fluorescence intensity and standard deviation from two experiments are reported in the table below.



T Cells	Spacer Length (iCAR)	MFI FLAG-PE CAR	MFI HA-APC iCAR	ABC (CAR)	ABC (iCAR)	iCAR:CAR
Untransduced	N/A	110.50 ± 14.85	42.30 ± 5.94	19270 ± 2653	2395 ± 362	0.13 ± 0.04
CAR	N/A	6611.00 ± 410.12	41.45 ± 14.21	447146 ± 22907	2346 ± 866	0.01 ± 0.00
CAR + H11-TROP2-PD1 iCAR	Long	6507.50 ± 1120.76	5982.50 ± 31.82	440630 ± 56993	503484 ± 19900	1.15 ± 0.10
CAR + B11-TROP2-PD1 iCAR	Long	5931.50 ± 2081.02	5426.00 ± 436.99	408210 ± 110142	453879 ± 54423	1.14 ± 0.17
CAR + C3-TROP2-PD1 iCAR	Long	6590.50 ± 1290.47	4733.50 ± 379.72	444704 ± 65597	391627 ± 46466	0.88 ± 0.03
CAR + B11-TROP2-PD1 iCAR	Short	6531.00 ± 858.43	2572.00 ± 19.80	442204 ± 43234	202313 ± 7364	0.46 ± 0.03
CAR + C3-TROP2-PD1 iCAR	Short	6415.00 ± 736.81	3533.50 ± 287.79	436281 ± 37143	285562 ± 33700	0.65 ± 0.02

Fig. S4. TROP2-targeting iCARs with short spacers inhibit CAR-T cell cytotoxicity less efficiently than long spacer counterparts.

A) Representative flow cytometry plots confirm that CAR$^+$/iCAR$^+$ cells are relatively pure and express both CAR and iCAR.

B) Representative histograms of CAR and iCAR with the H11, C3, and B11 TROP2 scFv chains show stable surface expression. CAR surface expression is measured by an antibody that recognizes a FLAG-tag on the N-terminus of the CAR. iCAR surface expression is measured by an antibody that recognizes a HA-tag on the N-terminus of the iCAR. The estimated values of both CAR and iCAR on the surface of the cell were measured by quantitative flow cytometry and averaged over two independent experiments. Geometric mean fluorescence intensity of each population was measured and compared to standard curves generated by Quantum Simply Cellular beads (Bangs Laboratories, Inc.) for Anti-Mouse or Anti-Rat antibodies to extrapolate the number of CAR and iCAR molecules on the surface of the cell. Estimations were made since the MFI of the population exceeded the quantitative range of the microspheres. The iCAR:CAR ratio was calculated based on the estimates.

C) Regardless of the scFv chain used in the iCAR, TROP2-targeting iCARs with short spacers (IgG4 hinge) are less efficient at inhibiting cytotoxicity compared to their long spacer counterparts. Representative cytotoxicity curves are displayed from one experiment where the total green

object area (μm/well) of GFP^+ DU145 target cells that express CEACAM5 and/or TROP2 were measured over approximately 160 hours.

D) Area under the curve analysis of cytotoxicity curves. The delay in inhibition was measured by calculating the area under each cytotoxicity curve. The AUC was normalized against the AUC calculated for untransduced T cells co-cultured with the target cells. The normalized AUC quantified is the mean $\pm$ s.d. (n=2) from two independent experiments. The significance values shown are comparisons between a control group. For the $CEA^-/TROP2^-$ cell line, values are compared to the untransduced control. For the $CEA^+/TROP2^-$ cell line, values are compared to the CAR control. For the $CEA^{LO}/TROP2^{HI}$ or $CEA^{HI}/TROP2^{LO}$ cell lines, values are compared to the $CAR^+/iCAR^+$ (H11-Long) group.

Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value $\leq$ 0.05, **P value $\leq$ 0.01, ***P value $\leq$ 0.001

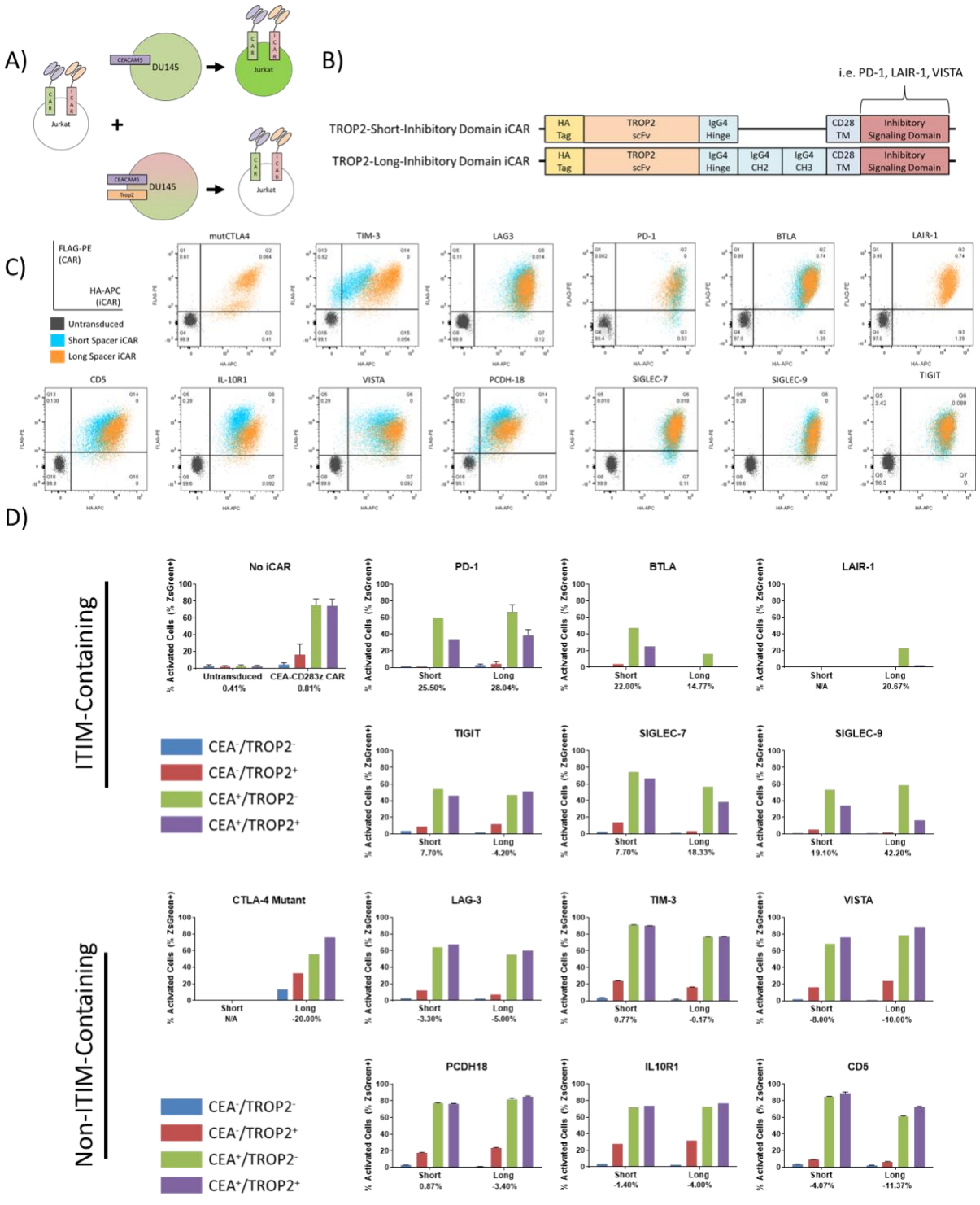


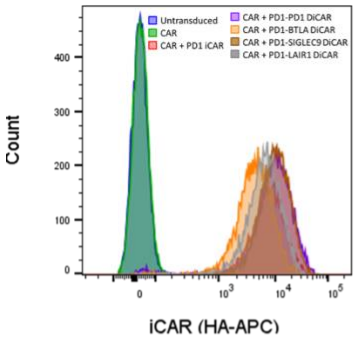
Fig. S5. ITIM-containing iCARs can inhibit CAR T cell activation in a Jurkat reporter assay.

A) An engineered Jurkat-NFAT-ZsGreen reporter cell line was transduced with the CEACAM5-Long-CD28-3z CAR and a TROP2-iCAR that contains the corresponding inhibitory signaling domain. The iCAR was transduced at a MOI of 25 and the CAR at a MOI of 1.

B) Inhibitory CAR structures are composed of a TROP2 scFv chain, a short or long spacer, a CD28 TM, and the corresponding intracellular signaling domain of the protein listed (Supplementary Table 1).

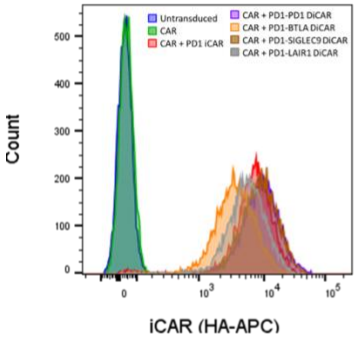
C) Jurkat-NFAT-ZsGreen cell lines transduced and sorted for $CAR^+/iCAR^+$ populations express the iCAR on the surface of the cell. Each group is compared to an untransduced control population (black). CAR and iCAR surface expression was detected using an antibody targeting the FLAG-tag or HA-tag on the construct's N-terminus respectively.

D) ITIM-containing iCARs are able to inhibit CAR T cell activation in a Jurkat-NFAT-ZsGreen reporter assay. Jurkat-NFAT-ZsGreen cell lines were transduced with CEACAM5-Long-CD28-3z CAR and one of the various TROP2-iCARs. Each cell line was sorted and co-cultured with engineered DU145 target cells at an effector:target ratio of 1:1. The percent of cells that are considered activated are measured by % $ZsGreen^+$ cells after gating for the CD3+ population. Percentage values under each set of graphs represent Δ Target to Non-target: Calculated difference between percent activated cells when co-cultured with target cell line ($CEA^+/TROP2^-$) versus the non-target cell line ($CEA^+/TROP2^+$). N/A were not tested.



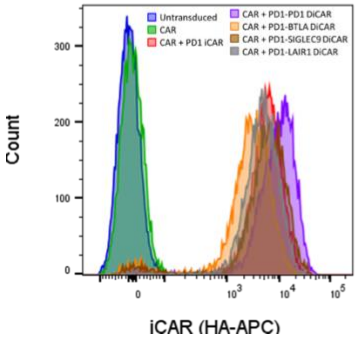
Experiment #1

T Cell	MFI (FLAG-PE) CAR	MFI (HA-APC) iCAR	ABC (CAR)	ABC (iCAR)	iCAR:CAR
Untransduced	N/A	N/A	N/A	N/A	N/A
CAR	6440	N/A	320005	N/A	N/A
CAR + PD1-iCAR	6215	6986	312088	533000	1.71
CAR + PD1-PD1 iCAR	7432	9072	353988	707200	2.00
CAR + PD1-BTLA iCAR	7958	4064	371459	296542	0.80
CAR + PD1-SIGLEC9 iCAR	8747	9080	397039	707875	1.78
CAR + PD1-LAIR1 iCAR	6755	6028	330954	454359	1.37



Experiment #2

T Cell	MFI (FLAG-PE) CAR	MFI (HA-APC) iCAR	ABC (CAR)	ABC (iCAR)	iCAR:CAR
Untransduced	N/A	N/A	N/A	N/A	N/A
CAR	9086	N/A	348069	N/A	N/A
CAR + PD1-iCAR	8620	6755	337249	530991	1.57
CAR + PD1-PD1 iCAR	10024	8297	369198	664037	1.80
CAR + PD1-BTLA iCAR	9286	3397	352645	251448	0.71
CAR + PD1-SIGLEC9 iCAR	10597	8133	381716	649776	1.70
CAR + PD1-LAIR1 iCAR	10145	5166	371865	396671	1.07



Experiment #3

T Cell	MFI (FLAG-PE) CAR	MFI (HA-APC) iCAR	ABC (CAR)	ABC (iCAR)	iCAR:CAR
Untransduced	N/A	N/A	N/A	N/A	N/A
CAR	6809	N/A	409939	N/A	N/A
CAR + PD1-iCAR	9059	5293	510309	523628	1.03
CAR + PD1-PD1 iCAR	6437	8676	392648	896803	2.28
CAR + PD1-BTLA iCAR	6511	2830	396105	264828	0.67
CAR + PD1-SIGLEC9 iCAR	6846	5675	411647	564903	1.37
CAR + PD1-LAIR1 iCAR	6469	4335	394144	421319	1.07

Fig. S6. DiCAR surface expression is similar to that of the TROP2-Long-PD1 iCAR.

Histograms of DiCARs from three independent experiments were generated to compare the surface expression of the DiCARs. Surface expression was measured by the HA-tag located on the N-terminus of each inhibitory CAR. Geometric Mean Fluorescence Intensity (MFI) was calculated for each population and reported in the tables above. The amount of CAR and iCAR (ABC – Antibody Binding Capacity) on the surface of T cells was estimated using Quantum Simply Cellular microspheres. Estimations were made since the MFI of the population exceeded the quantitative range of the microspheres. The iCAR:CAR ratio was calculated based on the estimates.

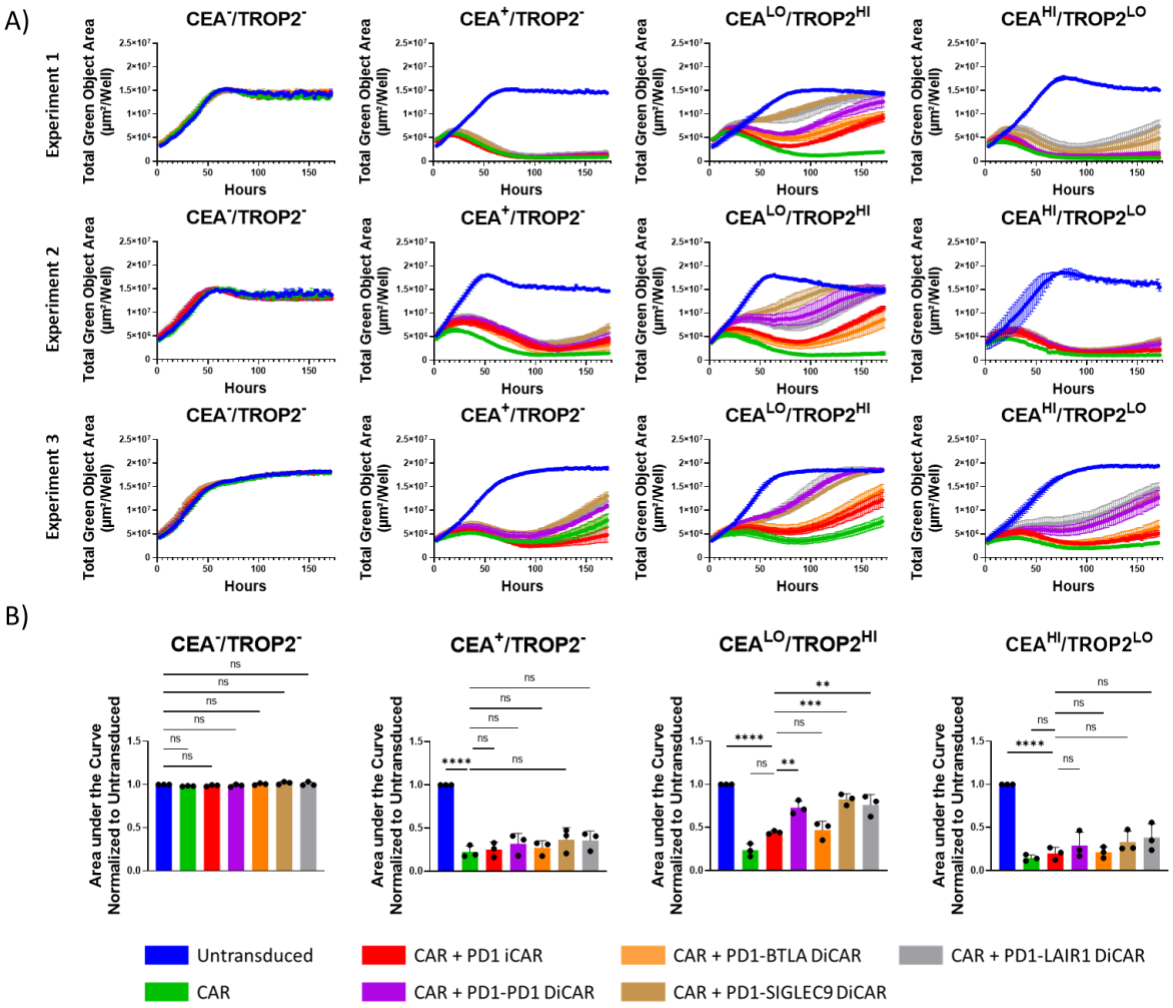


Fig. S7. Cytotoxicity curves of CAR T cells that contain a DiCAR are consistent in three experiments.

A) Cytotoxicity curves were generated by Incucyte live cell image analysis of co-cultures of T cells with GFP+ DU145 target cells that express CEACAM5 and/or TROP2. Three independent experiments were performed with three replicates using the same donor PBMCs. Presence of target cells was measured by total green object area (μm^2/well) over 150 hours.

B) The delay in inhibition of the iCAR/DiCAR was measured by area under the curve analysis of each cytotoxicity curve and normalized to the co-culture with the untransduced T cell group. The AUC displayed is an average of the three independent experiments shown above.

The significance values shown are comparisons between a control group. For the $CEA^-/TROP2^-$ cell line, values are compared to the Untransduced control. For the $CEA^+/TROP2^-$ cell line, values are compared to the CAR control. For the $CEA^{LO}/TROP2^{HI}$ or $CEA^{HI}/TROP2^{LO}$ cell lines, values are compared to the CAR + TROP2-PD1 iCAR group.

Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value $\leq$ 0.05, **P value $\leq$ 0.01, ***P value $\leq$ 0.001

Characteristics				Short Spacer	Long Spacer
Domain	Uniprot	Amino Acids for Intracellular Signaling Domain	ITIM Containing	Specific Inhibition Δ% ZsGreen+ ($CEA^+/TROP2^+$ - $CEA^+/TROP2^-$)	Specific Inhibition Δ% ZsGreen+ ($CEA^+/TROP2^+$ - $CEA^+/TROP2^-$)
PD1	Q15116	192-288	Yes	25.50	28.04
mutCTLA4	P16410	183-223 mutate (Y201G)	No	N/A	-20.00
BTLA	Q7Z6A9	179-289	Yes	22.00	14.77
LAIR1	Q6GTX8	187-287	Yes	N/A	20.67
TIGIT	Q495A1	163-244	Yes	7.70	-4.20
LAG3	P18627	472-525	No	-3.30	-5.00
TIM3	Q8TDQ0	224-301	No	0.77	-0.17
SIGLEC7	Q9Y286	377-467	Yes	7.70	18.33
SIGLEC9	Q9Y336	370-463	Yes	19.10	42.20
VISTA	Q9H7M9	216-311	No	-8.00	-10.00
PCDH18	D6RIG4	503-914	No	0.87	-3.40
IL10R1	Q13651	257-578 mutate (S319A, S323A, S370A)	No	-1.40	-4.00
CD5	P06127	403-495	No	-4.07	-11.37

Table S1. Structure of inhibitory CARs containing alternative inhibitory signaling domains and their ability to inhibit CAR T cell activation.

Inhibitory CARs are generated by combining the TROP2 scFv chain with a hinge/spacer, a CD28 TM, and the intracellular signaling domain of the listed protein. Each intracellular domain contains the amino acids listed defined by the Uniprot protein code. Short spacer – IgG4 Hinge; Long Spacer – IgG4 Hinge + CH2 + CH3 constant domains. Specific inhibition = The percentage difference of $ZsGreen^+$ cells between co-cultures of Jurkat cells with the $CEA^+/TROP2^+$ cell line subtracted by those co-cultured with the $CEA^+/TROP2^-$ only cell line.

Antibody	Source	Identifier
CD3 Monoclonal Antibody (SK7), APC-eFluor™ 780, eBioscience™	Invitrogen	47-0036-42
CD4 Monoclonal Antibody (OKT4 (OKT-4)), FITC, eBioscience™	Invitrogen	11-0048-42
PE/Cyanine7 anti-human CD271 (NGFR) Antibody	Biolegend	345110
PerCP/Cyanine5.5 anti-human EGFR Antibody	Biolegend	352914
PE anti-DYKDDDDK Tag Antibody	Biolegend	637310
HA Antibody, APC	Miltenyi Biotec	130-123-553
Human TROP-2 APC-conjugated Antibody	R&D Systems	FAB650A
Human CEACAM-5/CD66e Alexa Fluor® 750-conjugated Antibody	R&D Systems	FAB41281S-100UG
CD45 Monoclonal Antibody (HI30), FITC, eBioscience™	Invitrogen	11-0459-42

Table S2. Antibodies for flow cytometry staining.

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Chapter 3: Testing the specificity of DiCAR inhibition *in vivo*

Chapter 3: Testing the specificity of DiCAR inhibition *in vivo*

Introduction

In vivo validation of CAR T cell efficacy and toxicity must be performed before advancing a CAR T cell strategy into the clinic. Experiments performed in an *in vivo* setting can provide valuable insights into the ability of CAR T cells to persist in the blood, to traffic to the tumor, and to kill an established large tumor.[1] If modelled properly in transgenic and immunocompetent models, it can even give insights into toxicity against normal tissues and potency to overcome an immunosuppressive tumor microenvironment.[1] Important discoveries like how specific costimulatory domains increase persistence[2] and how certain spacers lead to FcγR binding and CAR T cell sequestration in the lungs[3] might not have been made without *in vivo* testing.

The AND-NOT Boolean logic gate, which utilizes an inhibitory CAR (iCAR) to reduce CAR T cell on-target, off-tumor toxicity has been tested in an *in vivo* capacity in a limited manner. The proof-of-concept model which paired a CD19-targeting CAR and a PSMA-targeting iCAR utilized a hematological tumor model. NALM/6 cells engineered to express CD19 or CD19 and PSMA were injected intravenously into mice followed by $CAR^+/iCAR^+$ T cells. Mice that received $CD19^+$ $PSMA^+$ cells had more tumor burden than those that received $CD19^+$ cells, indicating that the PSMA-targeting iCAR could inhibit CD19-targeting CAR killing when the iCAR antigen was expressed.[4] However, this model did not accurately reflect the conditions where debilitating on-target, off-tumor toxicity is seen – solid normal tissues.

On-target, off-tumor toxicities that have led to termination of CAR T clinical trials have occurred in the lung with both the CEACAM5 and HER2-targeting CARs and the liver with the CAIX-targeting CAR.[5–7] To better mimic a solid normal tissue environment, A2 Biotherapeutics used a subcutaneous tumor model, where tumor cells representing normal tissues that express both the CAR and iCAR antigen were engrafted into the flanks of mice. The $CAR^+/iCAR^+$ T cells were then delivered into the mice intravenously, and tumors observed for reduction.[8] They further modified

the protocol to a dual-tumor model, in which both the on-target tumor and the on-target "normal" cells were engrafted into the same mouse.[9] This model best mimicked a clinical setting, where CAR T cells would encounter tumor and normal tissue in the same patient.

As shown in Chapter 2, DiCARs are inhibitory CARs that contain two inhibitory signaling domains from receptors like BTLA, LAIR-1, SIGLEC-9, and PD-1. These engineered DiCARs were shown to inhibit CAR T cell cytotoxicity more efficiently *in vitro* than an iCAR with a single PD-1 domain. The question that now needed to be answered was whether these DiCARs could inhibit CAR T cell "normal" tissue damage *in vivo*. This chapter is focused on a preliminary experiment where CAR^+ $DiCAR^+$ T cells were assessed for their ability to specifically inhibit CAR T cell tumor killing.

Results

DiCARs with a 42NQ spacer can inhibit CAR T cell cytotoxicity

The DiCARs tested in Chapter 2 all utilized the long extracellular spacer that contained the hinge, CH2, and CH3 constant domains from IgG4. Since this spacer could lead to sequestration of CAR T cells in the lung due to FcγR binding,[3] we sought to replace it with the 4/2NQ spacer developed by Hudecek *et al*.[3] that would prevent this event from occurring. Three inhibitory constructs targeting TROP2 that contained the H11 TROP2 antibody, the long extracellular spacer, the CD28 transmembrane domain, and the inhibitory domains PD1, PD1 and LAIR1, or PD1 and SIGLEC9 had the long spacer replaced with the 4/2NQ spacer. These iCARs and DiCARs will hereby be referred to by only their signaling domains (i.e. TROP2-42NQ-PD1-LAIR1 DiCAR → **PD1-LAIR1 DiCAR**) (Fig. 3.1A). To confirm that changing the spacer would not reduce inhibition efficiency, these TROP2-targeting iCARs and DiCARs were transduced with the CEACAM5-targeting CAR (CEACAR) described in Chapter 2 into primary T cells. The CAR^+/$iCAR^+$ T cells were then co-cultured with DU145 target cells engineered to express CEACAM5 and TROP2 and observed for cytotoxicity over time. The cytotoxicity curves confirmed that these constructs had similar or better inhibition efficiencies compared to their original counterparts (Fig. 3.1B). Interestingly, in additional experiments where the MOI of the CAR was 1 and the MOI of the

DiCAR was 3, the PD1-LAIR1 DiCAR inhibited more efficiently than the PD1-SIGLEC9 DiCAR, especially at a higher effector:target ratio (5:1 E:T) (Fig. 3.2A, B). These results confirmed that these inhibitory constructs were still potent inhibitors of CAR T cell cytotoxicity even when their spacers were modified.

CAR$^+$ DiCAR$^+$ T cells for in vivo studies are pure and functional

Since the PD1-LAIR1 DiCAR inhibited cytotoxicity most efficiently *in vitro*, we decided to test its ability to specifically inhibit tumor killing *in vivo.* In this preliminary study, we chose the CEACAR targeting CEACAM5 as our activating CAR and the PD1-LAIR1 DiCAR targeting TROP2 as our inhibitory CAR (Fig. 3.3A).

CAR T cells were prepared as described in the Materials and Methods section. Briefly, primary T cells were transduced with CAR and DiCAR lentivirus at a MOI of 1 and 3 respectively. The MOI of the iCAR was increased to three-fold higher than the CAR to increase its avidity and potential for *in vivo* inhibition. T cells were enriched for the CAR$^+$/DiCAR$^+$ population and confirmed to be >95% pure by flow cytometry (Fig. 3.3B). Due to the large number and long preparation time (11 days) for CAR T cells, functional confirmation of CAR T cell killing and inhibition was necessary before proceeding to mice. A subset of CAR T cells on day of injection were co-cultured with target cells engineered to express CEACAM5 and/or TROP2 and observed over a week for cytotoxicity and inhibition. CAR$^+$/DiCAR$^+$ T cells could kill CEA$^+$/TROP2$^-$ tumor cells and inhibit killing of CEA$^+$/TROP2$^+$ cells *in vitro* (Fig. 3.3C). T cells transduced with the CEACAM5-targeting CAR and TROP2-targeting PD1-LAIR1 DiCAR were pure and functional.

The TROP2-PD1-LAIR1 DiCAR can specifically inhibit CEACAR Tumor killing in vivo

To determine whether the PD1-LAIR1 DiCAR could selectively inhibit CAR T cell killing *in vivo*, NSG (NOD scid gamma) mice were engrafted with DU145 tumors that express the CAR target alone (CEA$^+$/TROP2$^-$) or the CAR and DiCAR target (CEA$^+$/TROP2$^+$). When tumors were palpable, mice were injected with one of four groups of T cells prepared in the section above: 1) 4 X 10^6 untransduced, 2) 2 X 10^6 CAR only, 3) 2 X 10^6 CAR$^+$ DiCAR$^+$, and 4) 4 X 10^6 CAR$^+$ DiCAR$^+$.

Two doses of $CAR^+/DiCAR^+$ T cells were used to compare the effect of dosage on *in* vivo tumor killing (Fig. 3.4A).

Tumor growth was observed over four weeks. In mice that received untransduced T cells, both $CEA^+/TROP2^-$ and $CEA^+/TROP2^+$ tumors grew out. In mice with CAR T cells alone, the opposite occurred with both tumors shrinking. Tumors in mice that received the 4×10^6 dose of $CAR^+/DiCAR^+$ T cells were killed at comparable levels as those that received CAR T cells alone regardless of TROP2 expression, suggesting that the dose of T cells was too high. However, in mice with the 2×10^6 dose of $CAR^+/DiCAR^+$ T cells, differences could be seen in tumor killing based on antigen expression (Fig. 3.4B) ($p = 0.0762$). The $CEA^+/TROP2^-$ tumors shrunk, while the $CEA^+/TROP2^+$ tumors grew out. Blood from mice collected four weeks after injection showed continued persistence of T cells in all groups (Fig. 3.4C), eliminating the possibility that this difference was due to T cell loss. This data from a single *in vivo* experiment indicated that T cells transduced with the TROP2-PD1-LAIR1 DiCAR could specifically inhibit CAR T cell killing when TROP2 was expressed.

Discussion

In the preliminary experiment shown, the TROP2-PD1-LAIR1 DiCAR could inhibit CAR T cell tumor killing when TROP2 was expressed, but the difference in tumor growth between the $CEA^+/TROP2^-$ tumor and the $CEA^+/TROP2^+$ tumor was not significant. We must note that in a separate experiment the difference in tumor killing was not observed even with the 2×10^6 T cell dose of $CAR^+/DiCAR^+$ T cells (Data not shown). The reason for this variation is unclear. Although T cell preparation is consistent between both experiments and performance is validated using our cytotoxicity assay, T cell counts, differentiation states, and exhaustion might differ and contribute to *in vivo* functionality.

An additional confounding factor is the TROP2 surface expression *in vivo* versus *in vitro*. We have observed that after tumor cells are implanted in mice, TROP2 surface expression is decreased compared to the engineered parental line used for injection (Data not shown). This

loss of surface expression may be due to cleavage by ADAM17,[10] matriptase,[11] and/or ADAM10.[12] As described in Chapter 2, avidity plays a major role in iCAR inhibition efficiency. Due to the loss of TROP2 surface expression *in vivo*, the amount of DiCAR necessary for inhibition may need to be increased based on the amount of cleavage occurring in each mouse.

Figure 3.4B also shows an additional parameter that must be optimized – T cell dose. When 4 X 10^6 T cells were injected into mice no inhibition was seen, but when 2 X 10^6 T cells were introduced, some specific inhibition occurred. Although, in concept, all CAR^+/$DiCAR^+$ T cells should be inhibited when encountering the TROP2 antigen, CAR signaling is dynamic and changes over time. Although we have reported that DiCARs are more efficient at inhibition than iCARs with a single PD-1 domain, a kinetic delay in inhibition still occurs when the DiCAR target antigen density is low. It may be at high T cell doses enough CAR T cells are still activated in the population that a threshold needed for killing is reached. If too few CAR T cells are injected to mitigate this problem, tumor killing may not occur even when CEACAM5 is expressed alone due to ligand-independent inhibition observed in the TROP2-PD1-LAIR1 DiCARs. Further work needs to be performed to optimize T cell dose.

This preliminary data shows promise that DiCARs can inhibit on-target, off-tumor toxicity *in vivo*. The complexity of this AND-NOT gating strategy is already challenging for *in vitro* studies, where the balance between CAR, DiCAR, and both target antigens must be achieved to obtain specificity. It only becomes more complex for *in vivo* studies, where tumor growth rates, T cell proliferation rates, trafficking, and persistence also contribute to activity. Optimization of all these parameters will be necessary to achieve the desired specificity. Future work will also be focused on comparing these DiCARs against each other and an iCAR with a single PD1 domain.

Materials and Methods

Vectors and cell lines

The vectors and cell lines used in these experiments were described in Chapter 2's Materials and Methods section. The CEACAR used in this study contains an scFv derived from the CEACAM5-

targeting antibody Labetuzumab, an 4/2NQ spacer as described by Hudecek *et al*[3], a CD28 transmembrane domain (TM), a 41BB costimulatory domain, and a CD3ζ activation domain. The DiCAR used in this study contains a TROP2-targeting antibody generated by the lab as previously described in Chapter 2, an extracellular spacer (IgG4 Hinge + CH2 + CH3 or 4/2NQ spacer[3]), a CD28 transmembrane domain (TM), a PD-1 inhibitory signaling domain, a LAIR-1 inhibitory signaling domain, and/or a SIGLEC-9 inhibitory signaling domain. These receptors were expressed in a third-generation lentiviral vector pCCL-c-MNDU3 generously given by Dr. Gay Crooks and Dr. Donald Kohn. The DU145 prostate cancer cell lines were engineered as described in Chapter 2 to express CEACAM5 and/or TROP2, as well as, GFP and YFP-Luciferase.

Lentivirus Production

Lentivirus for the CARs and iCAR were generated as listed in Chapter 2 using a previously published protocol.[13] Concentrated virus was frozen and titered on 293T cells.

Primary CAR T cell generation, enrichment, and characterization

Peripheral blood mononuclear cells (PBMCs) were purchased from All Cells, LLC from a single donor. T cells and PBMCs were grown in TCM Base supplemented with the listed cytokines (TCM Base = AIM-V Media (Thermo Fisher, 12055) supplemented with 5% human heat-inactivated AB serum (Omega Scientific, HS-25), Glutamax (Thermo Fisher, 35050-061), and 55uM of Beta-mercaptoethanol). PBMCs were thawed and activated with Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher, 11132D) at a 1:1 Cell:Bead ratio and plated overnight at 37°C at a concentration of 1x10^6 cells/mL in TCM Base + 50U/mL IL-2 (Peprotech, 200-02). The following day activated cells were collected, resuspended in fresh TCM + 50U/mL IL-2, and counted. Cells were diluted to a concentration of 0.5x10^6 cells/mL and transduced with lentivirus containing the iCAR at the desired MOI if applicable. Infections were supplemented with Protamine Sulfate at a concentration of 100ug/mL. Following six hours of iCAR lentiviral transduction at 37°C, CAR lentivirus was added with fresh Protamine Sulfate at the desired MOI and incubated at 37°C overnight. The next day an additional 1mL of media was added to each well. Dynabeads were

removed seven days after activation, and T cells were grown in TCM Base + 50U/mL IL-2 + 0.5ng/mL IL-15 (Peprotech, 200-15) media at a concentration of 1x10^6 cells/mL. Two days later (day 9), T cells were enriched for CAR+, iCAR+ T cells using magnetic bead enrichment using the EasySep Release Human PE Positive Selection Kit and Easy Sep APC Positive Selection Kit (Stemcell, 17654 and 17681). CAR^+ cells were enriched using the N-terminal FLAG-tag followed by enrichment of $iCAR^+$ cells using the N-terminal HA-tag. On day 11, magnetic beads used for enrichment were removed. On day 12, T cells were characterized by flow cytometry. T cells were also confirmed to be functional by cytotoxicity assay. Cytotoxicity was measured by performing a co-culture of T cells and engineered target cells as described in Chapter 2 "T Cell Kinetic Cytotoxicity Assay."

Xenograft model for DiCAR inhibition of tumor killing

Animal experiments were conducted according to a protocol approved by the Division of Laboratory Medicine at the University of California, Los Angeles. NSG mice were obtained from The Jackson Laboratory at six-eight weeks of age. Engineered DU145 lines that express CEACAM5 and/or TROP2 were mixed with Matrigel at a 1:1 ratio and engrafted into mice subcutaneously on the right flank. T cells were prepared as described in Primary CAR T cell Generation, Enrichment, and Characterization. Approximately, three weeks after engraftment, when tumors were measurable (10-100 mm^3), T cells were injected into mice via teil-vein at various doses (2 X 10^6, 4 X 10^6). Weekly caliper measurements were obtained of the tumors. Presence of T cells in the peripheral blood of the mice were confirmed by flow cytometry of blood obtained by retroorbital bleed at least four weeks after T cell injection.

Acknowledgments

Planning and advice for these in vivo assays came with the help of Dr. Liang Wang and Dr. Yvonne Chen. *In vivo* experiments were performed with assistance from Lisa Ta and Donny Johnson. Lisa Ta helped prepare tumors, and Donny performed the bleeds.

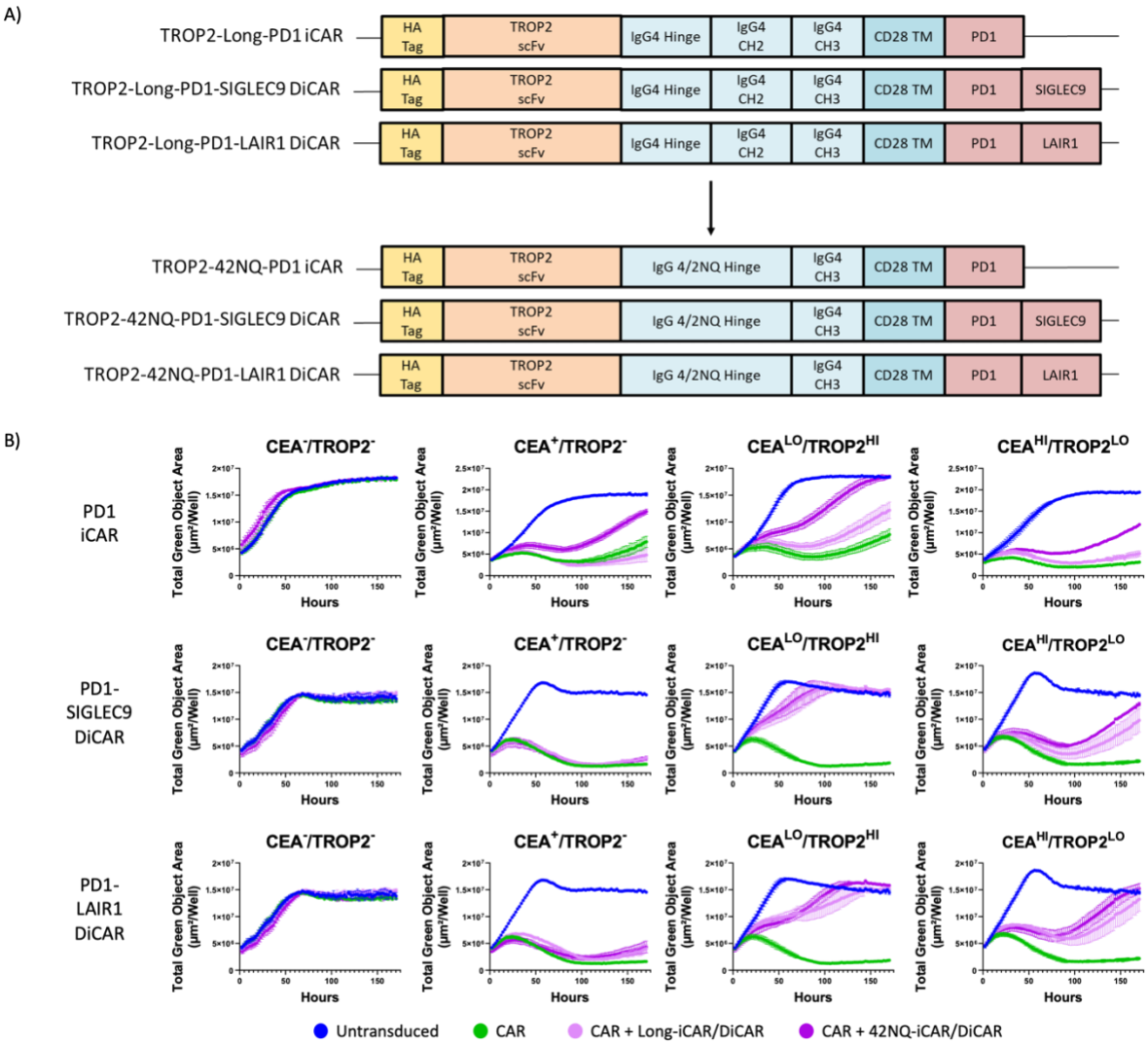

Fig. 3.1. Inhibitory constructs that contain the 4/2NQ spacer have similar or better inhibition efficiency than the IgG4 spacer.

A) Schematic of inhibitory CARs and DiCARs that contain the listed spacer. Constructs with a Long spacer contain a IgG4 hinge, a CH2 constant domain, and a CH3 constant domain. The Long spacer was replaced with the 4/2NQ modified spacer which contains the 4/2NQ spacer, which replaced the IgG4 hinge and CH2 constant domain. It continues to contain the IgG4 CH3 constant domain.

B) CAR T cells that contain the CEACAR and a PD1, a PD1-SIGLEC9, and a PD1-LAIR1 iCAR/DiCAR can inhibit CAR T cell activity regardless of the extracellular spacer used. Long – IgG4 Hinge + CH2 + CH3. 4/2NQ – IgG 4/2NQ modified hinge + IgG4 CH3 constant domain. Cytotoxicity was measured for approximately 170 hours by quantifying the target cell area by target cell GFP expression by Incucyte live cell image analysis. Standard error was calculated for three triplicate wells.

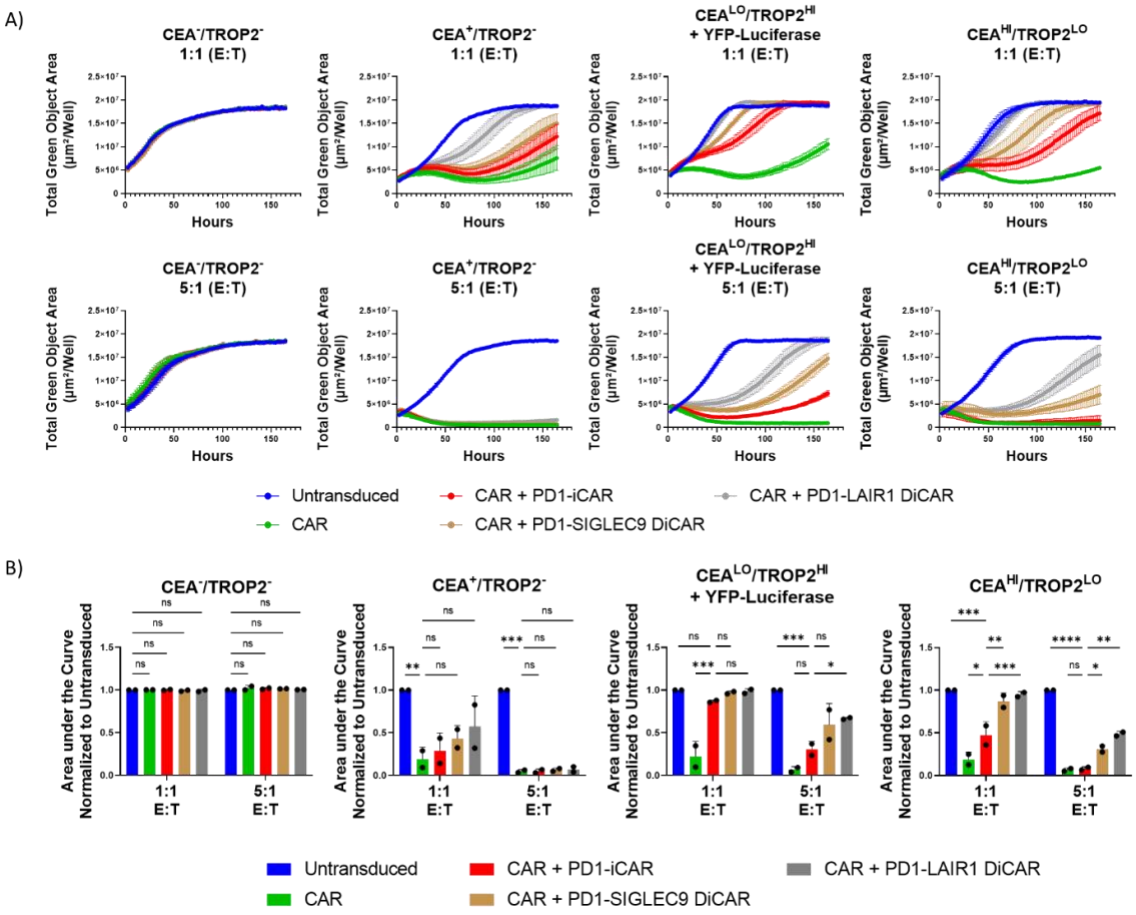

Fig. 3.2. DiCARs can inhibit CAR T cell cytotoxicity at 5:1 effector:target ratios.

A) DiCARs and iCARs can inhibit CAR T cell cytotoxicity at a 1:1 and 5:1 effector:target ratio as measured by total green object area (µm^2/well) of DU145 target cells that express CEACAM5 and/or TROP2 over 150 hours. Images were obtained and quantified by Incucyte live cell image analysis approximately every two hours. The cytotoxicity curves are a representative from one experiment. Standard error was determined by three replicate wells.

B) The delay in inhibition of the iCAR was measured by area under the curve analysis of each cytotoxicity curve. AUC was normalized against the untransduced T cell control in each experiment. The AUC displayed is the mean ± s.d. (n = 2).

The significance values shown are comparisons between a control group. For the CEA$^-$/TROP2$^-$ cell line, values are compared to the untransduced control. For the CEA$^+$/TROP2$^-$ cell line, values are compared to the CAR control. For the CEA^{LO}/TROP2^{HI} or CEA^{HI}/TROP2^{LO} cell lines, values are compared to the CAR + TROP2-PD1 iCAR group.

Statistics are performed using 1-way ANOVA analysis with Tukey multiple comparison correction. *P value ≤ 0.05, **P value ≤ 0.01, ***P value ≤ 0.001

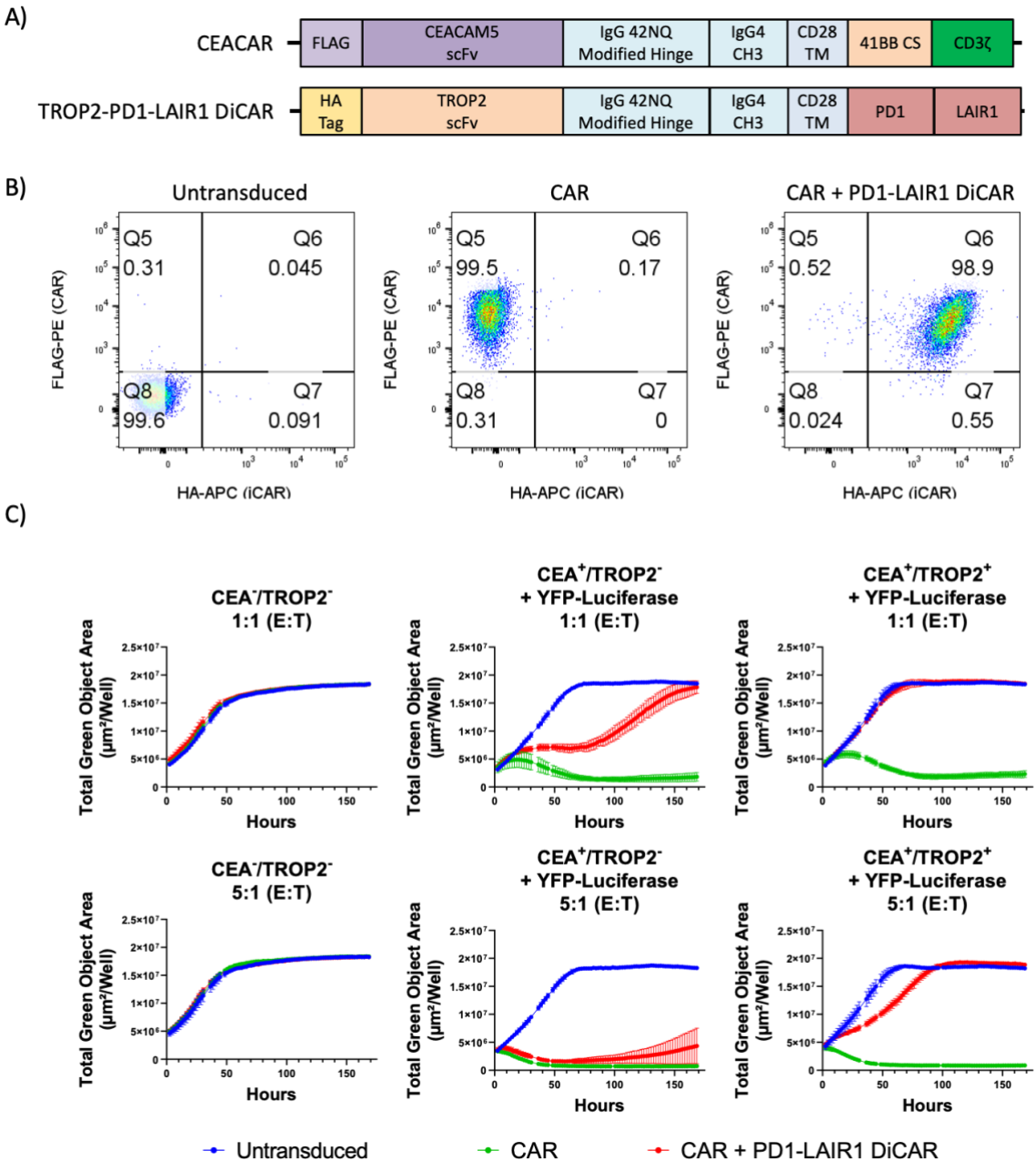

Fig. 3.3. CAR T cells injected into mice are functional.

A) Schematic of the CAR (CEACAR) and DiCAR (Trop2-PD1-LAIR1 DiCAR) used in this study. TM – transmembrane domain. CS – Costimulatory domain.

B) T cells injected into the mice are at least 98% percent of the purified population. CAR and DiCAR surface expression were measured by flow cytometry for the corresponding N-terminal tags FLAG (for CAR) and HA (for iCAR).

C) CAR T cells can efficiently kill target cells that express the appropriate antigens. The engineered DU145 target cells injected into mice as tumors were used as target cells in the cytotoxicity assay. These cells expressed GFP, YFP, and luciferase. Cytotoxicity was measured

by the Total Green Object Area (μm^2/well) of the target cells quantified over 150 hours by Incucyte live cell image analysis every two hours. The cytotoxicity curves are generated from three replicate wells. Effector to target ratios of both 1:1 and 1:5 were used.

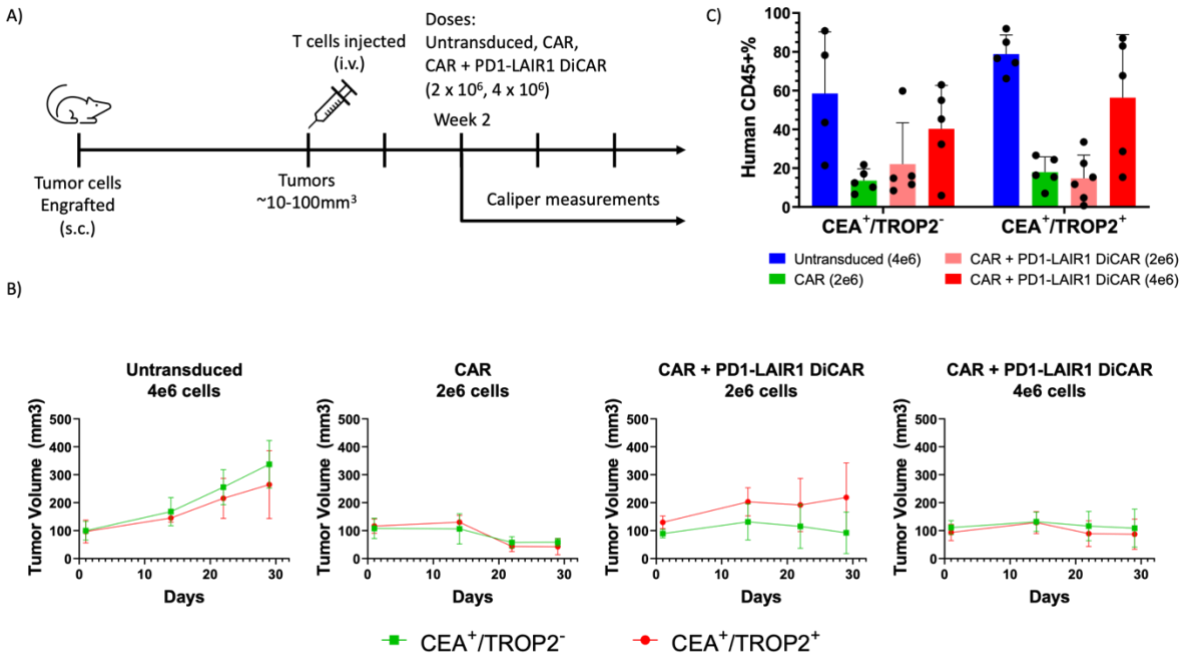

Fig. 3.4. The Trop2-PD1-LAIR1 DiCAR can reduce CAR T cell "on-target, off-tumor" toxicity *in vivo*.

A) Schematic diagram of CAR T cell injections in mice bearing CEACAM5+ or CEACAM5+, TROP2+ DU145 tumors. Mice were injected subcutaneously (s.c.) with tumors cells approximately three weeks before T cell injection. Once tumors are at ~10-100mm^3, CAR T cells are injected into mice at doses of 2 X 10^6 or 4 X 10^6 cells intravenously (i.v.). Tumors were measured by caliper measurements weekly starting two weeks after T cell injection.

B) Tumor growth curves of CEA$^+$/TROP2$^-$ or CEA$^+$/TROP2$^+$ tumors after CAR T cell injection at varying dosages. The tumor volume was calculated by using both the length and width obtained from caliper measurements of the mouse. The tumor growth curves are an average of 4-5 mice/group.

C) CAR+/DiCAR+ T cells are present in mice four weeks after injection. Peripheral blood from mice was obtained from the retroorbital cavity. Persistence of T cells was measured after gating for live cells using 7AAD and the human marker CD45. The average percentage of CD45+ cells and standard deviation was calculated for each group.

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Chapter 4: Conclusion and Future Directions

Chapter 4: Conclusion and Future Directions

In 2013, the *Science* journal named cancer immunotherapy "the Breakthrough of the Year." It was declared so due to major strides in the research and clinical application of checkpoint inhibitors and CAR T cell therapy.[1] Although promising in clinical trials, CAR T cells had yet to be approved by the U.S. Food and Drug Administration (FDA). They could not have imagined the remarkable developments that would be made throughout the next decade.

CAR T cell therapy has changed drastically since 2013. Not only have six different CAR T cell products been approved by the FDA for the treatment of hematological malignancies, but many more clinical trials directed at solid tumors are currently being performed.[2] CAR targets are being extended from CD19 and BCMA to CEACAM5, HER-2, GD2, PSMA, and Mesothelin.[2] And research is still being done to further extend that list of potential antigens. CAR T therapy has evolved from equipping a T cell with a single receptor for killing one specific tumor to converting a T cell into a killing machine. CAR T cells can now target multiple antigens through more than one receptor, transform the immunosuppressive tumor microenvironment through recruitment of other immune cells, and even self-regulate through Boolean logic gates. Every year, more advancements are made to enhance CAR T cell persistence, trafficking, and function. CAR T cells are only getting stronger.

But with that increase in strength, there has also been an increase in toxicities like cytokine release syndrome, neurotoxicity, and on-target, off-tumor toxicity, which have all limited CAR T cell use.[3] Although groups have developed ways to mitigate this problem, much work still needs to be done. Our study has contributed to this endeavor. This thesis has been focused on better understanding the mechanism behind which inhibitory CARs can regulate CAR T cell activity through an AND-NOT-gating strategy. We have found that avidity and the internal signaling domains of an iCAR can affect the kinetics of inhibition. For this reason, regulating CAR T cell activity will not only require optimizing the CAR but also the iCAR. The balance of signaling

strength from both receptors will be critical. Additionally, we engineered a dual-inhibitory domain iCAR (DiCAR) by combining two inhibitory signaling domains into one receptor that inhibits more efficiently than an iCAR with a single domain. This second-generation iCAR, which replicates the dual co-stimulation of a third-generation CAR, can expand the therapeutic dynamic range of the AND-NOT gating strategy, which has been limited by its inefficient delayed inhibition.

Future work will still need to be performed to further optimize these DiCARs for clinical application. There are three hurdles that need to be overcome. First, these DiCARs must be validated for their ability to specifically inhibit on-target, off-tumor toxicity *in vivo*. Although Chapter 3 has illustrated the potential that DiCARs have to inhibit CAR T cell toxicity, more refined experiments optimizing the amount of CAR, DiCAR, and T cells need to be performed. Additionally, transgenic mouse models that express the DiCAR target antigen in the same normal tissues as found in humans will provide a layer of complexity that is critical to determine safety of this strategy. It also would be beneficial to test alternative DiCARs in these *in vivo* assays for improved function.

Second, DiCARs must be engineered to enhance their efficiency and specificity. One problem we observed with these DiCARs is the high level of ligand-independent tonic signaling. This led to undesired inhibition and weakening of CAR T cell killing. Our DiCARs only used one type of extracellular spacer (4/2NQ),[4] transmembrane domain (CD28), and order of inhibitory domains (PD1 followed by the alternative domain). Since spacers have been shown to contribute to tonic signaling[5] and transmembrane domains are suspected to contribute to it,[6] experimenting with alternative domains may reduce this problem. Additionally, with CARs, the location of the costimulatory domains in respect to the transmembrane domain have contributed to their efficacy.[7–9] Because the order is normally empirically determined, future work should look to optimize the order and distance of these inhibitory domains from the transmembrane. With these changes, we hope to make DiCARs more specific.

Third, the universality of improved DiCAR inhibition must be tested. Our proof-of-concept study has only shown that a TROP2-targeting DiCAR can inhibit a CEACAM5-targeting CAR. Whether

the improved DiCAR inhibition efficiency is limited to TROP2 scFv's is unknown. Single-chain Fv's against alternate targets in combination with CARs targeting other tumor antigens should be examined. If this principle holds true for multiple combinations of targets, DiCAR's can be considered the next-generation of inhibitory receptors.

Although our study has given us insights on how to improve iCAR inhibition efficiency, it has also introduced a series of questions about the mechanism behind which it functions. One interesting finding was that only inhibitory domains that contained an ITIM could inhibit CAR T cell cytotoxicity. TIM-3, LAG-3, and CTLA-4 – all known checkpoint inhibitors – when incorporated into an iCAR did not reduce CAR T cell activation in our assays. This result could be due to two things: 1) the constructs that incorporated these domains are non-functional or 2) CARs can only be inhibited through the recruitment of phosphatases via ITIMs. If the latter is true, it can revolutionize how we understand CAR signaling. Another question is whether ITIMs are sufficient for inhibition or whether other portions of the LAIR-1 and SIGLEC-9 signaling domains are necessary for inhibition. If ITIMs are sufficient, smaller constructs could be made that only incorporate these motifs. Like all good science, this dissertation has introduced more questions than those answered.

There are still clear limitations preventing the use of CAR T cell therapy for all cancer patients. Limitations include high production costs, immunosuppression, and on-target toxicity of normal tissues. This thesis work has contributed to overcoming the toxicity limitation through the development of dual-inhibitory domain iCARs (DiCARs). These DiCARs are a new class of receptors that can be engineered to improve CAR T cell specificity through AND-NOT Boolean logic gates. By utilizing DiCARs, we envision that CAR T cells can be made safer without compromising strength. This important balance between T cell activation and inhibition will be critical to making CAR T cells applicable for solid tumors.

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