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SELECTION METHODS FOR GENETICALLY-MODIFIED T CELLS: IN SUPPORT OF TRANSLATIONAL THERAPY

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SELECTION METHODS FOR GENETICALLY-MODIFIED T CELLS:

IN SUPPORT OF TRANSLATIONAL THERAPY

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SELECTION METHODS FOR GENETICALLY-MODIFIED T CELLS:

IN SUPPORT OF TRANSLATIONAL THERAPY

A

DISSERTATION

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of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By

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DEDICATION

כִּי אֲנִי יְהֹוָה רֹפְאֶךָ

“Because I G-d am (your) healer” Exodus 15: 26

To the true healer. May we be so blessed as to know the eloquence of your creation.

To my wife Amber who supports and encourages me.

ACKNOWLEDGEMENT

I am indebted to the wisdom gained from my undergraduate mentors. Dr. Yuri Blednov accepted me into his lab as a freshman college student with no prior experience and only a basic understanding of biology. The experiences I gained there taught me my love of science, for which I am truly grateful. Dr. David Crews, my research mentor from sophomore through senior year of college shared with me his knowledge, understanding, and scientific reasoning. The experience gained there enabled me to reach my goal of entering the physician/scientist training program at the University of Texas Health Science Center at Houston MD/ PhD dual degree program. I am reminded almost daily of the wisdom that he gave to me with the help of his former graduate student Dr. Brian Dias in how to appropriately design experiments and utilize statistics. Without this prior training I feel I would have been lost.

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Which leads me to the laboratory of Dr. Cooper; Dr. Cooper graciously responded to my initial email in 2008 to rotate through his laboratory, and has been very kind to make room and personnel available for me to join his laboratory since that time. In 2010, when I officially entered the laboratory, Dr. Cooper took a great deal of time to listen to my naïve experimental plans, entertain my unusual sense of humor, and provide guidance on my overly ambitious projects. It is difficult to express the gratitude I have that he took the time to help me with writing and project design when he clearly was very busy. Likewise, Dr. Cooper made available to me a seemingly unlimited amount of resources and encouragement to study whatever most captured my interest. I doubt I will ever have such an amazing opportunity again to pursue science without the traditional limitations of research such as specific deadlines,

reports, or monetary concern. Many of my findings would not have been possible if it were not for Dr. Cooper's flexibility and willingness to allow me to test my hypotheses. A jumble of varied projects came together unexpectedly to yield new tools and insights into the biology of T cells. The experience has been very valuable in teaching me how to organize my time, data, and to interpret results. Many other members of the Cooper Lab taught me valuable lessons about conducting scientific investigations in T cell biology: Thank you to Dr. Harjeet Singh for taking the time to train and re-train me on much of the lab equipment and lab techniques. Thank you to Margaret Dawson and Matthew Figliola for teaching me the basics of T cell culture on top of their busy clinical schedules. Thank you to Simon Olivares for taking the time to teach me the basics of molecular biology. Thank you to Ling Zhang who repeatedly taught me how to perform western. Thank you to Dr. Sonny Ang and Dr. Colleen O'Conner for teaching me valuable interpersonal skills. Thank you to Dr. Hiroki Torikai for teaching me important lessons on work ethic and organization.

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In the development of Anti-thymidylate resistance (AThyR) transgenes for expression in T cells, I thought I happened upon a completely original idea. I later found that Dr. Cooper and Simon Olivares had been developing DHFR^{FS} possibly two years

before I had thought of it. Simon's DNA sequence for non-codon optimized $DHFR^{FS}$ matched mine exactly. Great minds think alike! Unfortunately, the constructs Simon designed and built were never tested. The AThyR+ T cell studies hinged on the availability of clone.4 aAPC, and I would especially like to thank Tiejuan Mi who maintained the clone.4 aAPC for the lab and provided stock for me to propagate and modify with OKT3 antibody on my own. She even provided me with OKT3-loaded clone.4 when I had none. Thank you to Amber Mathews for her continuous efforts to make bad cells look good in performing western blots on T cells subjected to high doses of toxic drug. Thank you to Dr. David Spencer for providing the sequence of inducible caspase 9. Thank you to Dr. Brian Rabinovich for providing for interesting discussions and sometimes even DNA or protein sequences that was vital to the progress of this and other projects.

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SELECTION METHODS FOR GENETICALLY-MODIFIED T CELLS: IN SUPPORT OF TRANSLATIONAL THERAPY

By David Daniel Rushworth, BS

Advisory Professor: Laurence Cooper, M.D., Ph. D.

T cells are blood cells which organize the immune system of the host. These cells are necessary for the host to respond appropriately to threats from foreign organisms and cancerous growth. However, in the case of certain infections and cancer, T cells are unable to respond appropriately to a threat and establish immunity. This leads to disease when the infection or cancer is not sufficiently eliminated. On the other hand, T cells can lack tolerance for healthy tissue and perceive healthy tissue as infected. The ensuing over-reactive immune response also leads to disease. A delicate balance must exist between immunity and tolerance to prevent these diseases. Small molecules have been developed to ameliorate human diseases resulting from the failure of T cell immunity or T cell tolerance, but these small molecules rarely lead to cure. This has driven investigators to develop approaches where T cells are modified to target disease in order to restore the balance between immunity and tolerance. The results have been promising and include long-term cure of disease. Furthermore, genetic modification of T cells has the potential to provide supra-physiological capacities to T cells, including targeting infection or cancer in ways that T cells could never achieve naturally within the host. These gene therapy approaches are hindered by technical challenges such as selecting for genetically-modified T cells and against unwanted T cell phenotypes. Here we describe novel methods utilizing unique transgenes and small molecules

aimed at improving the selection of genetically-modified T cells for the treatment of disease in humans.

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Chapter 1: INTRODUCTION

The immunobiology of T cells

Vertebrate animals have evolved a dedicated system capable of removing foreign organisms from within the animal. This organ is called the immune system. The immune system is vital to the survival of an animal, and genetic deficiencies within the immune system typically lead to disease and death at a young age. The immune system operates in a characteristic way: Large molecules typically found on bacteria, fungi, or viruses, are recognized by receptors on innate immune cells in the animal. Innate cells and proteins do not change over the life of the animal. These innate cells are activated by a foreign threat to secrete proteins called cytokines and chemokines which mobilize the innate and adaptive immune system. Adaptive immune cells develop an immune response tailored to remove the foreign threat. Both adaptive and innate immune cells are typically found circulating in the blood or resting in immune organs, but when cytokines or chemokines are recognized, these immune cells travel through the body to the site where the hormones are being secreted.

The early innate immune response involves proteins such as complement and pattern recognition receptors, as well as immune cells, such as macrophages and neutrophils, which traffic to these sites.[1] For example, *Staphylococcus epidermidus* is relatively benign and found on the skin while *Staphylococcus aureus* frequently causes infections and disease,[2] but each activate the innate immune system similarly. One of the first immune cells to arrive in an innate

immune response is a macrophage. Macrophages engulf foreign organism and cell debris, but also carry out a crucial role in further activating the immune system.

Macrophages and similar cells called dendritic cells engulf foreign matter associated with the pathological organism and degrade the foreign matter into smaller components, called antigens. Macrophages and dendritic cells then present the foreign matter antigens on their surface bound to a family of proteins known as the major histocompatibility complex or MHC. The advantage of MHC is that antigens on MHC are recognized by a specialized type of immune cell known as a T cell. T cells and B cells constitute the portion of the immune system able to adapt to variations in pathological organisms which the innate immune system cannot. T cells and B cells therefore constitute the adaptive immune system. Cells like macrophages and dendritic cells that present this antigen are called antigen presenting cells (APCs).[3]

When T cells recognize antigen: MHC complex on APC, then the T cell become activated and releases cytokines that activate other immune cells to begin an immune response. The T cells also begin to replicate and produce more T cells with the same capacity to respond to the same antigen.[1] Cytotoxic T cell (T_{cyt}), also known as $CD8^+$ T cells, have the capacity to kill cells that are expressing target antigen on class I MHC, and this is crucial for the removal of intracellular pathological organisms such as bacteria and viruses.[4] When B cells are activated or receive cytokine signal from activated T cells, then B cells begin an immune response towards antigen present on the surface of foreign organisms. B cells respond to antigens by generating proteins called antibodies, which bind a specific portion of a given antigen. Antibodies are an important part of the immune system as they have the capacity to detect

extracellular and cell surface antigens, and binding of antibodies to foreign matter activate the innate immune system to bind and destroy foreign organisms.[1, 3]

Antigen response in T cells

The ability of B cells and T cells to bind unique antigens associated with pathogens is due to a capacity of these cells to reorganize their DNA, an uncommon phenomenon in animals. The DNA of B cell receptors (BCR) and T cell receptors (TCR) is designed and systematically reorganized in such a way that the DNA encodes millions of possible BCRs and TCRs, but after reorganization only one of those possibilities is present in an individual B cell or T cell. Thus, when a T cell responds to antigen on an APC, the T cell is restricted to that antigen presented on MHC and will not recognize other antigens or the same antigen when unbound to MHC. [1]

T cells require the presence of the appropriate antigen presented on MHC to begin activation, but more signals are needed to fully stimulate the T cell. In fact, if those other signals are absent, a T cell recognizing antigen on MHC may undergo death or be otherwise inhibited from normal activation.[3] Cytokines are one of these signals which is important to T cell survival and activation. Cytokines such as IL-7 and IL-15 help T cells to continue to survive independent of antigen. However, when T cells recognize antigen, cytokines such as IL-2, IL-4, IL-12, IL-17, and TGF-β can strongly influence the activation and differentiation of the T cell.[5] T cell naïve to antigen must receive activation through the TCR as well as costimulatory receptor activation. The presence of the appropriate costimulatory ligands on the APC is a crucial component in the activation of T cells.

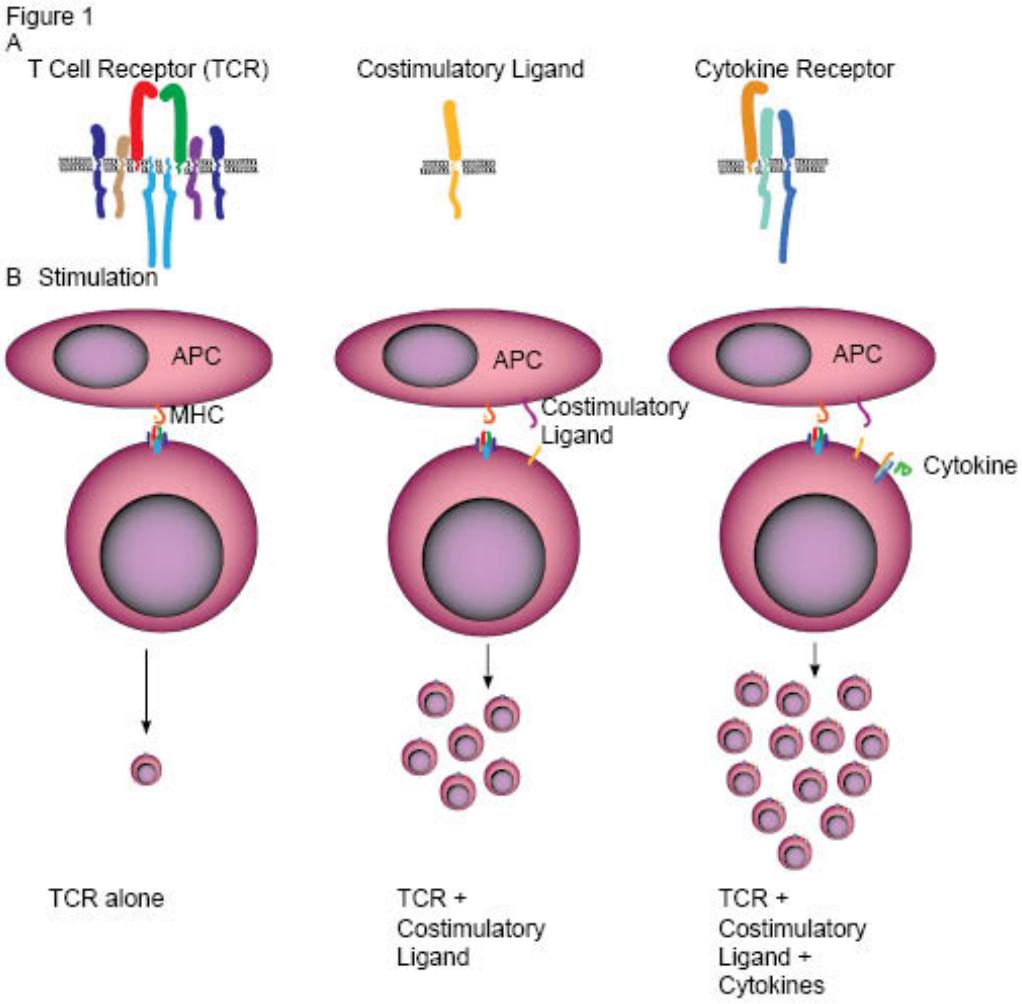

Figure 1 Factors influencing T cell activation and propagation

A) The proteins on T cells involved in transducing external signal into internal signals are shown. Antigen on MHC of APC stimulates TCR, costimulatory receptor detects costimulatory ligand on APC, and cytokine receptors detect cytokines. **B)** The strength of stimulation of TCR alone is insufficient to activate and propagate T cells. Consequently, costimulatory receptors and cytokines strongly influence the capacity of a T cell to propagate in response to antigen.

A costimulatory molecule like CD28 has the capacity to fully activate a T cell and initiate an immune response when CD86 is present on the APC or inhibit the T cell from responding when CTLA-4 prevents binding of CD28 to CD86.[6] A strong activation signal has the potential to promote activation, proliferation, and survival. T cells survival after infection can last many years and prepare the animal to respond more rapidly to a future infection of the same pathogen. This phenomenon of T cell survival and enhanced response is called T cell memory. T cell memory is clearly advantageous as many pathogens are ever-present in the environment of an animal, and survival depends on the ability to deter an initial infection and prevent repeated infections.[7]

Natural suppression mechanisms of T cells

T cells are quite powerful effectors of the immune system, but T cell activity can be deleterious if inflammation persists too long or in the absence of an actual pathogen. It is hypothesized that one of the evolutionary reasons for using multiple signals to fully activate T cells is that an inappropriately activated T cell could recognize antigens that naturally occur in the body of an animal. Typically T cells develop in such a way that many of these self-reactive T cells (T cells responding to self as though it were a foreign pathogen, also called auto-reactive) are deleted in the thymus before the auto-reactive T cell is capable of leaving the thymus to target self-antigen on healthy tissues. However, some auto-reactive T cells continue to persist within the body usually in a state of suppressed activity.[8] While multiple mechanisms of immune system suppression exist, suppression mediated by a subset of T cells called

regulatory T cells – T_{reg} - is considered one of the most effective means of T cell suppression within the body. [9]

T_{regs} responds to antigen via TCR like other effector T cells (T_{eff}) and T_{cyt}, but, unlike other T cells, the TCR of T_{reg} is reactive towards self and induces suppression of inflammation when activated. While T_{reg} previously were considered a controversial topic in immunology, these cells have come to be understood as critical for the maintenance of immune homeostasis in the body. As an understanding of T_{regs} developed it was found that loss of a specific transcription factor, FoxP3, leads to the absence of T_{reg} and, subsequently, severe autoimmunity in mice and humans from an early age. T_{reg} suppress immune responses through a variety of mechanisms listed in **Table 1**. These suppressive mechanisms each inhibit T_{eff} and T_{cyt} function to prevent autoimmunity.[10]

T_{eff}, commonly called $CD4^+$ T cells in the literature, often coordinate initiation of an immune response including innate immune cells and B cells, in order to remove pathogen.[3, 5] T_{eff} and T_{reg} are both $CD4^+$ and differ from T_{cyt} by responding to antigen presented on class II MHC rather than class I MHC. T_{reg} inhibit the inflammatory state from persisting to the point where it becomes deleterious. Equally, T_{reg} inhibit T_{eff} from initiating and propagating an immune response towards self-antigen which would be deleterious under healthy conditions. However, an immune response towards self is at times warranted in the case of somatic mutations that initiate and propagate cancer. In the case of cancer, T_{reg} suppression of autoimmunity is deleterious as it prevents a cancer-specific immune response.[11] Clearly, the ratio of T_{eff} to T_{reg} can be crucial to the prevention of pathology. As such, many clinicians and

Table 1 Suppressive mechanisms of T_{reg}

Mediator	Abbreviation	Mechanism
Adenosine		Signaling via the A2a receptor to generate cAMP[12, 13]
3`-5` Cyclic adenosine monophosphate	cAMP	Inhibitory secondary signal to effector lymphocytes during intercellular contact.[14]
Transforming growth factor - β	TGF - β	Inhibitory signaling to multiple cell types[15]
Cytotoxic T Lymphocyte Antigen -4	CTLA – 4	Inhibitory costimulation to T cells.[16]
CD25		This portion of the IL-2 receptor is overexpressed and acts as a sink for the T cell activator IL-2[17]
Interleukin-10	IL-10	Suppressive cytokine[17]
Indoleamine – 2,3 - deoxygenase	IDO	Depletion of Tryptophan and arylhydrocarbon receptor activation[18]

researchers have begun to use T cell therapies to treat diseases of inflammation or immune suppression.

Application of T cells in the treatment of disease

The clearance of infections in humans requires functional adaptive immunity. Vaccines co-opt functional T cells and B cells to develop immunologic memory towards dangerous pathogens. The memory established by vaccines leads to an immune response during early signs of infection from a pathogen. This use of T cells and B cells has prevented an untold amount of morbidity and mortality related to overwhelming pathogenic infections, by early suppression of infection or disease pathology. The success of vaccines has motivated researchers to develop therapeutics for patients with dysfunctional immune responses. Researchers have successfully targeted immune suppression in cancer or chronic infections with cytokines and antibodies. This has proven quite effective in reducing the burden of many forms of chronic disease due to a weak immune response. In the case of an excessive immune response, such as autoimmunity, clinicians have also utilized cytokines and antibodies to inhibit the abnormal immune response.[19] With much development in the way of immunologic modulation, adoptive transfer of T cells remains elusive in the treatment of disease beyond a small number of Phase I and II clinical trials. Although these cells are crucial for mounting, maintaining, and suppressing an immune response, no adoptive T cell therapy has been formally approved for use in the United States.[20]

T cells to treat cancer

Targeting T cells to human disease has been in progress for more than 25 years.[21] The initial aim of clinical trials was to direct T cells to target and kill diffuse cancers such as metastatic melanoma and leukemia.[21, 22] With the objective of targeting unusual antigens present only on the cancer. Antigens on cancers are often times overexpressed or mutated versions of proteins found on normal cells. Although cancer antigens ideally demarcate only the cancer, this is rarely the case and the risk of off-tumor toxicities can occur and cause serious complications that many times have led to morbidity and death. The powerful nature of T cell therapies is one of the reasons that T cells continue to be sought as a therapeutic, but have not yet reached FDA approval in the United States for any form of disease.

T cell therapies are consistently improving and begun to incorporate more complex ideas such as transgenic modifications that perform supraphysiological actions. An early idea of combining the activating domain of a TCR with the antigen specificity of an antibody (**Figure 2**) developed a protein called a chimeric antigen receptor (CAR) which activates a T cell expressing CAR when antigen is recognized on the surface of a cell.[23] This approach is advantageous as it extends the capacity of T cells to detect antigens not present within MHC. However, the risk of off-tumor toxicity continues for the same reason. CAR expression on T cell is a technological challenge that has improved with advances in gene therapy. Now, technical aspects of T cell culturing techniques and gene therapy are at a point where multiple clinical trials generate tumor-specific T cells or genetically-modified CAR^+ T cells to target cancers.[24-26]

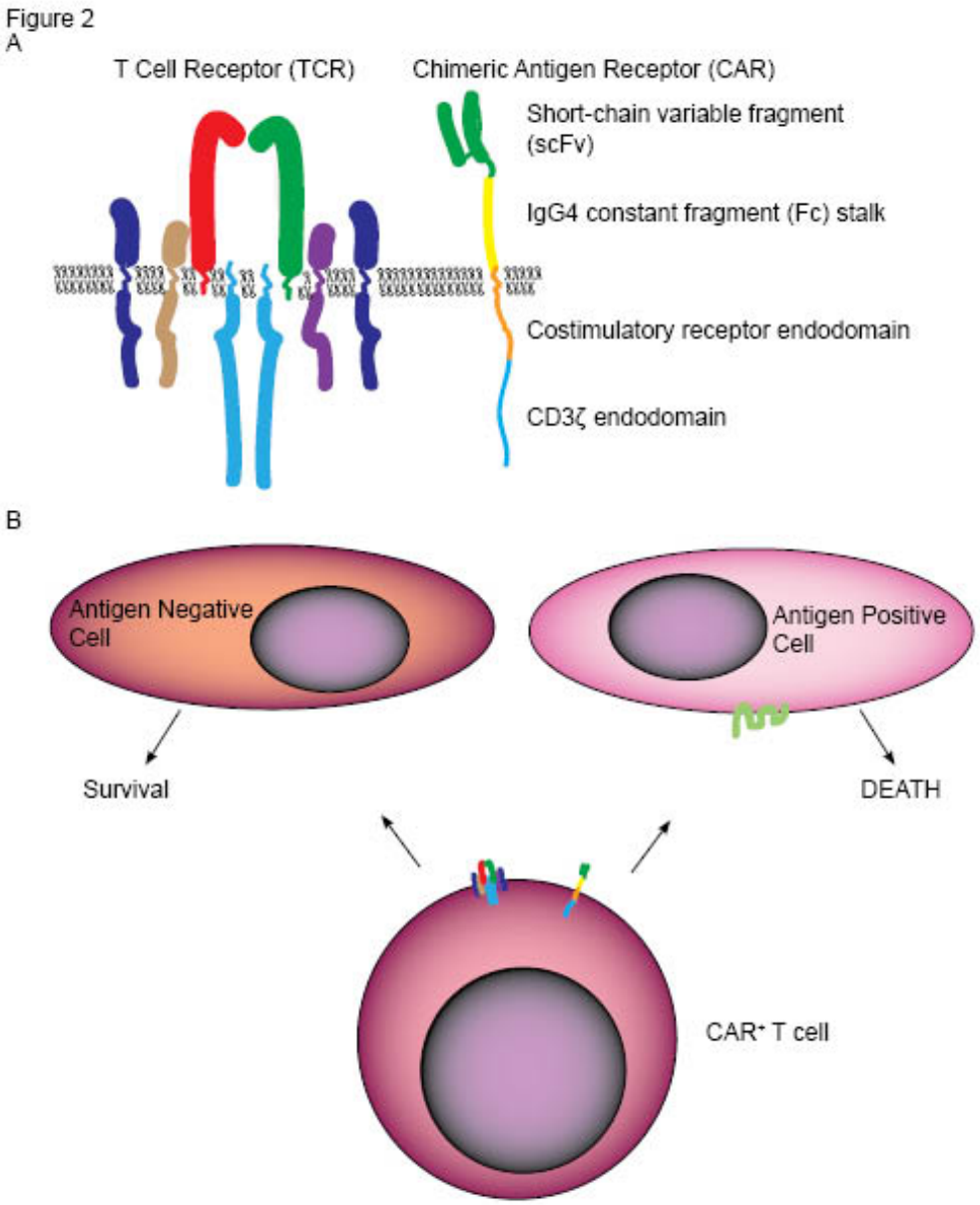

Figure 2 Comparison of chimeric antigen receptor (CAR) to TCR.

A) The structural differences between a second-generation CAR and endogenous TCR are displayed with identification for each domain. **B)** Selective targeting of CAR to surface antigens independent of MHC is represented. CAR activates T cells to kill cells containing the target antigen, without killing cells that do not express antigen.

While many of the T cell clinical trials are showing strong benefit over standard of care, the cost of producing a T cell therapy and risk to the patient continues to hamper development of these technologies beyond a few specialized centers. Further limitations exist due to the complex immunosuppressive environment of the tumor, and difficulty of identifying appropriate tumor antigens.[25] It should be noted that T cell therapeutics in cancer were initially developed for the treatment of melanoma and leukemia, and in the intervening quarter century have not significantly deviated from those cancer targets. Further improvements in the technical aspects of T cell therapy as well as continuing research and development of immune-modulatory drugs will continue to promote T cell cancer therapies for cancer and potentially broaden the applicability of these therapeutics.

T cells to treat infection

A competent immune system can often develop an appropriate immune response to bacterial and viral antigens leading to initial clearance of the pathogen and long term immunity. However, not every patient has a competent immune system. In fact, patients who receive a transplanted organ are chronically immunosuppressed. Patients on immune-suppressive regimens are at risk for infection from organisms not typically pathogenic in the normal population. Also, viral infections, which previously led to mild and moderate illness, can be life-threatening. This risk is most severe in the hematopoietic stem cell transplant (HSCT) setting where depletion of innate immune cells combines with immune-suppression to prevent MHC mismatching between bone marrow graft and the body of the host. High doses of immunosuppressive drugs put the patient at risk for viral, bacterial, and fungal infections, but

are necessary to prevent deadly graft versus host disease (GvHD). In this case, the appropriate memory T cells may not exist in sufficient quantities to fight off infection following the high dose chemotherapy and immunosuppression used to condition the patient for HSCT. This clinical need was addressed with T cell therapy, and T cells specific to viral infections began development more than 20 years ago. While this approach of T cell therapy has faced technical challenges, the risk of off-target toxicity and decreased efficacy are not as great as targeting cancer. T cells targeting common viral infections such as CMV, EBV, or adenovirus are up to Phase III clinical development at multiple institutions in the United States and at a similar stage in Europe.[27]

T cells to treat inflammation

Diseases of excessive inflammation are currently targeted by immune-modulatory or immune-suppressive medications. These therapies are often effective, but have untoward side effects as discussed in the above section. Better targeted immunosuppression may be possible using T_{regs}. As T_{regs} are better understood and culturing techniques become more advanced, cell therapies based on reconstituting T_{regs} will likely move toward clinical trials more rapidly. The use of T_{regs} in clinical trials has been limited to preventing GvHD following HSCT for the most part. It is likely that the number of uses for T_{reg} will expand as many other forms of inflammation have been targeted in preclinical models. Technical challenges related to the isolation and propagation of T_{reg} is currently limiting the advance of this T cell therapy.[10]

Selection for T cells in the treatment of disease

A common theme in section **1.2** was the technical limitations of T cell therapies. While development of more effective and safer T cell therapies at times awaits an improved understanding of immunobiology, there is still a great deal of improvements to be made in the technical knowledge of T cell isolation, propagation, and re-infusion. Technical knowledge and expertise continues to advance the field. It often permits biological studies that advance knowledge as well as new and improved applications for T cell therapies. Here we consider in further detail methods for the isolation and propagation of T cells.

Antigen-specificity dependent selection (ASDS)

The isolation of T cells based on antigen specificity can be considered an advantage of working with T cells. A T cell that recognizes an antigen of interest can activate and propagate itself to larger numbers. However, the MHC restriction of T cell antigens often limits the feasibility of this approach. Thus, APC must be derived from the patient, or MHC matched donor, to stimulate their T cells, which was the original ASDS method.[28] Methods using APC involves isolation and infection of donor APC with virus or activation and pulsing of antigenic peptide on derived APC. The infected or activated APC then presents antigen to T cells which activates and propagates antigen specific cells.[28] Alternatively, if the patient has a common MHC then other approaches are available. Artificial APCs (aAPC) made by genetically-modifying tumor cells to express specific MHC and peptides can be used to propagate T cells targeting the MHC - peptide combination.[29, 30] Similarly, synthetic multimer proteins containing a specific MHC – peptide combination can be used to isolate T cells specific to that MHC

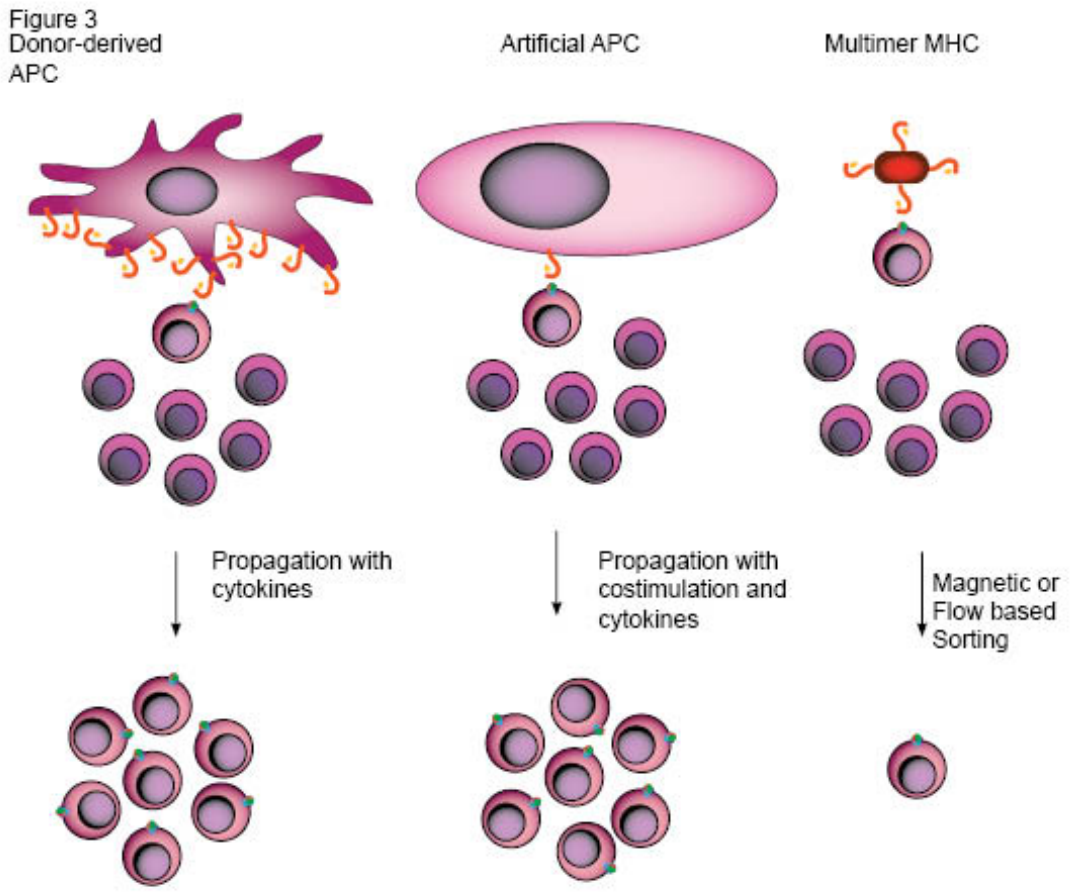

Figure 3 Common ASDS techniques for T cell selection.

The selection of T cells using ASDS techniques occurs through three common practices. Shown on the left is ASDS of T cells using donor derived APC. These APCs present antigen on every MHC the donor expresses – hence there are 12 MHC on the donor-derived APC. In the center, is an artificial APC (aAPC) expressing one MHC, as aAPC are typically manufactured expressing one MHC. The same selective propagation of T cells is achieved by only one MHC peptide complex, which decreases the variety of antigen-specific T cells that are propagated. Similar to aAPC, on right, T cells can be selected with a multimer (seen as a tetramer), that can bind T cells specific to a certain MHC-peptide combination and then be purified via this method.

peptide combination.[31] ASDS methods are quite effective, but are limited by the specificity of the TCR. Selection of T cells using APC from the same donor propagate T cells specific to multiple antigens of the peptide presented on multiple MHC (There are up to 12 different MHCs expressed by the same individual), but extracting and modifying host APC adds technical challenges to T cell therapy production. Artificial APC selection does not require APC modification for each donor, but generation of aAPC is a costly task, which results in a restricted array of MHC choices for presenting antigen to T cells. The use of multimer MHC technology does not require artificial or donor APC, but producing a multimer is a costly task and multimer MHC peptide complexes are typically limited to common MHC presenting a common peptide. Consequently, the T cells generated using aAPC and multimer MHC techniques target T cells to antigen within a limited population of donors and with a limited number of antigen targets. This restricted antigen specificity of aAPC and multimers increases the risk that the T cell response will be insufficient to eradicate disease. Subsequently, MHC restriction of T cells has become a limitation in generating T cell therapies, and many groups have focused on propagating cells by antigen-specificity independent selection.

Antigen-specificity independent selection (ASIS)

The development of MHC independent T cell propagation methods has been a great technical advance for T cell therapies. Growing T cells by ASIS generates large numbers of T cells for reinfusion to a patient. While it might seem counterintuitive to grow T cells without direct selection for specificity, the large number of T cells typically includes an activated and propagated subset of T cells that are specific to the antigen targeted. A possible advantage

over ASDS is that T cells grown by ASIS may have many different types of T cells specific to the same disease. In the case of T cells derived from tumor, also known as tumor infiltrating lymphocytes (TIL), the *in vivo* development of multiple tumor-specific T cells led to better suppression of disease than a single tumor specific T cell.[32] Also, the use of ASIS permits T cells to be genetically modified and propagated without selection. This is commonly used in TIL and CAR therapies where the specificity of the T cell is not selected *in vitro* but once infused leads to ASDS within the patient.[33] The use of *ex vivo* expansion via ASIS and *in vivo* expansion by ASDS could be considered the current standard in T cell therapies.

Generating T cells by ASIS requires stimulators of T cell activation and propagation such as stimulation, costimulation, and / or cytokine signals.[26] The propagation of T cells independent of MHC increases the risk for expanding deleterious T cells that might cause toxicity.[22] In the case of cancer, ASIS techniques propagate T_{reg} along with T_{cyt}. Consequently, a T cell therapeutic that is developed to target and destroy tumor may propagate cells that prevent T cell activity at the site of tumor.[34] On the opposite spectrum of T_{cyt} activity, T_{cyt} may begin to recognize antigen on healthy tissue and target vital organs for destruction.[8] These are just a few of the examples of poorly modulated T cell activity. The issues of poor efficacy and off-target toxicity are critical considerations for the field of T cell therapeutics to move forward. Some of the issues have biological solutions, such as the use of IL-7 or IL-15 rather than IL-2 to decrease T_{reg} ASIS.[26] However, other issues require a more complex approach.

Adoptively transferred host-reactive T cells can become life-threatening when T cells begin to react to host antigens on vital organs. Therefore, selective depletion of adoptively

transferred host-reactive T cells is needed in arguably any ASIS propagated T cell therapy.[25] The field has found no unanimous solution to selectively deplete host-reactive T cells. Most groups seriously addressing this issue have determined genetic modification of T cells to be the most effective means to selectively deplete these cells.[35, 36] As host-reactive T cells have the same potential as tumor-reactive T cells to undergo ASDS in the host, an *ex vivo* selection method is needed before adoptive transfer of T cells. Transgenic CAR or TCR did not require *in vitro* selection by ASIS, as these approaches develop *in vivo* ASDS. However, transgenes not involved in direct targeting of pathology do require *in vitro* selection by an ASIS method. Thus, groups have genetically modified T cells for selective depletion in the case of host-reactivity using *in vitro* ASIS. Currently, few transgenic ASIS methods have been described to uniformly select a transgene. Sorting for the desired transgene co-expressed with a surface marker is the typical methodology with the surface marker selected using magnetic beads.[37-39]

While magnetic bead based sorting has shown efficacy, it is time consuming and costly as the number of antigens to be sorted increases. Fluorescence based flow sorting techniques could avoid some of the technical pitfalls of magnetic sorting, but this approach is even more costly and time consuming.[40] Sorting cell products through large machines, possibly several times, increases the risk of contamination, which is a serious concern in immune-compromised patients receiving these therapies. It is apparent that another form of *in vitro* ASIS for transgene expression is needed. Hence, we have determined that there are technical limitations in transgenic selection methods, which are preventing T cell therapeutics from advancing to a more consistent and safe form of treatment. We have found potential methods that could be

modified to develop safe, non-immunogenic [41] *in vitro* ASIS selection for genetically modified T cells. Our objective is to develop *in vitro* ASIS methods to select for the expression of transgene in T cells. The long-term goal is to disseminate these approaches in order for the field to develop better transgenic methods to control activation, propagation, and depletion of genetically modified T cells *in vivo*. We have developed the following hypothesis for this purpose:

Hypothesis

We find the lack of *in vitro* ASIS methods for T cell therapeutics to be a major limitation in the development of higher quality and more complex disease modifying T cell therapies. Novel ASIS methods are sought to enhance the selection of transgenic T cells and desirable T cell phenotypes. While *in vitro* ASIS using chimeric cytokine receptors [42] is a recently reported method of non-immunogenic selection, it only utilizes the third signal in T cell activation – cytokine signaling. We hypothesized that aAPC can complete this *in vitro* ASIS methodology by utilizing the first and second signals of T cell activation (CD3 and costimulatory signaling) of human genes to activate and propagate T cells independent of antigen specificity.

Following our above argument, a single selection method (*i.e.* surface-expressed marker) is limiting the development of more advanced T cell therapeutics. Hence, we sought another *in vitro* ASIS method independent of surface-expression. The original *in vitro* ASIS techniques, selection with toxic drug by expressing a drug resistance transgene,[43, 44] suffered from the immunogenicity of the transgene origin – bacteria.[45] Here we hypothesize that the

lower immunogenicity of mutated human transgenes will establish resistance to anti-thymidylate drugs as a new *in vitro* ASIS method. This method will have the advantage of potentially allowing for *in vivo* ASIS to a whole class of FDA approved drugs.[46] It is our overall hypothesis that these non-immunogenic *in vitro* ASIS techniques will guide development of better *in vitro* and *in vivo* ASIS of T cell phenotypes such as T_{reg}, T_{eff}, or T_{cyt} and the capacity to better control the activity of the T cell.

Specific Aims

Specific Aims 1

We sought an AaPC that could select for and propagate T cells independent of antigen specificity by targeting a conserved exo-domain of CAR^+ T cells. This AaPC would ligate and propagate T cells through CAR independent of antigen specificity.

Specific Aims 2

We determined that transgenic selection with toxic drug would best be achieved with non-genotoxic drugs. The thymidine synthesis pathway was targeted as it is well described, non-genotoxic, and often used clinically to suppress T cell proliferation and cancers. Two enzymes in the thymidine synthesis pathway, dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS) have an entire class of drugs designed to target these enzymes. We developed anti-thymidylate resistant mutants of DHFR and TYMS to achieve *in vitro* ASIS of transgenic T cells.

Specific Aims 3

Utilizing the transgenic *in vitro* ASIS techniques above, we sought a novel ASIS method to select for or against T_{regs}. We dissected the T cell suppressive effects of the anti-thymidylate methotrexate (MTX) to develop a drug-based method for selecting T_{reg}. This improved understanding of T_{reg} physiology led to the finding that a common antibiotic drug class - aminoglycosides - depletes T_{reg}. Hence, transgenic *in vitro* ASIS techniques advanced the understanding of selection for T cell phenotypes

CHAPTER 2: *In vitro* ASIS of CAR$^+$ T cell for testing and clinical expansion using aAPC

This chapter is presented without substantial modification from the following publication:

Rushworth D, Jena B, Olivares S, Maiti S, Briggs N, Somanchi S, Dai J, Lee D, Cooper LJ: Universal artificial antigen presenting cells to selectively propagate T cells expressing chimeric antigen receptor independent of specificity. *Journal of immunotherapy* 2014, 37(4):204-213.

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Introduction:

The adoptive transfer of antigen-specific T cells is a rapidly developing field of cancer immunotherapy with innovative approaches to their manufacture being tested and new antigens being targeted. T cells can be genetically-modified for immunotherapy to express chimeric antigen receptors (CARs) that recognize tumor-associated antigens (TAAs) independent of HLA (editorial note: HLA is the human version of MHC) expression. Recent results from early-phase clinical trials demonstrate that CAR$^+$ T-cell (CART) therapies can lead to partial and complete remissions of malignant diseases, including in some recipients with advanced/relapsed B-cell tumors.[47, 48]

Currently, many CART therapies are based upon *ex vivo* propagation from the donated T cells obtained from steady-state apheresis or venipuncture.[49-54] Approaches for numeric expansion typically use either CAR-independent T-cell proliferation based upon cross-linking CD3 and CD28 with antibodies[50-52, 55] or CAR-dependent propagation using TAA expressed on

artificial antigen presenting cells (aAPC).[56-59] Other methods to selectively propagate T cells to constitutively express CAR include co-expression with transgenes for selection under cytocidal concentrations of drug and/or sorting, such as using magnetic beads that recognize introduced proteins co-expressed with CAR.[56],[37] After electro-transfer of DNA plasmids derived from *Sleeping Beauty* (SB) system, we employ CAR-mediated expansion to selectively propagate T cells that stably express the introduced single-chain immunoreceptor by repeated additions of γ-irradiated K562 cells genetically modified to co-express costimulatory molecules and the TAA targeted by the introduced CAR.[49, 57, 60] However, this necessitates that each aAPC design must be manufactured to express the TAA targeted by a given CAR. Furthermore, some TAA that are biochemically or structurally complex, such as glycosphingolipids, are difficult to recapitulate on the surface of aAPC.[61]

Here, we describe an approach to achieve CAR-mediated expansion that avoids the requirement for cytotoxic drugs, magnetic selection, or TAA-specific proliferation. A monoclonal antibody (mAb, clone 2D3), previously reported by our laboratory, [57] was shown to bind to the conserved exodomain (derived from modified human hinge and Fc region of IgG4) [62] of a CAR. The antigen-specificity of this mAb was constructed as a single-chain variable fragment (scFv) and expressed on K562 cells to serve as an aAPC.[49, 63] This scFv on the cell surface of aAPC is able to ligate a panel of CARs with diverse specificities that contain the IgG4 extracellular scaffold, leading to selective expansion of genetically modified T cells that have redirected specificity for multiple TAAs. This scFv serves as a ligand for CAR (designated

CARL) that can substitute for TAA and thus provides investigators with one source of aAPC that may be used to generate populations of CAR^+ T cells with any specificity.

Materials and Methods:

Cells and culture conditions

K562 cells (European Collection of Cell Cultures through Sigma-Aldrich, St. Louis, MO; Cat. No. 89121407), noted for expression of desired endogenous adhesion molecules and the absence of most HLA class I and all class II molecules, [63] were used to derive $CD19^+$ and $CARL^+$ K562 that served as aAPC. Immortalized tumor targets $CD19^{neg}$, $GD2^+$ EL-4 murine thymoma (Cat. No. TIB-40) and $CD19^+$, $GD2^{neg}$ NALM-6 (pre-B cell leukemia, Cat. No. CRL-1567) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Identity of cell lines was validated by the MDACC Cancer Center Support Grant Characterized Cell Line Core using short tandem repeat DNA fingerprinting. Peripheral blood was donated by consenting healthy volunteer adults at Gulf Coast Regional Blood Center (Houston, TX). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus density centrifugation (GE Healthcare Biosciences, Piscataway Township, NJ; Cat. No. 17-1440-02) before freezing in a mixture of 10% DMSO (Sigma, Allentown, PA; Cat. No. D2650), 50% heat-inactivated fetal bovine serum (FBS-Thermo Scientific Hyclone, Bridgewater, NJ, Cat. No. SH30070.03), and 40% RPMI 1640 (Thermo Scientific Hyclone; Cat. No. SH30096.01). All cells were cultured in a 37º C humidified incubator with complete media (CM) prepared from

RPMI 1640, 10% heat-inactivated FBS, and 2 mM GlutaMAX supplement (Life Technologies, Grand Island, NY; Cat. No. 35050061).

DNA expression plasmids

Codon-optimized CD19RCD28mZ (CoOp)/pSBSO, [57] which codes for CAR between transposition sites mediated by SB transposase, [64] was used as the vector backbone for cloning of the following transgenes. The DNA plasmid 19G4CAR (also designated CD19RCD28, [65] **Figure 4A**) codes for a 2nd generation CD19-specific CAR containing a modified IgG4 exodomain, CD28 transmembrane, and CD28/CD3ζ endodomain. The synthesis of DNA plasmid GD2G4CAR (**Figure 4B**), specific to sphingolipid GD2, utilized the same 19G4CAR backbone. The GD2-specific scFv derived from murine mAb (clone 14G2a) [66] was commercially synthesized (Geneart, Life Technologies) as codon-optimized cDNA with *Nhe*I and *Xmn*I restriction enzyme (RE) sites flanking the scFv. The 19G4CAR plasmid backbone and GD2-specific scFv cDNA were excised using these REs and ligated to replace CD19-specific scFv with GD2-specific scFv. A DNA plasmid (**Figure 4C**) coding for a control CAR that contains no scFv region, designated G4CAR, but does contain an Igκ-FLAG peptide sequence (METDTLLLWVLLLWVPGSTGDYKDEGTS), was derived from 19G4CAR using primer-directed PCR amplification from the beginning of the IgG4 domain hinge (primer 5'GGTACCTCTGGGGGGCAGGGCCTGCATG3') to the terminus of the CD3□ domain (primer 5'GGGCCCAGCGCTGAGAGCAAGTACGGCCCTCCC3') and sequence verified. The G4CAR was ligated into the 19G4CAR backbone *Apa*I and *Kpn*I RE sites. DNA plasmid coding for a CD19-specific CAR with no IgG4 (**Figure 4D**), designated 19CAR, encodes from amino to

carboxyl termini a GM-CSF (amino acid 1-22; NP_758452.1), CD19-specific scFv (245 amino acids), CD8α extracellular domain and hinge (amino acids 136-203; NP_001759) followed by the same CD28 transmembrane and CD28 and CD3ζ domains as other CARs.[65] The full length of this transgene was synthesized by GeneArt, cut with *Cla*I and *SpeI* REs, and ligated into the 19G4CAR backbone replacing the 19G4CAR codon, which had been excised using *EcoRV* and *SpeI*. The scFv sequence of CARL was derived from the cDNA library of the 2D3 hybridoma.[57] This was achieved by extracting RNA from $5x10^6$ cells using the RNeasy Mini Kit (Qiagen, Gaithersburg, MD; Cat No. 74104) according to manufacturer's protocol. A cDNA library was generated by reverse transcription using oligo-dT primers per the protocol provided in the Superscript III First Strand kit (Invitrogen; Cat No. 18080-051). PCR (using Amplitaq Gold) was performed on the cDNA using the degenerate primers for the FR1 region [67] to amplify the mouse V_H and V_L regions. The V_H and V_L amplified products were ligated into the TOPO TA vector and sequenced. The CARL construct for surface expression on aAPC was composed of GM-CSF leader peptide (amino acid 1-22; NP_758452.1) fused to the 2D3-derived scFv, and tethered by CD8α (amino acid 136-182; NP_001759.3) to the transmembrane and intracellular portions of CD28 (amino acid 56-123; NP_001230006.1) followed by CD3ζ (amino acid. 48-163; NP_ 000725.1) intracellular domain. Design of all transgenes utilized Vector NTI Advance™ 11 software (Invitrogen). All transgenes were human codon optimized before synthesis at GeneArt. The CARL construct was excised and ligated into a SB expression plasmid, designated Zeo-2A-CARL (**Figure 4E**), to co-express a zeocin resistance gene linked via a modified T2A peptide sequence- (amino acids

Figure 4

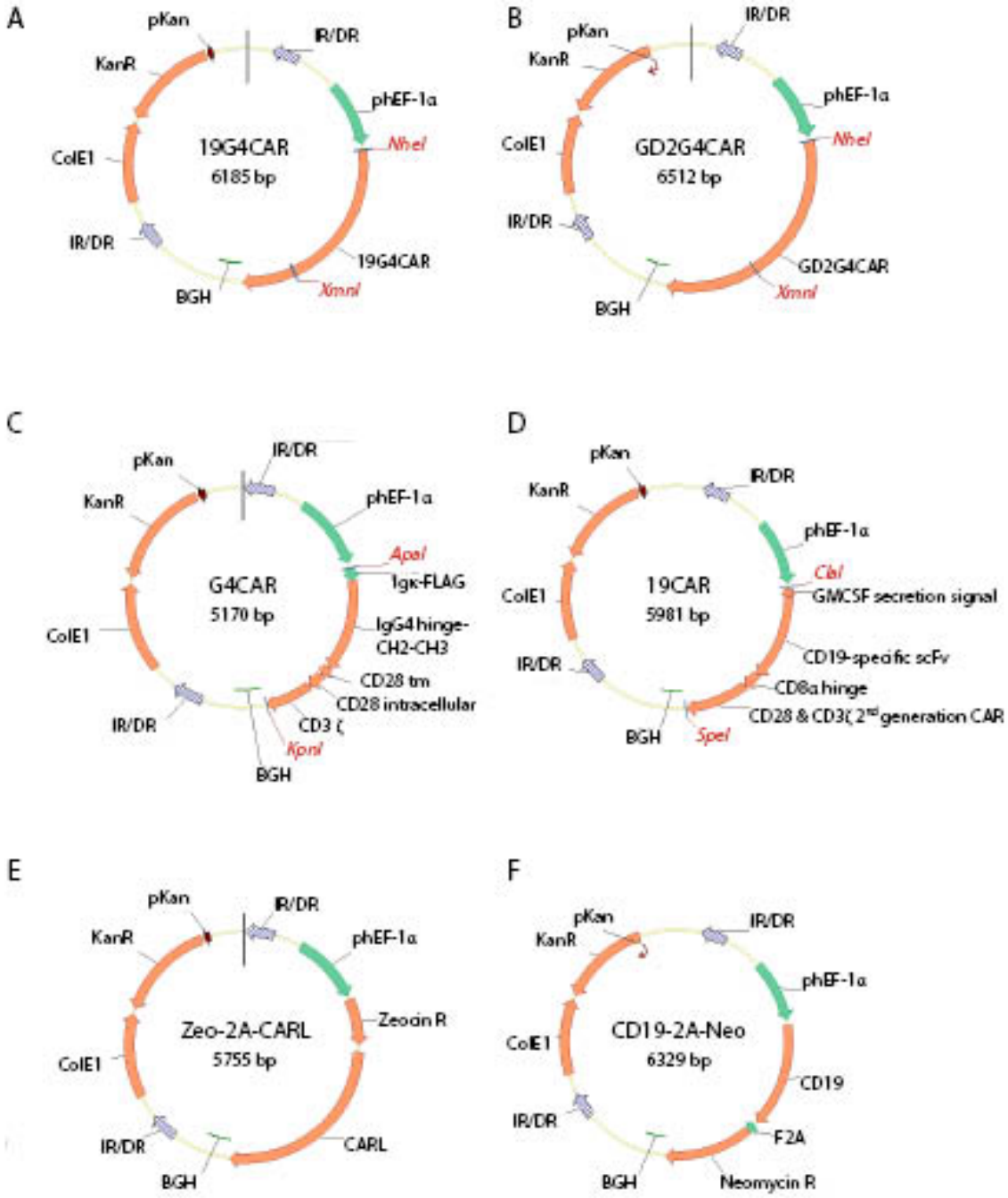

A
pKan
KanR
IR/DR
phEF-1α
NheI
19G4CAR
6185 bp
ColE1
IR/DR
19G4CAR
BGH
XmnI
B
pKan
KanR
IR/DR
phEF-1α
NheI
GD2G4CAR
6512 bp
ColE1
IR/DR
GD2G4CAR
BGH
XmnI
C
pKan
KanR
IR/DR
phEF-1α
ApaI
IgK-FLAG
G4CAR
5170 bp
IgG4 hinge-
CH2-CH3
ColE1
CD28 tm
CD28 intracellular
IR/DR
CD3 ζ
KpnI
BGH
D
pKan
KanR
IR/DR
phEF-1α
ClaI
GMCSF secretion signal
19CAR
5981 bp
ColE1
CD19-specific scFv
IR/DR
CD8α hinge
CD28 & CD3ζ 2nd generation CAR
BGH
SpeI
E
pKan
KanR
IR/DR
phEF-1α
Zeocin R
Zeo-2A-CARL
5755 bp
ColE1
IR/DR
CARL
BGH
F
pKan
KanR
IR/DR
phEF-1α
CD19-2A-Neo
6329 bp
ColE1
CD19
IR/DR
F2A
BGH
Neomycin R

Figure 4 Vector maps for expression of transgenes. Each DNA plasmid expresses a transgene of interest under promoter human Elongation Factor 1 alpha (phEF-1α), using the beta hemoglobin poly-adenylation signal (BGH) to terminate transcription. The indirect repeats / direct repeats (IR/DR) allow for transgene transposition into the genome using SB11. All plasmids were propagated in bacteria using the origin of replication ColE1 and Kanamycin resistance (KanR) under the promoter pKan. **A**) 19G4CAR demonstrates the original plasmid design used in these studies and shows *NheI* and *XmnI* restriction enzyme (RE) sites used to generate **B**) GD2G4CAR from PCR-directed truncation of CD19-specific scFv on 19G4CAR which led to the generation of **C**) G4CAR and final ligation using *ApaI* and *KpnI* REs. **D**) 19CAR was designed without an IgG4 exodomain, instead expressing the CD8α hinge and exodomain. **E)** Zeo-2A-CARL, expressing CARL, and **F**) CD19-2A-Neo, expressing truncated human CD19 (tCD19), were designed to express CARL or CD19 on aAPC under drug selection conditions.

ATGEGRGSLLTCGDVEEPGP). Truncated human CD19 was synthesized by GeneArt containing the extracellular and transmembrane portions of human CD19 (amino acid 1-313; NP_001171569.1). This gene was excised and ligated into SB DNA plasmid, designated CD19-2A-Neo (**Figure 4F**), to co-express with neomycin phosphotransferase linked via a modified F2A peptide sequence (amino acids $(G)_4S(G)_4$SVKQTLNFDLLKLAGDVESNPGP).

Artificial antigen presenting cells (aAPC)

$CARL^+$ and $CD19^+$ aAPC were derived by the independent electroporation of parental K562 cells with Zeo-2A-CARL or CD19-2A-Neo and SB11 transposase DNA plasmids using the Amaxa 2D nucleofector under program T-16 with Kit V (Lonza, Allendale, NJ; Cat No. VCA-1003). After 3 days incubation, each transfection was placed under drug selection in a 6-well plate using either 0.5 mg/mL Zeocin or 1 mg/mL G418 for Zeocin resistance or Neomycin resistance, respectively (Invivogen, San Diego, CA; Cat. No. ant-zn-1 and ant-gn-1). This was achieved by dispersing 10,000 cells with drug in 3 mL semi-solid Methocult H4230 media (StemCell Technologies, Vancouver, BC, Canada; Cat No. 04230). After 10 days visually-perceptible individual (clonal) colonies were transferred to individual flasks and grown in CM. Each clone was tested for uniform expression of CARL or CD19 using flow cytometry. Clones of $CARL^+$ (designated Zeo-2A-CARL MC5) and $CD19^+$ (designated CD19-2A-Neo MC2) aAPC were grown to large numbers, γ-irradiated at 100 cGy, and cryopreserved. Before freezing, the aAPC were routinely tested for the presence of transgenes, absence of mycoplasma, and absence of endotoxin.

Propagation of CAR$^+$ T cells (CART)

The designs of each CAR and antigen (CARL and CD19) as expressed on the respective T cell or aAPC are shown in **Figure 5A**. The propagation of CART is depicted in **Figure 5B.** Each CAR from **Figure 5A.II** was co-cultured with aAPC from **Figure 5A.I**. At the initiation of the experiment (defined as Day 0), thawed PBMC were washed twice, and maintained in CM for 3 to 4 hours before electroporation using the Amaxa 2D Nucleofector under program U-14 with human T cell Nucleofector kit (Lonza Biosciences; Cat No. VPA-1002). After resting overnight in CM, viable PBMC (counted by exclusion of 0.1% Trypan Blue) were resuspended in CM and mixed at a 1:2 ratio (mononuclear cell to γ-irradiated aAPC) using thawed aAPC that were washed twice and counted. The co-culture contained 10^6 total cells/ mL in CM and 50 IU/mL recombinant human IL-2 (Proleukin, Prometheus Labs, San Diego, CA). The live-cell counts were determined by Trypan Blue exclusion on Days 1, 7, 14, and 21 of co-culture. Flow cytometry for CD3, CD4, CD8, and human IgG (to assess CAR expression) occurred on Days 1, 7, 14, and 21, and for CD45RO, CD62L, and CD28 occurred on Days 14 and 21. Irradiated aAPC were re-added to co-cultures on Days 7 and 14 by re-stimulating mononuclear cells with γ-irradiated aAPC at 1:2 ratio. On Day 21 products of propagation were assessed for specific killing, and DNA and RNA were extracted. Each experiment was repeated at least 4 times using 5 donors.

Flow cytometry

We used a FACSCalibur (BD Biosciences, Billerica, MA) to acquire samples prepared in FACS staining solution as previously described.[68] After washing once in FACS staining solution, cells were stained for 30 minutes at 4º C without blocking in FACS staining solution containing a 1:33 dilution of antibody. When anti-human Fc antibody was used, the anti-Fc stained sample was washed and re-stained for alternative surface markers before re-suspension in FACS buffer for flow cytometer analysis. Measurement of intracellular cytokine used the same protocol for cell surface staining followed by 20 min. fixation using BD cytofix/cytoperm kit fixative (BD Biosciences; Cat No. 554714), followed by washing twice in perm/wash buffer containing 20% FBS and staining with a 1:33 dilution of antibody in perm/wash buffer. Antibody incubation lasted 30 minutes at 4ºC before samples were washed in perm/wash buffer and resuspended in FACS staining solution for acquisition. FlowJo v 10.0.5 (Tree Star Inc., Ashland, OR) was used for analysis of flow cytometry data. See **Table 2** for antibodies used.

Chromium release assay (CRA)

CRA was performed as previously described.[57] In brief, on Day 21 of T-cell co-culture on aAPC, the tumor targets (i) EL-4, (ii) NALM-6, and (iii) K562 were loaded with ^{51}Cr for 3 hours, and, after washing, co-cultured with effector T cells for 6 hours at 37º C using a ratio of 5 T cells to 1 target cell.

Abundance and diversity of TCR repertoire

The direct TCR expression assay (DTEA), as previously reported,[69] was used to measure the abundance of mRNA transcripts coding for 45 TCR α alleles, 46 TCR β alleles, 13 TCR γ alleles, and 5 TCR δ alleles from RNA obtained on Day 0 (T cells in PBMC before electroporation) and Day 21 (from T cells after electroporation/ propagation). Day 0 samples were negatively sorted for CD56 (Miltenyi Biotec, Cambridge, MA; Cat. No. 130-050-401) and then positively sorted for CD3 (Miltenyi Biotec; Cat. No. 130-050-101). The resulting $CD3^{+}CD56^{neg}$ T cells (2 to 3 x 10^6 from each sample) were snap frozen as were 2 x 10^6 cells directly harvested at Day 21 of co-culture. RNA was extracted from thawed samples using the ALLprep DNA/RNA mini kit (Qiagen; Cat. No. 80204).

Statistics

Statistical analysis was performed using Prism v6.0 (Graph Pad Software Inc.). Student's *t*-test (unpaired) was used to perform two sample comparisons. One- or two-way ANOVA F–test was used to perform group comparisons, and if found significant ($p < 0.05$); a *t*-test (unpaired) was undertaken to assess and report differences. Spearman's nonparametric correlation was performed on housekeeping gene normalized DTEA transcript counts to assess the divergence of the TCR repertoire in T cells from an experimental group and autologous Day 0 PBMC. If the Spearman correlation coefficient was greater than or equal to 0.8 ($\rho \geq 0.8$) within the 95% confidence interval of the correlation coefficient, then the two TCR repertoires were considered to be highly correlated.

Results:

Figure 5

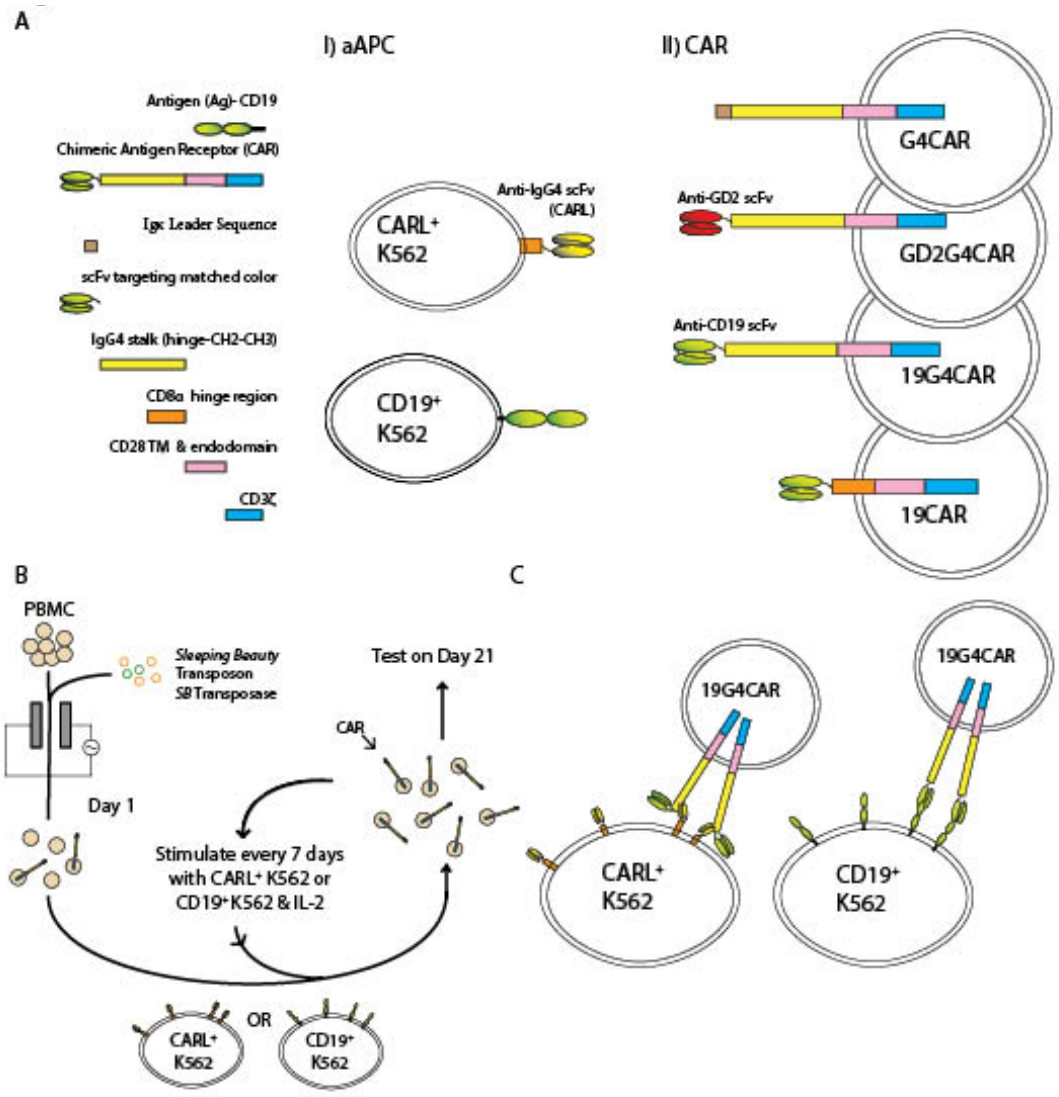

A
I) aAPC
II) CAR
Antigen (Ag)- CD19
Chimeric Antigen Receptor (CAR)
Igκ Leader Sequence
scFv targeting matched color
IgG4 stalk (hinge-CH2-CH3)
CD8α hinge region
CD28TM & endodomain
CD3ζ
Anti-IgG4 scFv (CARL)
CARL+ K562
CD19+ K562
G4CAR
Anti-GD2 scFv
GD2G4CAR
Anti-CD19 scFv
19G4CAR
19CAR
B
PBMC
Sleeping Beauty Transposon
SB Transposase
Day 1
Stimulate every 7 days with CARL+ K562 or CD19+K562 & IL-2
Test on Day 21
CAR
OR
CARL+ K562
CD19+ K562
C
19G4CAR
19G4CAR
CARL+ K562
CD19+ K562

Figure 5: Study design to compare ability of chimeric antigen receptor (CAR) ligand (CARL) versus CD19 TAA on K562 cells for the selective propagation of CAR^+ T cells (CART). **A)** Artificial antigen presenting cells (aAPC) demonstrated in **I)** were derived from parental K562 cells following transgene transfer, stable integration, and clonal selection. Each aAPC clone expresses either CARL, a scFv derived from 2D3 mAb that binds IgG4 exodomain of CAR, or truncated human CD19. **II)** CART used to evaluate specificity towards CARL or CD19 are shown. SB-derived DNA plasmids coding for a panel of CARs were individually electro-transferred into PBMC and recursively stimulated with $CD19^+$ K562 or $CARL^+$ K562 in the presence of soluble recombinant human IL-2. Each CAR follows a modular design. 19G4CAR contains the IgG4 scaffold and targets CD19 through the same scFv as 19CAR which lacks IgG4 scaffold and instead uses CD8α hinge and extracellular domain. GD2G4CAR contains the IgG4 scaffold and targets GD2. G4CAR contains the IgG4 scaffold, but has no scFv. All CARs employ of a 2^{nd} generation design containing CD28 and CD3□ signaling endodomains. **B)** On Day 0, synchronous electroporation of PBMC was undertaken with DNA plasmid coding for SB transposase (SB11- green) and SB DNA plasmids coding for CAR species (orange). To achieve outgrowth of T cells stably expressing CARs, the genetically modified cells were co-cultured, beginning on Day 1, upon γ-irradiated $CD19^+$ or $CARL^+$ K562 in the presence of 50 IU/mL IL-2. Cytokine was added with stimulation or during media change. Re-stimulation of CAR with aAPC occurred every 7 days until Day 21. **C)** Diagram of docking between $CARL^+$ K562 cells and $19G4CAR^+$ T cells as compared with $CD19^+$ K562 cells with $19G4CAR^+$ T cells.

Table 2 Fluorochrome-conjugated antibodies used for flow cytometry.

Antibody	Vendor	Catalogue number
mouse anti-human Fc-PE*	Invitrogen	Cat. No. H10104
CD3-FITC	BD	Cat. No. 349201
CD4-APC	BD	Cat. No. 340443
CD8-PerCP-Cy5.5	BD	Cat. No. 341051
CD28-PerCP-Cy5.5	BD	Cat. No. 337181
CD45RO-APC	BD	Cat. No. 559865
CD62L-PE	BD	Cat. No. 555544
IL-2-APC	BD	Cat. No. 554567
IL-4-PE	BD	Cat. No. 340451
Ifn-γ-FITC	BD	Cat. No. 554700
goat anti-mouse Fab-FITC**	Jackson Immunoresearch	Cat. No. 555415
CD19-APC	BD	Cat. No. 555415

*Used to detect CAR containing IgG4 exodomain; ** Used to detect CARL

Numeric expansion of CAR$^+$ T cells upon K562 cells expressing CARL or CD19

Mouse mAb clone 2D3 was obtained by repeated footpad injections of NSO cells expressing 19G4CAR into BALB/c mice and blocking studies defined the specificity of the mAb to the human IgG4 exodomain of 19G4CAR.[57] We hypothesized that this mAb may be used to cross-link CAR and activate genetically modified T cells for sustained proliferation. Therefore, the scFv of 2D3 (designated CARL) was expressed on the cell surface to compare with human truncated CD19 TAA on K562 cells. The CARL and CD19 transgenes were cloned into DNA plasmids for co-expression with drug-selection genes between SB transposable elements. The SB transposon DNA plasmids for 2D3-derived scFv or CD19 were electro-transferred with SB11 transposase DNA plasmid into K562 cells in separate experiments. Genetically modified cells were propagated under drug selection from a single cell for homogeneous expression of CARL (as detected by antibody against mouse Fab) or CD19 (**Figure 6A)**. A comparison of the γ-irradiated K562-derived aAPC to selectively propagate CART was undertaken following electroporation (defined as Day 0) of the panel of CAR constructs (**Figure 5A.II)** into PBMC using SB system. On Day 1, initial expression of CAR in T cells was evaluated by flow cytometry using antibody specific for human Fc (**Figure 6B)**. The expression of CARs and number of total viable T cells were measured weekly for 21 days of co-culture with CD19$^+$ K562 or CARL$^+$ K562 with the following immunoreceptors on T cells; (i) 19G4CAR with specificity for CD19 and containing the IgG4 exodomain, (ii) 19CAR with specificity for CD19 and absence of IgG4 exodomain, (iii) G4CAR without scFv, but containing an IgG4 exodomain, and (iv) GD2G4CAR with specificity for GD2 and containing the IgG4 exodomain. All CAR species

Figure 6

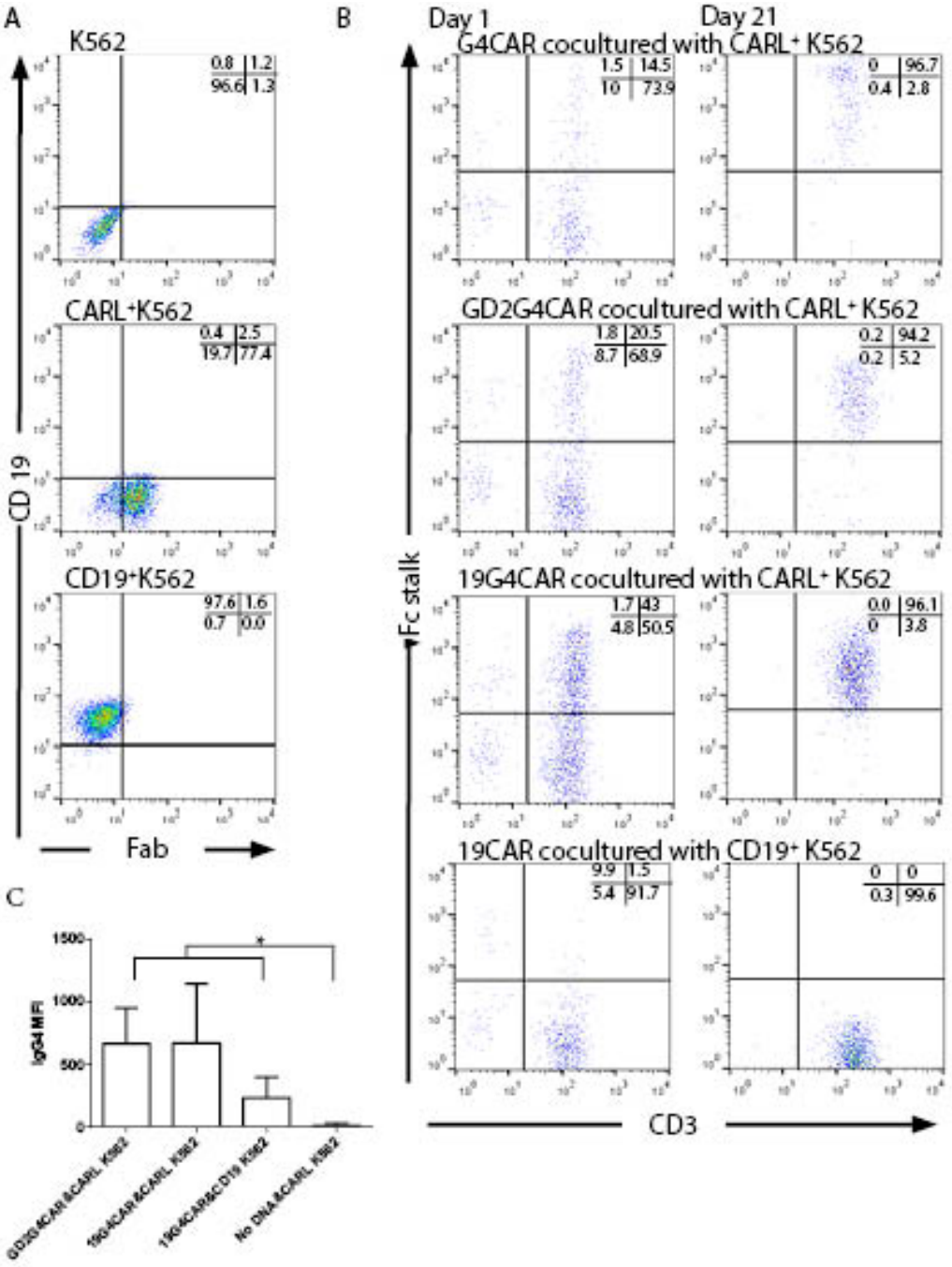

A
K562
0.8 1.2
96.6 1.3
CARL+K562
0.4 2.5
19.7 77.4
CD19+K562
97.6 1.6
0.7 0.0
CD 19
Fab
B
Day 1
Day 21
G4CAR cocultured with CARL+ K562
1.5 14.5
10 73.9
0 96.7
0.4 2.8
GD2G4CAR cocultured with CARL+ K562
1.8 20.5
8.7 68.9
0.2 94.2
0.2 5.2
19G4CAR cocultured with CARL+ K562
1.7 43
4.8 50.5
0.0 96.1
0 3.8
19CAR cocultured with CD19+ K562
9.9 1.5
5.4 91.7
0 0
0.3 99.6
Fc stalk
CD3
C
IgG4 MFI
1500
1000
500
0
*
GD2G4CAR &CARL K562
19G4CAR &CARL K562
19G4CAR&CD19 K562
No DNA &CARL K562

Figure 6: Characterization of aAPC and CAR$^+$ T cells. **A)** CD19 and CARL as SB transposons were integrated into parental K562 cells using SB11 transposase and clonally expanded for homogeneous expression of CD19 or CARL. Dot plots depict the expression of CD19 and CARL on parental K562 and derived clones. The stable expression of CARL is shown using antibody that detects mouse Fab. **B)** The expression level of CAR species as determined by flow cytometry is shown on Days 1 and 21 of co-culture with aAPC. Expression of chimeric IgG4 revealed CAR expression in all constructs except 19CAR which was determined using an antibody against human Fc. The percentage of cells in each flow plot quadrant is provided as an inset. **C)** The effect of aAPC design on abundance of CAR expression was assessed on Day 21 by measuring mean fluorescent intensity (MFI) of IgG4 signal by flow cytometry. The experiments are designated [CAR & aAPC] with unmodified mock electroporated T cells (No DNA plasmid) used as a control. Each experimental group contained 4 or 5 separate donor-derived PBMC. Statistical comparison was undertaken by One-way ANOVA followed by unpaired *t*-tests between each experiment (* = $p < 0.05$).

contained the same transmembrane and intracellular domains (CD28/CD3ζ) as the 2nd generation 19G4CAR.[57] The co-cultures of CART with the two types of aAPC were found to have significantly different amounts of T cells by Day 21 depending on the choice of aAPC ($p < 0.05$) using two-way ANOVA followed by un-paired *t*-tests. The $19CAR^+$ T cells proliferated upon co-culture with $CD19^+$ K562 and $G4CAR^+$ T cells proliferated upon co-culture with $CARL^+$ K562 in an exponential fashion, whereas 19CAR on $CARL^+$ K562 and G4CAR on $CD19^+$ K562 did not numerically expand (**Figure 7A** top panel).

These data indicate that, as expected, the CD19 TAA on aAPC selectively supports the outgrowth of CD19-specific CART. Furthermore, they demonstrate that CARL can activate T cells to proliferate that contain a CAR species with an IgG4 exodomain. Next, the ability of $CD19^+$ K562 and $CARL^+$ K562 were assessed for ability to sustain the proliferation of T cells expressing 19G4CAR to evaluate how two modes of crosslinking (**Figure 5C**) can activate T cells. There were no significant differences in the accumulated number of viable T cells on Day 21 of co-culture based on the type of aAPC used (**Figure 7A** bottom panel), the expression of CAR as a percent of the population (**Figure 7B** bottom panel), or the mean fluorescence intensity (MFI). A trend ($p = 0.09$) towards a difference in MFI of CAR expression resulting from aAPC employed to expand 19G4CAR was noted (**Figure 6C)**. There were no significant differences (**Figure 7C & D**) between the two aAPC types for propagating $19G4CAR^+$ T cells co-expressing cell-surface proteins associated with memory phenotype ($p = 0.82$),[70] or other co-receptors ($p = 0.26$), as well as the specific lysis by $19G4CAR^+$ T cells ($p = 0.16$). Therefore,

Figure 7

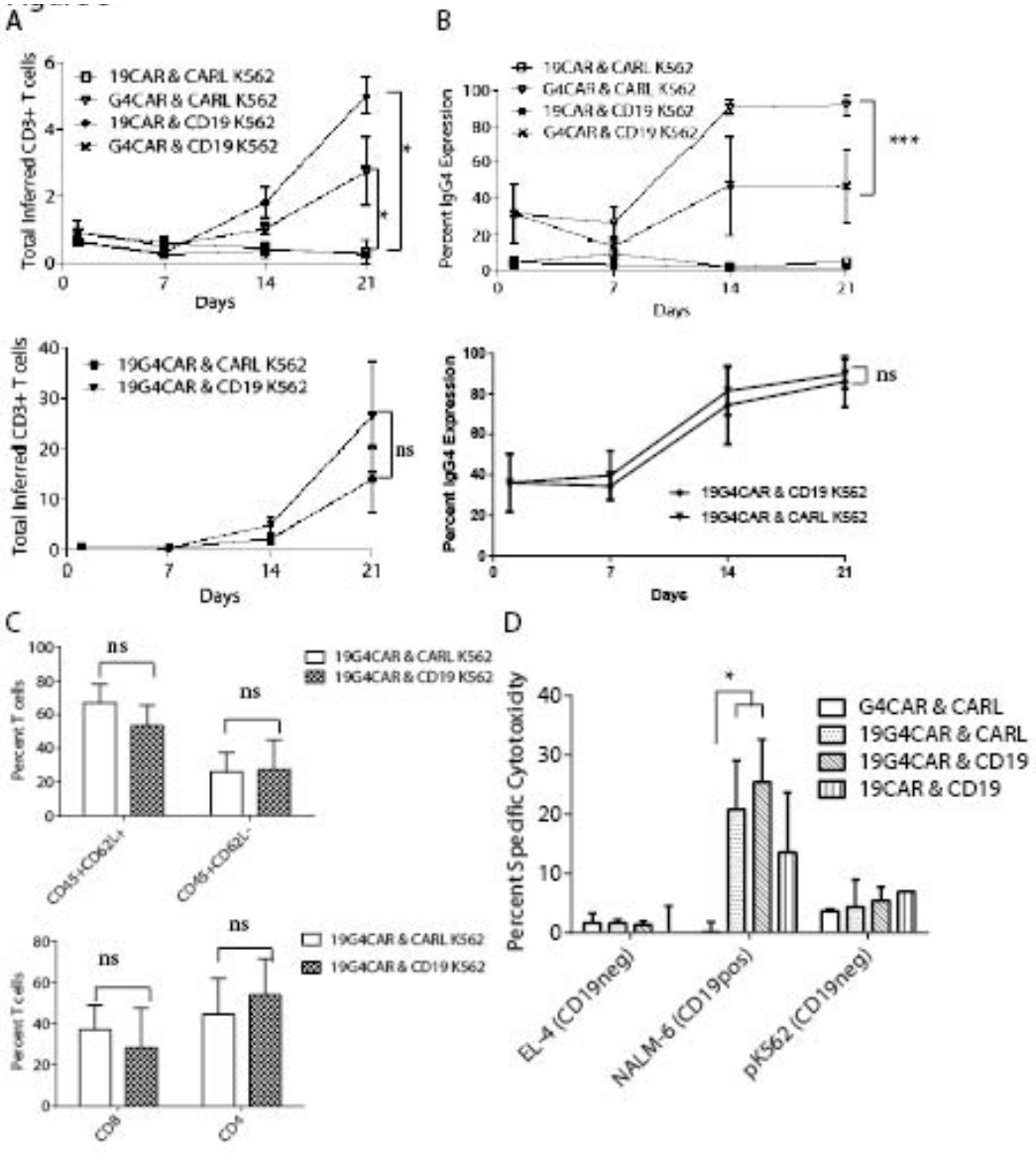

A
19CAR & CARL K562
G4CAR & CARL K562
19CAR & CD19 K562
G4CAR & CD19 K562
Total Inferred CD3+ T cells
Days
B
Percent IgG4 Expression
***
19G4CAR & CARL K562
19G4CAR & CD19 K562
ns
C
Percent T cells
CD45+CD62L+
CD45+CD62L-
CD8
CD4
D
Percent Specific Cytotoxicity
G4CAR & CARL
19G4CAR & CARL
19G4CAR & CD19
19CAR & CD19
EL-4 (CD19neg)
NALM-6 (CD19pos)
pK562 (CD19neg)

Figure 7: Comparison of CAR$^+$ T cells propagated on CD19$^+$ or CARL$^+$ aAPC. **A)** Total inferred T-cell number and **B)** CAR (IgG4) expression for each CART was measured every 7 days for 5 donors. Top panel: 19CAR$^+$ or G4CAR$^+$ T cells were numerically expanded on either CD19$^+$ or CARL$^+$ aAPC. Bottom panel: 19G4CAR$^+$ T cells were propagated on either CD19$^+$ or CARL$^+$ aAPC. **C)** After 21 days of co-culture on CD19$^+$ or CARL$^+$ aAPC, 19G4CAR$^+$ T cells from 5 donors were assessed for expression of markers associated with memory (top panel) or T cell co-receptors (bottom panel). **D)** Specific killing by electroporated/propagated T cells expressing 19CAR, G4CAR, and 19G4CAR, by CRA at a ratio of 5 effectors to 1 target. The tumor targets were EL-4 (murine thymoma- GD2$^+$, CD19^{neg}), NALM-6 (human B cell ALL- GD2^{neg}, CD19$^+$), and K562 (a human CML- GD2^{neg}, CD19^{neg}). Up to 5 donors were tested in 4 independent experiments. ns- No significance, * = $p<0.05$, *** = $p<0.001$

CD19-specific CAR$^+$ T cells can be propagated in similar quantity and quality by K562-derived aAPC expressing CAR or CD19.

CARL$^+$ K562 can numerically expand CAR+ T cells independent of specificity

The 2D3-derived scFv on aAPC was evaluated for ability to propagate not just CD19-specific T cells, but CAR$^+$ T cells of alternative specificities. The CD19 and GD2 [71] TAAs are not present on parental K562 cells to propagate T cells expressing GD2G4CAR, 19G4CAR, and G4CAR. Nonetheless, T cells bearing these three CARs numerically expanded on CARL$^+$ K562. The number of total viable T cells on Day 21 of co-culture with CARL$^+$ K562 cells did not significantly differ between 19G4CAR, G4CAR, and GD2G4CAR ($p = 0.16$, **Figure 8A**). Similarly, the percentage of each CAR expressed on T cells at Day 21 did not significantly differ among the three populations of genetically modified T cells ($p = 0.68$, **Figure 8B**). Finally, the electroporated and propagated T cells exhibited specific lysis of CD19 and GD2 TAAs recognized by CD19-specific and GD2-specific CARs. EL-4 cells, previously reported to express GD2, [66] were specifically killed by GD2G4CAR$^+$ T cells and not with T cells expressing G4CAR or 19G4CAR. As anticipated, CD19$^+$ NALM-6 cells were targeted by T cells expressing 19G4CAR (**Figure 8C**). In summary, genetically modified T cells can be selectively propagated by CARL$^+$ K562 cells resulting in T cells that retain specificity for TAA and stable expression of CAR.

Figure 8

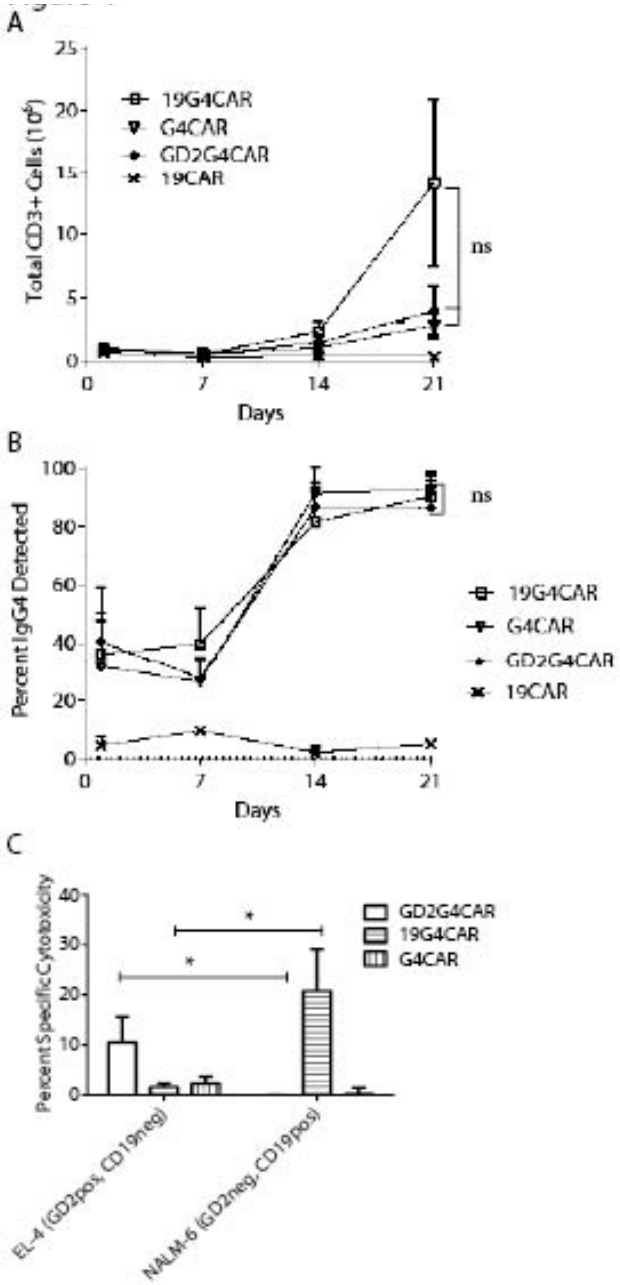

A
Total CD3+ Cells (10^6)
25
20
15
10
5
0
19G4CAR
G4CAR
GD2G4CAR
19CAR
ns
0
7
14
21
Days
B
Percent IgG4 Detected
100
80
60
40
20
0
ns
19G4CAR
G4CAR
GD2G4CAR
19CAR
0
7
14
21
Days
C
Percent Specific Cytotoxicity
40
30
20
10
0
*
*
GD2G4CAR
19G4CAR
G4CAR
EL-4 (GD2pos, CD19neg)
NALM-6 (GD2neg, CD19pos)

Figure 8: Numeric expansion of CAR^+ T cells using $CARL^+$ aAPC. **A)** Total inferred T-cell number and **B)** CAR (IgG4) expression for each CART was measured every 7 days from 4 to 5 donors for 21 days of co-culture on aAPC. The differences between Day 21 total T-cell number and percent CAR expression was assessed using One-way ANOVA. **C)** The specific killing by panel of T cells expressing GD2G4CAR, 19G4CAR, and G4CAR, were tested using CRA at a ratio of 5 effectors to 1 target. The targets were EL-4 ($GD2^+$, $CD19^{neg}$), NALM-6 ($GD2^{neg}$, $CD19^+$), and parental K562 ($GD2^{neg}$, $CD19^{neg}$). Two-way ANOVA followed by unpaired *t*-tests was performed for 4 to 5 donors tested in 4 independent tests on Day 21 of co-culture on aAPC. ns- No significance, * = $p<0.05$.

The choice of aAPC does not skew the TCR repertoire for numerically expanded CART

Each T cell in peripheral blood bears a distinct pair of αβ or γδ TCRs which can be analyzed using the direct TCR expression assay (DTEA) to determine the abundance of TCR chains. This assay was employed to determine whether CARL$^+$ or CD19$^+$ K562 influenced the distribution of TCR alleles after 21 days co-culture on aAPC. TCR variants were assayed on the nCounter Analysis System using a set of 111 TCR α, β, γ, and δ transcripts.[68, 69] By measuring the distribution of TCR alleles we could determine if the aAPC design preferentially supported the numeric expansion of some, but not all genetically modified T cells. The starting TCR distribution of T cells on Day 0 was ranked from the most to least frequent TCR usage and the rank order compared for T cells harvested on Day 21 of co-culture with aAPC (**Figure 9A)**. This revealed no apparent monoclonal or oligoclonal outgrowth of electroporated T cells propagated on CARL$^+$ or CD19$^+$ K562 cells. The ranks of TCR frequencies on Day 0 and Day 21 from each experiment were compared using Spearman's rank correlation test and found to significantly correlate ($p < 0.0001$; **Table 3)**. The statistical comparison of TCR abundance and type from Day 0 and 21 indicated that all correlation coefficients (ρ) had values greater than 0.8 within the 95% confidence interval of ρ which is consistent with a strong correlation, indicating no change in TCR frequency. The measurement of TCR abundance demonstrates that CARL or TAA on aAPC do not skew the outgrowth of sub-populations of propagated T cells, but rather that both 2D3-derived scFv and CD19 on K562 cells can sustain the outgrowth of CAR$^+$ T cells that maintain a polyclonal repertoire.

Figure 9

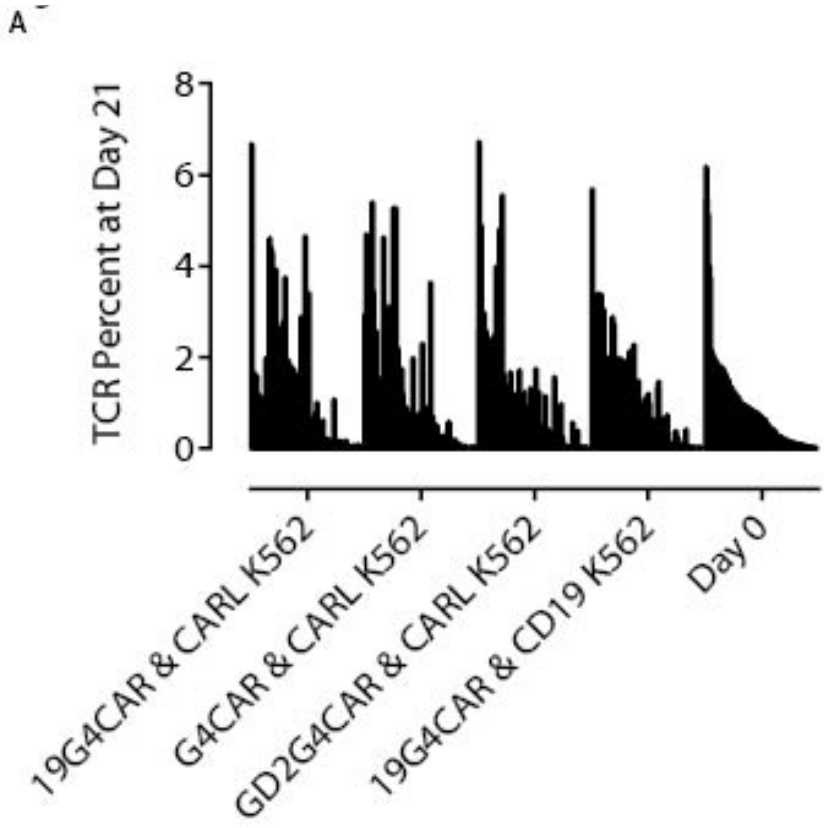


Figure 9: Comparison of TCR repertoire changes induced by CAR-mediated expansion on aAPC. **A)** TCR repertoire was measured for 111 TCR α, β, γ, and δ alleles using DTEA. [69] TCR abundance was organized from the most to the least frequently occurring transcripts based on sorted $CD3^{+}CD56^{neg}$ cells from Day 0. The set is visually represented next to TCR repertoire expressed by T cells at Day 21 of co-culture on $CARL^{+}$ K562 cells and $CD19^{+}$ K562 cells. Analysis was performed on 2 donors and a representative plot of one donor is shown.

Table 3 Comparison of TCR abundance harvested from T cells before versus after propagation on aAPC*

Day		0	21			
			19G4CAR & CARL$^+$ K562	G4CAR & CARL$^+$ K562	GD2G4CAR & CARL$^+$ K562	19G4CAR & CD19$^+$ K562
0			0.748 (0.65-0.82)	0.857 (0.80-0.90)	0.867 (0.81-0.91)	0.912 (0.87-0.94)
21	19G4CAR & CARL$^+$ K562	0.752 (0.65-0.83)		0.805 (0.72-0.86)	0.706 (0.59-0.79)	0.734 (0.63-0.81)
	G4CAR & CARL$^+$ K562	0.899 (0.85-0.93)	0.71 (0.61-0.79)		0.816 (0.74-0.87)	0.839 (0.77-0.89)
	GD2G4CAR & CARL$^+$ K562	0.825 (0.75-0.88)	0.72 (0.61-0.80)	0.801 (0.72-0.86)		0.881 (0.83-0.92)
	19G4CAR & CD19$^+$ K562	0.916 (0.87-0.94)	0.69 (0.57-0.78)	0.887 (0.84-0.92)	0.808 (0.73-0.87)	

*Analysis DTEA data from two donors was normalized using housekeeping genes and assessed using Spearman correlation coefficient to compare distributions of TCR usage for two donors. The upper right of the table contains the correlation between experimental groups for one donor. The lower left of the table contains the second donor subjected to the same analysis. Each cell in the table contains the Spearman correlation coefficient (ρ) and within the brackets the 95% confidence interval. (A strong correlation is considered to be $\rho \geq 0.8$.)

Discussion

This study demonstrates that a ligand directed against a conserved extracellular domain on CARs can function to numerically expand CART while preserving redirected specificity of genetically modified T cells for TAA. This differs from other methods to select or sort for CART such as magnetic sorting, [37] selection with cytotoxic drug, [56] or TAA-mediated numeric expansion. A recent report demonstrated antigen-independent CAR-mediated T-cell activation using antibody binding to an extracellular Myc-tag of ErbB2-specific T cells.[72] Our study differs as CARL recognizes a determinant native to an extracellular scaffold to induce proliferation of CART. This provides an apparent advantage, as the use of epitope tags may alter antigen recognition or increase immunogenicity. Our data demonstrate that a mAb-derived scFv sequence directed against conserved extracellular CAR domains can be used for cross-linking, activation, and propagation of CAR species on genetically modified T cells.[50-52] Thus, CARL-mediated numeric expansion of CART will be useful to laboratories seeking to augment the selective outgrowth of CART within a tissue culture environment after gene transfer.

A benefit of our approach is that one CARL design could functionally substitutes for multiple TAAs. Specifically, the CARL in this report enables K562 cells to function as aAPC to propagate T cells expressing a panel of CARs to specifically lyse tumor cells expressing multiple TAAs. Alternatively, our technology allows for CARs to be designed which impart no specificity. This was demonstrated here as a proof-of-concept with G4CAR activating T cells to proliferate during co-culture with $CARL^{+}$ K562 cells without ligating endogenous CD3. Implicit in

this finding is that any T cell bearing an introduced CAR, or other immunoreceptor containing the CARL-binding domain, may be propagated upon cross-linking by CARL.

Recent studies have demonstrated that reducing the length of an IgG4 exodomain improved cytokine secretion, cytotoxicity, and proliferation of ROR1-specific CART [73] and removal of IgG1 scaffold from a CAR appeared to improve killing of $CD22^+$ targets.[74] These improvements in potency support modifying the scFv distance from the T-cell membrane to enable a candidate CAR design to provide a fully-competent T-cell activation signal. The identification of the peptide recognized by 2D3-derived scFv is ongoing, and may enable us to alter the length of the extracellular domain to tune CAR^+ T cells for optimal activation by TAA while preserving the ability of CARL to propagate genetically modified T cells.

One measure of redirected specificity is the ability of CAR to mediate T-cell killing of TAA^+ targets. The cytotoxicity of CARL-propagated CART appears to be moderately reduced based on prior publications.[64, 75] This may be accounted by the design of the $CARL^+$ and $CD19^+$ aAPC, which were not engineered to express costimulatory molecules such as CD86, CD137L and membrane-bound IL-15, as are present on aAPC (designated clone 4) we previously used to generate CD19-specific 19G4CAR^+ T cells.[49] Furthermore, we used aAPC clone 4 in the presence of soluble recombinant IL-2 and IL-21 whereas $CARL^+$ K562 cells were co-cultured with genetically modified T cells with only IL-2. Future studies will help elucidate the effect of costimulation on CART performance.

It is possible that the aAPC used to activate CAR may selectively propagate a sub-population of genetically modified T cells over the co-culture period. However, we observed

that both CARL$^+$ and CD19$^+$ K562 cells numerically expanded T cells bearing a similar percentage expression and density of CAR, a comparable immunophenotype. In addition, there were no significant differences in TCR repertoire expression and abundance before versus after propagation on aAPC indicating that the starting population of T cells matched the population present at the end of the co-culture period. These findings justify investigating whether CARL$^+$ aAPC might be used to generate CART for human application. Furthermore, it is our expectation that expression of CARL on a single source of clinical-grade aAPC can be used to generate panels of CAR$^+$ T cells, overcoming the current need to produce panels of aAPC with each expressing a given TAA for a given specificity of CART.

In summary, we report the development of an aAPC based on a CAR-specific mAb for the CAR-mediated propagation of CAR$^+$ T cells with multiple specificities.

CHAPTER 3: Establishing anti-thymidine resistance in T cells

INTRODUCTION

Certain chemotherapies used to treat cancer are able to activate the immune system. One of the ways chemotherapy does this is by causing the cancer to die in a way that activates the immune system. This is called immunogenic cell death.[76] Integrating immunogenic cell death with newer forms of immunotherapy, such as adoptive transfer of T cells, could potentially improve patient outcomes by improving the immunologic response of adoptively transferred T cells towards cancer.[77, 78] However, the toxicity and immunosuppressive nature of many of these chemotherapies prevents the concurrent use of chemotherapy with adoptive transfer of T cells. To utilize the immunogenicity of chemotherapy without affecting the efficacy of adoptively transferred T cells there are two strategies: 1) adoptively transfer T cells following the clearance of each dose of chemotherapy, or 2) adoptively transfer T cells genetically-modified to resist chemotherapy-induced toxicity before the administration of multiple rounds of chemotherapy.

Genetically-modifying T cells to resist toxicity from chemotherapy appears to be a desirable strategy, but has presented a technological challenge to the field. The depletion of T cells by chemotherapy is most apparent in the aftermath of allogeneic hematopoietic stem cell transplant (HSCT) where the conditioning chemotherapy and subsequent immunosuppressive chemotherapy, used to prevent graft versus host disease, prevents both anti-tumor[22] and anti-viral[27] immunity mediated by T cells. Various groups have attempted to address this issue by knocking down [79] or inserting a gene which confers resistance [80] to the commonly used

immunosuppressive drugs tacrolimus and cyclosporine. Genetic-modification of T cells to resist other immunosuppressive drugs have followed including temsirolimus, [81] mycophenolate mofetil, [46] and immunosuppressive doses of methotrexate (MTX).[82] These modifications have been made using mutated human proteins (muteins) resistant to higher doses of the toxic chemotherapy. Unfortunately, only one mutein has been described to resist a chemotherapeutic agent typically used outside of the HSCT setting. That is resistance to temozolomide for concurrent use with T cell therapy in the treatment of brain cancer.[83]

Muteins resistant to chemotherapeutics targeting the most common cancers - lung, breast, colon, and pancreas,[84] – are lacking. In seeking a mutein for these cancers we considered leading candidate drugs which were non-genotoxic. Hence, the chemotherapy resistant T cell would receive no genotoxic insult and would not be at an increased susceptibility for malignant transformation. Anti-thymidylates (AThys) appeared to be the optimal candidate as the AThy 5-fluorouracil (5-FU) is used to treat breast, colon, and pancreatic cancer, [85] and lung cancer is treated with the AThy pemetrexed (Pem).[86] All AThys inhibit the synthesis of thymidine from uridine by blocking the activity of thymidylate synthase (TYMS) and/ or co-enzyme dihydrofolate reductase (DHFR) (See **Figure 10A**). The inhibited synthesis of thymidine prevents DNA synthesis, and ultimately leads to death in rapidly replicating cells such as activated T cells or cancer cells.[85, 86] Thus, AThy resistance (AThyR) conferred by a mutein TYMS and DHFR should permit resistance to 5-FU and Pem, and this was shown in murine bone marrow cells.[87, 88] Here, we propose AThyR is feasible in human T cells using a mutein of DHFR resistant to MTX - $DHFR^{FS}$, [89] in combination with a mutein of

TYMS. The human mutein of TYMS - $TYMS^{SS}$ – has not yet been described outside of the bacterial system in which it was developed.[90]

To test the ability of $DHFR^{FS}$ and $TYMS^{SS}$ to resist toxic levels of AThys, selective outgrowth assays were developed where AThyR+ T cells co-expressed fluorescent proteins that were used to track improved survival by flow cytometry. We find that combining $DHFR^{FS}$ with $TYMS^{SS}$ confers survival advantages to AThyR+ T cells in toxic concentrations of MTX, 5-FU, and Pem, as expected. It is noted that $TYMS^{SS}$ also enhances the survival advantage of $DHFR^{FS}$ to MTX, likely through improved resistance of $TYMS^{SS}$ towards the anti-TYMS action of MTX.[86] Furthermore, we discover that a known biochemical phenomenon in which TYMS auto-regulates expression of TYMS[91] and DHFR auto-regulates expression of DHFR[92] is co-opted by $TYMS^{SS}$ and $DHFR^{FS}$. Our findings show that $DHFR^{FS}$ and its *cis*-expressed fluorescent protein are up-regulated by increasing doses of MTX and that TYMS *cis*-expressed fluorescence proteins are down-regulated at the same concentration of MTX. These findings further elucidate unknown aspects of the auto-regulatory biochemical pathway and demonstrate that AThyRs $DHFR^{FS}$ and $TYMS^{SS}$ can be used to increase or decrease the expression of *cis* transgene in a drug dependent manner. The ability of AThyRs to enhance survival in the presence of chemotherapy and inducibly change *cis* transgene makes these transgenes desirable for selecting intracellular transgenes, such as suicide genes. Thus, $DHFR^{FS}$ was used to select for the suicide gene inducible caspase 9 (iC9) [35, 93] *in vitro* as a demonstration of the utility of AThyRs in future studies involving adoptive T cell transfer.

MATERIALS AND METHODS:

Cells and culture conditions:

Cells: Peripheral blood mononuclear cells (PBMC) derived from healthy donors at the Gulf Coast Regional Blood Bank or MDACC Blood Bank, both in Houston, Texas, was subjected to density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway Township, NJ; Cat No. 17-1440-02). PBMC were washed once in CliniMACS Plus PBS/EDTA buffer (Miltenyi Biotec, Gladbach, Germany, Cat. No. 130-070-525) and twice in Dulbecco's PBS (D-PBS) (Sigma-Aldrich, St. Louis, Missouri, Cat. No. D8537) before resting in complete media (CM) made of RPMI 1640 (Thermo Scientific Hyclone, Bridgewater, NJ; Cat. No. SH30096.01), 10% heat-inactivated fetal bovine serum (FBS -Thermo Scientific Hyclone, Cat. No. SH30070.03), and 2 mM GlutaMAX supplement (Life Technologies, Grand Island, NY; Cat. No. 35050061). Alternatively, PBMC were frozen using a prepared mixture of 50% heat-inactivated FBS, 40% RPMI 1640, and 10% DMSO (Sigma-Aldrich, PA; Cat. No. D2650) - freeze media (FM) at 4 X 10^7 cells/ mL. The use of rested or frozen PBMC is outlined in each experiment. The Jurkat cell line, a human T cell acute lymphoblastic leukemia (American Type Culture Collection, Manassas, VA, Cat. No. TIB-152) was used and maintained in CM. The identity of this cell line was assured by short tandem repeat DNA fingerprinting performed by MDACC Cancer Center Support Grant Characterized Cell Line Core. Activating and propagating cells (AaPC) were used to stimulate T cells. The AaPC cell line K562 clone.4, expressing CD86, CD137, CD64, along with membrane bound IL-15, was modified to present OKT3 antibody for the polyclonal stimulation of T cells, as previously described.[94] For the propagation of chimeric antigen receptor $(CAR)^+$ T cells, the AaPC $CARL^+$ K562[95] was utilized.

All AaPC were rapidly thawed in a 37º C water bath and washed twice before stimulation of T cells.[94] Jurkat and AaPC were tested for the presence of mycoplasma before use. Cell counting was accomplished in a mixture of 0.1 % Trypan Blue (Sigma-Aldrich, T8154) with the Cellometer K2 Image Cyotmeter (Nexcelom, Lawrence, MA).

Chemical and biological agents:

Stimulation via CD3 and CD28 was achieved by the addition of 30 ng/mL OKT3 antibody (eBioscience, San Diego, CA, Cat. No. 16-0037-85), 100 ng/ mL anti-CD28 antibody (EMD Millipore, Temecula, CA, Cat. No. CBL517). T cell stimulation included recombinant human IL-2 (Proleukin, Prometheus Labs, San Diego, CA). When indicated, the following drugs were used: 5-FU, MTX, pemetrexed, raltitrexed, G418, and AP20187. Further information regarding each drug is given in **Table 4**.

DNA expression plasmids:

DNA plasmids for testing AThyR transgenes were generated using the previously described DNA plasmid G4CAR as a backbone.[95] Commercially synthesized FLAG-DHFR^{FS}, codon optimized (CoOp) DHFR^{FS}, FLAG-TYMS^{SS}, and CoOp TYMS^{SS} DNA (Life Technologies, Gene Art), and neomycin resistance gene (NeoR) DNA product were cleaved by *NheI* and *ApaI*. Reporter genes mCherry with N-terminus SV40 nuclear localization sequence (RFP), inducible suicide gene CoOp iC9 (both produced by GeneArt), and enhanced green fluorescent protein

Table 4 Chemical Agents

Agent	Manufacturer	ID No.
5-fluorouracil	APP Pharmaceuticals, Schaumburg, IL	NDC 63323-117-10
Methotrexate	Hospira, Lake Forest, IL	NDC 61703-350-38
Pemetrexed	Lilly, Indianapolis, IN	NDC 0002-7640-01
Raltitrexed	Abcam Biochemicals, Cambridge, MA	Ab142974
G418	Invivogen, San Diego, CA	Ant-gn-1
AP20187	Clontech, Mountain View, CA	635060

(eGFP) DNA were digested by *ApaI* and *KpnI.* The G4CAR backbone was restriction enzyme digested by *NheI* and *KpnI*. The G4CAR backbone was ligated with NheI and ApaI digested fragments and ApaI and KpnI digested fragments in a three component ligation. Enzyme digestion locations of *NheI, KpnI,* and *ApaI* are shown in **Figure 10B.** The drug resistant component [$DHFR^{FS}$, $TYMS^{SS}$, or NeoR] was permutated with the transgenes [RFP, CoOp iC9, and GFP] to make the following DNA plasmids: FLAG-$DHFR^{FS}$-2A-eGFP pSBSO (DG), FLAG-CoOp $DHFR^{FS}$-2A-eGFP pSBSO (CoOp DG) FLAG-$TYMS^{SS}$-2A-GFP pSBSO (TSG), FLAG-CoOp $TYMS^{SS}$-2A-GFP pSBSO (CoOp TSG), FLAG-$TYMS^{SS}$-2A-RFP pSBSO (TSR), NeoR-2A-GFP pSBSO (NRG), FLAG-$DHFR^{FS}$-2A-iC9 pSBSO (D^{FS}iC9). Codon optimization of $DHFR^{FS}$ and $TYMS^{SS}$ DNA was performed to avoid the mRNA transcript from being bound by DHFR and TYMS proteins, respectively. Known RNA binding motifs of DHFR and TYMS mRNA are recognized by DHFR[92] and TYMS[96], respectively. Codons of $DHFR^{FS}$ and $TYMS^{SS}$ were altered as much as possible while maintaining the amino acid sequence of each protein in order to avoid protein binding of the mRNA transcript. Previously described CD19-specific chimeric antigen receptor (CAR)[95] was utilized without modification.

Myc-ffLuc-NeoR pSBSO (NRF) was constructed using the backbone of CD19-2A-Neo pSBSO[95] isolated after restriction digestion with *NheI* and *SpeI. NheI* and *SpeI* digested Myc-firefly Luciferase (ffLuc) insert was ligated to CD19-2A-Neo backbone followed by digestion of the ligation product with *SpeI* and *EcoRV*. *SpeI* and *EcoRV* digested NeoR fragments were then ligated to the digested backbone to yield NRF. All constructs contain Sleeping Beauty (SB) indirect/ direct repeat (IR/DR) sites to induce genomic integration in the presence of SB

Table 5 Synthetic DNA/ protein sequences

FLAG- dmDHFR	atggactacaaggacgacgacgacaaggattacaaggatgatgatgataaggactataaagacgacgat gataaggacgtcgttggttcgctaaactgcatcgtcgctgtgtcccagaacatgggcatcggcaagaacggg gacttcccctggccaccgctcaggaatgaatccagatatttccagagaatgaccacaacctcttcagtagaa ggtaaacagaatctggtgattatgggtaagaagacctggttctccattcctgagaagaatcgacctttaaagg gtagaattaatttagttctcagcagagaactcaaggaacctccacaaggagctcattttctttccagaagtctag atgatgccttaaaacttactgaacaaccagaattagcaaataaagtagacatggtctggatagttggtggcag ttctgtttataaggaagccatgaatcacccaggccatcttaaactatttgtgacaaggatcatgcaagactttga aagtgacacgtttttttccagaaattgatttggagaaatataaacttctgccagaatacccaggtgttctctctgatg tccaggaggagaaaggcattaagtacaaatttgaagtatatgagaagaatgat
FLAG-CoOp dmDHFR	atggactacaaggacgacgacgacaaggattacaaggatgatgatgataaggactataaggacgatgatg acaaagacgtcgtgggcagcctgaactgcatcgtggccgtgtcccagaacatgggcatcggcaagaacgg cgacttcccctggccccctctgcggaacgagagccggtacttccagcggatgaccaccaccagcagcgtg gaaggcaagcagaacctcgtgatcatgggcaagaaaacctggttcagcatccccgagaagaaccggccc ctgaagggccggatcaacctggtgctgagcagagagctgaaagagccccctcagggcgcccacttcctga gcagatctctggacgacgccctgaagctgaccgagcagccagagctggccaacaaggtggacatggtgtg gatcgtgggcggcagctccgtgtacaaagaagccatgaaccaccctggccacctgaaactgttcgttaccc gtataatgcaggatttcgagagcgataccttcttccccgagatcgacctggaaaagtacaagctgcttcccga gtaccccggcgtgctgtccgatgtgcaggaagagaagggcatcaagtacaagttcgaggtgtacgagaag aatgac

FLAG- dmTYMS	atgtatccgtacgacgtaccagactacgcatatccgtacgacgtaccagactacgcagacgtccctgtggcc ggctcggagctgccgcgccggcccttgccccccgccgcacaggagcgggacgccgagccgcgtccgcc gcacggggagctgcagtacctggggcagatccaacacatcctccgctgcggcgtcaggaaggacgaccg ctcgagcaccggcaccctgtcggtattcggcatgcaggcgcgctacagcctgagagatgaattccctctgctg acaaccaaacgtgtgttctggaagggtgttttggaggagttgctgtggtttatcaagggatccacaaatgctaa agagctgtcttccaagggagtgaaaatctgggatgccaatggatcccgagacttttggacagcctgggattct ccaccagagaagaaggggacttgggaccagtttatggcttccagtggaggcattttggggcagaatacaga gatatggaatcagattattcaggacagggagttgaccaactgcaaagagtgattgacaccatcaaaaccaa ccctgacgacagaagaatcatcatgtgcgcttggaatccaagagatcttcctctgatggcgctgcctccatgc catgccctctgccagttctatgtggtgaacagtgagctgtcctgccagctgtaccagagatcgggagacatgg gcctcggtgtgcctttcaacatcgccagctacgccctgctcacgtacatgattgcgcacatcacgggcctgaa gccaggtgactttatacacactttgggagatgcacatatttacctgaatcacatcgagccactgaaaattcagc ttcagcgagaacccagacctttcccaaagctcaggattcttcgaaaagttgagaaaattgatgacttcaaagc tgaagactttcagattgaagggtacaatccgcatccaactattaaaatggaaatggctgtt
FLAG-CoOp- dmTYMS	atggactacaaggacgacgacgacaaggattacaaggatgatgatgataaggactataaggacgatgatg acaaagacgtccccgtggccggcagcgagctgcctagaaggcctctgcctcctgccgctcaggaaaggga cgccgaacctagacctcctcacggcgagctgcagtacctgggccagatccagcacatcctgagatgcggc gtgcggaaggacgacagaagcagcacaggcaccctgagcgtgttcggaatgcaggccagatacagcct gcgggacgagttccctctgctgaccaccaagcgggtgttctggaagggcgtgctggaagaactgctgtggtt catcaagggcagcaccaacgccaaagagctgagcagcaagggcgtgaagatctgggacgccaacggc agcagagacttcctggacagcctgggcttcagcaccagagaggaaggcgatctgggtcccgtgtacgggtt

	tcaatggcggcacttcggcgccgagtatcgggacatggagagcgactacagcggccagggcgtggacca gctgcagagagtgatcgacaccatcaagaccaaccccgacgaccggcggatcatcatgtgcgcctggaa ccccagagatctgcccctgatggccctgcctccatgtcacgccctgtgccagttctacgtcgtgaactccgagc tgagctgccagctgtaccagcggagcggcgatatgggactgggcgtgcccttcaatatcgccagctacgcc ctgctgacctacatgatcgcccacatcaccggcctgaagcccggcgactttatccacaccctgggcgacgcc catatctacctgaaccacatcgagcccctgaagattcagctgcagcgcgagcccagacccttcccaaagct gcggatcctgcggaaggtggaaaagatcgacgacttcaaggccgaggacttccagatcgagggctacaa cccccaccccacaatcaagatggaaatggccgtg
eGFP forward	5` cccgggcccggcgccatgccacctcctcgcctcctcttc 3`
eGFP reverse	5` ggtaccttgtacagctcgtccatgccgagagtgatcccggcggcggtcac 3`
NeoR forward	5’ gctagcacatgtgccaccatgattgaacaagatggattgcacgcaggttctccggccgcttgg 3`
Neo R reverse	5’ aagcttccgcggccctctccgctaccgaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaat c 3`
NLS	MAPKKKRKVGIHRGVP

transposase. Each transgene is promoted using elongation factor 1 alpha (EF1-α) promoter. Cartoon representations of frequently used constructs can be seen in **Figure 10 B** and **Figure 17A**. Select DNA and protein sequences can be found in **Table 5**.

Genetic Modification and Propagation of Cells:

The Amaxa Nucleofector® II (Lonza, Allendale, NJ) was used to electroporate both Jurkat and human PBMC. Electroporation of Jurkat cells utilized a modified buffer[97] containing 5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, and 50 mM DMSO, where 10^6 Jurkat cells per cuvette were electroporated using program T-14 before immediate transfer to CM. The addition of drug occurred 48 hours after electroporation and cell culture remained undisturbed until sampling for gene expression on days 10-12 post electroporation. Human PBMC electroporation followed a previously described protocol.[95] Briefly, 1 to 2 X 10^7 thawed PBMC per cuvette were electroporated in Amaxa T cell Nucleofector solution (Lonza Biosciences; Cat No. VPA-1002) using program U14. On the following day, PBMC were stimulated in fresh CM with AaPC at a ratio of 1: 1 including 50 IU/ mL IL-2, unless otherwise noted. The cellular co-culture concentration of 10^6 cells/ mL was maintained at each stimulation, and PBMC derived T cells were re-stimulated every 7 days using the same concentrations. IL-2 was added when media was changed between stimulations. Drug treatment initiated 48 hours after co-culture began and continued until day 14. Drug was only added with fresh CM.

Western blot:

10^6 T cells were centrifuged from culture, supernatant aspirated, and the pellet rapidly frozen in liquid nitrogen. Whole-cell extracts were harvested using 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 1mM phenylmethylsulfonyl fluoride, 150 mM p-nitrophenyl phosphate and 0.3 µM Aprotinin, pH 7.4. Proteins were separated by SDS-PAGE in reducing conditions and analyzed using specific primary antibodies indicated in **Table 6**. Detection was performed using an enhanced chemiluminescence detection system.

Flow cytometry:

Cultured cells were resuspended, and washed once in FACS staining solution.[95] If transgene expression alone was sought, the specimen was then analyzed on a flow cytometer. The BD LSRFortessa (BD Biosciences) was used to analyze RFP expression; otherwise, BD FACSCalibur (BD Biosciences) was used. Surface antibody staining was performed in FACS staining solution with fluorochrome-conjugated antibodies at 4º C for at least 30 minutes. Antibody targets, concentrations, and manufacturers are listed in **Table 7**. Analysis of flow cytometry data utilized FlowJo v 10.0.5 (Tree Star Inc., Ashland, OR).

Luciferase assay:

Cultured T cells were tested for the persistence of ffLuc transgene by the cleavage of D-luciferin (Perkin Elmer, Waltham, MA, Cat. No. 122796). Resuspended cells were plated and washed once in D-PBS before testing in a D-PBS solution of D-luciferin at 0.14 mg/ mL. After incubation at 37 º C for 10 min, the plate was analyzed on a TopCount NXT Luminescence Counter (Perkin Elmer).

Table 6 Western Blot Antibodies

Antibody	Manufacturer	Cat. No.	Dilution
Actin	Sigma	A2228	1:10000
Hsp-70	Santa Cruz Biotechnology, Dallas, TX	SC-24	1:5000
DHFR	Santa Cruz Biotechnology	SC-377091	1: 1000
TYMS	Millipore	MAB4130	1: 1000
Myc Tag	CST	2276S	1: 1000
DYKDDDDK Tag	Pierce	MA1-91878	1: 1000

Table 7 Flow Cytometry Antibodies

Antibody	Manufacturer	Cat. No.	Dilution
CD3-APC	BD Pharmingen	340661	1:33
CD3-PerCP-Cy5.5	BD Pharmingen	340949	1:33
CD4 FITC	BD Pharmingen	340133	1:33
CD4 -PE	BD Pharmingen	347327	1:33
CD4-PerCP-Cy5.5	BD Pharmingen	341645	1:33
CD8-APC	BD Pharmingen	340659	1:33
Annexin V-PE	BD Pharmingen	556422	1:20
7-AAD	BD Pharmingen	559925	1:20
Propidium Iodide	BD Pharmingen	556463	
Human Fc-PE	Invitrogen	H10104	1:40
Myc- AF488	MBL	M047-A48	1:33
FLAG-AF647	Cell Signaling	3916S	1:33

Chromium Release Assay:

Antigen specific cytotoxicity was assessed by chromium release assay (CRA). This assay was previously described.[95] Briefly, antigen positive $CD19^{+}$ EL-4 were compared to antigen negative $CD19^{neg}$ EL-4 after each cell line was loaded with ^{51}Cr for 3 hours and subsequently incubated with CD19-specific CAR^{+} T cells at a 1 target : 5 effector cell ratio for 6 hours. Release of ^{51}Cr from cell lysis was assessed by the TopCount NXT scintillation counter.

Statistical Analysis:

Statistical analysis and graphical representation of data was achieved using Prism v6.0 (Graph Pad Software Inc., La Jolla, Ca). Experiments of more than one variable were analyzed by multivariate analysis: Two-Way ANOVA was used when appropriate with Sidak's multiple comparison test, One-Way ANOVA was used when appropriate with Tukey's or Dunnett's multiple comparison tests as applicable, non-Gaussian distributions were assessed by the Kruskall-Wallis test followed by Dunn's multiple comparison test. Single variable tests (experimental vs. control) were made using the Mann-Whitney test. Statistical significance was designated as $\alpha < 0.05$.

RESULTS:

Testing AThyR transgene selection in Jurkats.

Jonnalagadda *et. al* recently developed $DHFR^{FS+}$ T cells resistant to MTX with *in vitro* and *in vivo* applications.[46, 82] The studies focused on demonstrating that $DHFR^{FS+}$ T cells continue to survive and function in a moderate dose of MTX used post-HSCT. We continued

using $DHFR^{FS}$ to determine whether T cells can be genetically-modified to resist toxic doses of AThys used in the initial treatment of malignancy. For this purpose, a combination of mutein human TYMS with $DHFR^{FS}$ was sought. 5-FU resistant TYMS muteins previously identified within a bacterial culture system[90] were tested in human cells (not shown) and $TYMS^{SS}$ was chosen for further study.

To test the enhanced survival of each AThyR, constructs individually expressing $DHFR^{FS}$, $TYMS^{SS}$, and NeoR were ligated into the same backbone containing Sleeping Beauty (SB) transposable elements upstream of eGFP (**Figure 10B**). eGFP was used to track the predominance of surviving genetically-modified T cells. Jurkat cells were co-electroporated with each construct and SB11 transposase [57] which mediated genomic integration of each construct. Cytotoxic drugs were added two days after electroporation. Jurkat were assessed for eGFP expression in viable cells by propidium iodide (PI) exclusion on day 10-12 (**Figure 10C)**. Increased percentage expression of eGFP was sought as a measure for transgene selection in the presence of drug. Overall survival and mean fluorescence intensity (MFI) of eGFP are also given in **Figure 11AI and AII,** respectively. Overall, we demonstrate that $DHFR^{FS}$ has much better selection than the traditional drug-resistance transgene NeoR. We also demonstrate that $TYMS^{SS}$ has no independent capacity to enhance Jurkat survival.

More specifically, we found that $DHFR^{FS}$ confers resistance to MTX at concentrations range of 0.01-0.5 μM, and codon optimization of $DHFR^{FS}$ enhanced the drug resistance range of CoOp $DHFR^{FS}$ to 0.01-1 μM (**Figure 10C**). Codon optimization removed potential endogenous DHFR binding to the $DHFR^{FS}$ mRNA as well as possible micro RNA binding

domains.[92] Notably, gating on eGFP^{+} cells demonstrated that DHFR^{FS} constructs lead to a MTX dependent increase in eGFP MFI. Hence, eGFP expression within a single cell increased based on the addition of MTX. This finding occurred independent of mRNA regulation until 5 μM MTX where endogenous codon DHFR^{FS} expression significantly decreased compared to CoOp DHFR^{FS} ($p < 0.0001$) (**Figure 11A-II**). Drug inducible transgene expression is a rare phenomenon. This phenomenon, although rare, is not novel and the capacity of DHFR to increase cis-expressed eGFP in an MTX dependent manner was previously described for native DHFR. However, this was attributed to MTX binding DHFR, DHFR releasing DHFR mRNA, and free DHFR mRNA leading to increased translation of DHFR protein.[98] Here we note that the phenomenon also occurs with MTX resistant DHFR^{FS}, and with DHFR^{FS} occurs independent of mRNA regulation from 0.01 – 1 μM MTX. Hence, we postulate the regulation of DHFR expression occurs partially through an mRNA independent mechanism, which has not been described to our knowledge. We further explore this mechanism in later sections

As noted, there was no drug selective advantage for TYMS^{SS} expressing Jurkat when tested with 5-FU (**Figure 10C**). Native codon TYMS^{SS} had no expression advantage over No DNA Jurkat at any concentration of 5-FU. Further analysis of eGFP^{+} cells for eGFP MFI revealed that TYMS^{SS} expressed at a lower eGFP MFI compared to CoOp TYMS^{SS} **(Figure 11A)**. We conclude that lower expression of TYMS^{SS} due to mRNA based suppression contributed to the lack of TYMS^{SS} survival advantage. When mRNA regulatory mechanisms are ablated by codon optimization, TYMS^{SS} has a significant

Figure 10

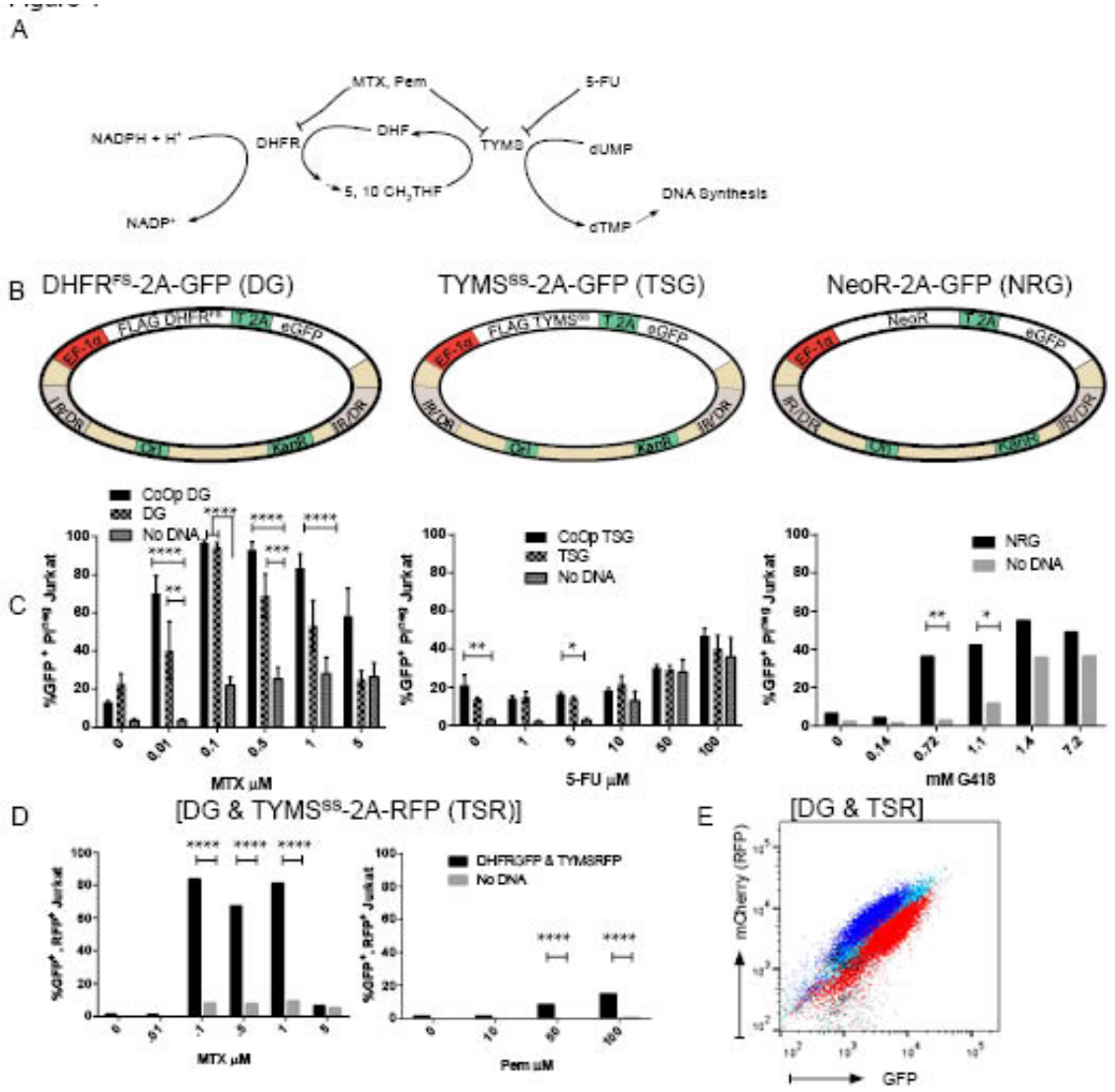

A
MTX, Pem
5-FU
NADPH + H+
DHFR
DHF
TYMS
dUMP
5, 10 CH2THF
DNA Synthesis
NADP+
dTMP
B
DHFRFS-2A-GFP (DG)
TYMSSS-2A-GFP (TSG)
NeoR-2A-GFP (NRG)
EF-1α
FLAG DHFRFS
T2A
eGFP
FLAG TYMSSS
NeoR
IR/DR
CR1
KanR
C
CoOp DG
DG
No DNA
CoOp TSG
TSG
NRG
%GFP+ PI neg Jurkat
MTX µM
5-FU µM
mM G418
D
[DG & TYMSSS-2A-RFP (TSR)]
DHFRGFP & TYMSRFP
%GFP+, RFP+ Jurkat
Pem µM
E
[DG & TSR]
mCherry (RFP)
GFP

Figure 10 Testing enhanced survival by selection of anti-thymidylate (AThy) resistance (AThyR) transgenes in Jurkats. (**A**) The synthesis of thymidine is crucial to DNA replication and cell survival. It has long been a target of AThy chemotherapeutic agents like methotrexate (MTX), Pemetrexed (Pem), and 5-fluorouracil (5-FU) in many common forms of cancer. (**B**) Putative AThyR transgenes resistant to AThy toxicity were designed in order to confer resistance to T cells that might be used in a combination therapeutic with AThy chemotherapy. AThyRs were co-expressed with a fluorescent protein to indicate that surviving cells contained the transgene. These transgene utilized the Sleeping Beauty transposon/ transposase system to induce stable transgene expression in Jurkat. Human muteins $DHFR^{FS}$ - resistant to MTX (**left**), human mutein $TYMS^{SS}$ - resistant to 5-FU (**center**), and the gold-standard Neomycin resistance gene (NeoR) drug resistance gene - resistance to G418 (**right**) were used in this study. Codon optimized (CoOp) versions of $DHFR^{FS}$ & $TYMS^{SS}$ replaced native codon $DHFR^{FS}$ & $TYMS^{SS}$ to test whether known post-transcriptional regulatory mechanisms were affecting AThyR selection or survival. (**C- left**) $DHFR^{FS}$-2A-GFP (DG), CoOp DG, and no DNA, were electroporated into Jurkat and subjected to MTX after 2 days. (**C- center**) $TYMS^{SS}$-2A-GFP (TSG), CoOp TSG, and No DNA electroporated Jurkat were treated on day 2 with 5-FU. (**C- right**) NeoR-GFP and No DNA electroporated Jurkat were treated on day 2 with G418. For each experiment in C the percentage of $eGFP^{+}$ viable Jurkat is given after testing on day 8-10 after the addition of drug. (**D**) MTX and Pem are known to inhibit native DHFR and TYMS, DG and $TYMS^{SS}$-2A-RFP (TSR) were co-electroporated into Jurkat to determine whether combination $DHFR^{FS}$ & $TYMS^{SS}$ confer enhanced survival to MTX (**left**) or Pem (**right**). (**E**)

Following 2 weeks of selection in 1 µM MTX, [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat displayed a uniform and repeatable pattern of correlated expression. Shown here, four separate [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat experiments are overlaid in different colors. Experiments were independently repeated at least twice with 4-6 replicates. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. ; Dihydrofolate (DHF); DHF reductase (DHFR); deoxyuridine monophosphate (dUMP); deoxythymidine monophosphate (dTMP); 5, 10 – methylenetetrahydrofolate (5,10 CH_2THF); nicotinamide adenine dinucleotide phosphate (NADP).

Figure 11

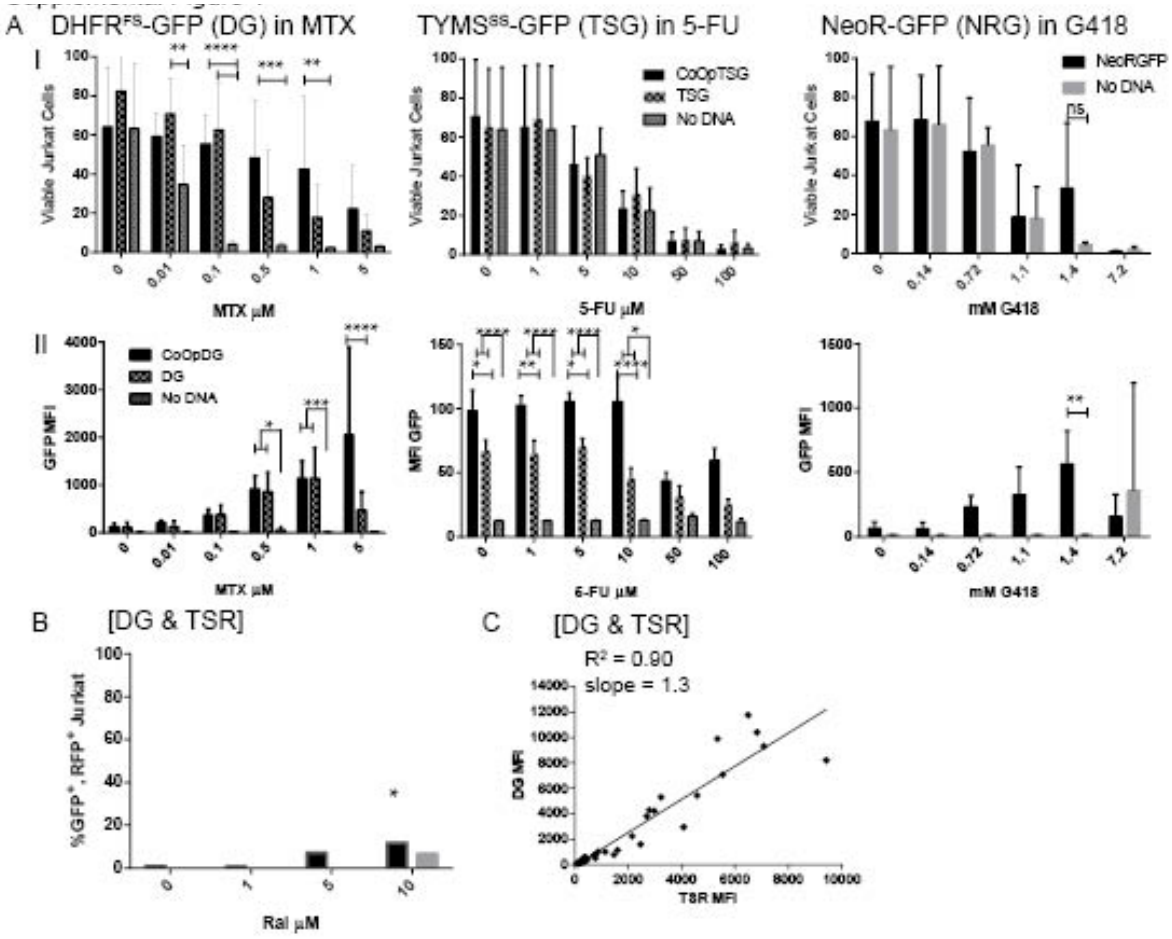

A DHFR^FS-GFP (DG) in MTX
TYMS^SS-GFP (TSG) in 5-FU
NeoR-GFP (NRG) in G418
I
Viable Jurkat Cells
MTX µM
CoOpTSG
TSG
No DNA
5-FU µM
NeoRGFP
No DNA
ns
mM G418
II
GFP MFI
CoOpDG
DG
No DNA
MTX µM
MFI GFP
6-FU µM
GFP MFI
mM G418
B
[DG & TSR]
%GFP+, RFP+ Jurkat
Ral µM
C
[DG & TSR]
R² = 0.90
slope = 1.3
DG MFI
TSR MFI

Figure 11 Contributory findings in the testing of individual and combined AThyR selection for Jurkats. Jurkat were electroporated with AThyR or NeoR drug resistance transgenes and treated from day 2 until days 10-12 with appropriate drug. The enhancement in survival of Jurkat expressing transgene is noted in **Figure 1**. Here the enhanced survival, as determined by PI exclusion (**I**), and alterations in the mean fluorescent intensity (MFI) of eGFP (**II**), are given for $DHFR^{FS}$ (**left**), $TYMS^{SS}$ (**right**), and NeoR (**center**). (**B**) Raltitrexed (Ral) is an anti-folate AThy known to primarily inhibit native TYMS. $DHFR^{FS}$ & $TYMS^{SS}$ were co-electroporated into Jurkat treated with Ral to determine whether this transgene combination enhanced survival. (**C**) Observations suggested that cells expressing $DHFR^{FS}$ & $TYMS^{SS}$ as independent plasmids have correlated expression of each plasmid. This could have implications in the co-regulation of $DHFR^{FS}$ with $TYMS^{SS}$. Hence, the MFI of eGFP and RFP were correlated for treatments with multiple concentrations of MTX, Pem, and Ral. The linear regression data is included in the figure. Each experiment was independently repeated at least twice with 4-6 replicates. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

expression advantage over mock electroporated Jurkat, and a weak survival advantage in 5 μM 5-FU. The lack of significantly enhanced survival is likely due to an alternative mechanism of 5-FU contributing to toxicity, which is likely the known inhibition of mRNA and rRNA synthesis by 5-FU.[85, 99]

NeoR is one of the first drug resistance transgenes utilized in mammalian cells.[45] Here, NeoR was used to select for enhanced survival of Jurkat in the presence of G418. This was intended to serve as a standard to gauge the utility of $DHFR^{FS}$ and $TYMS^{SS}$. Electroporation of NeoR into Jurkat improved survival in the presence of G418, as expected, at 0.72-1.1 mM G418 (**Figure 10C**). The survival advantage of NeoR over No DNA was not significant due to variability (**Figure 11A**), but a G418 dependent increase in GFP MFI was noted. The GFP MFI significantly increased above No DNA Jurkat at 1.4 mM G418 (**Figure 11A-II**). These results reinforce that $DHFR^{FS}$ and NeoR are capable of providing dose-dependent transgene selection advantage in surviving Jurkat. However, only $DHFR^{FS}$ conferred reliable survival advantages to Jurkat in this experiment (**Figure 11A-II**).

The next experiment combined $DHFR^{FS}$ and $TYMS^{SS}$ by co-electroporating each plasmid into Jurkat. The capacity of the combined transgenes to resist commonly used anti-folate AThys: MTX, Pem, and Raltitrexed (Ral), were tested. As before, drug was added on day 2 and cells were tested on day 10-12. There was clear selection for [$DHFR^{FS}$ & $TYMS^{SS}$] expressing Jurkat in 0.1 - 1 μM MTX when compared to similarly treated No DNA or untreated [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat (**Figure 10D)**. It should be noted that endogenous codon $DHFR^{FS}$ was used in these experiments and the resistance to MTX was enhanced from 0.5 (**Figure**

10C) to 1 μM MTX (**Figure 10D**) by the addition of $TYMS^{SS}$ with no other changes to the experimental conditions. Selection was also noted for 50-100 μM Pem (**Figure 10D).** Moderate selection was also noted with 10 μM Ral when compared to untreated [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat (**Figure 11B**). Ral primarily targets TYMS, whereas MTX and Pem target both DHFR and TYMS, [86] hence the improved selection for MTX and Pem over Ral in [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat. After 2 weeks within 1 μM MTX, surviving [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat were refreshed in untreated media and grown for 3-5 weeks. Subsequently, the stability of transgene expression of [$DHFR^{FS}$ & $TYMS^{SS}$]$^{+}$ Jurkat was tested by flow cytometry with the co-expression of eGFP representing $DHFR^{FS}$ expression and RFP representing $TYMS^{SS}$ expression as seen in **Figure 10E**. Each color represents a separate experiment and is overlaid to represent the trend that $DHFR^{FS}$ and $TYMS^{SS}$ co-express in a correlated fashion. In fact, analysis of GFP MFI representing $DHFR^{FS}$ expression and RFP MFIs representing $TYMS^{SS}$ expression over multiple anti-folate drugs, at multiple concentrations demonstrated that $DHFR^{FS}$ & $TYMS^{SS}$ co-express with a strong Pearson's correlation (R^2 = 0.9) (**Figure 11C**). This finding suggests that expression of $DHFR^{FS}$ is somehow regulated by the expression of $TYMS^{SS}$, or *vice versa*. These findings begin to suggest a more complex mechanism for the expression of DHFR and TYMS that is inter-related.

Selective propagation of primary human T cells resistant to MTX and/ or 5-FU.

As demonstrated, $TYMS^{SS}$ enhances the ability of Jurkat expressing $DHFR^{FS}$ to survive in the presence of MTX and Pem, which both target endogenous DHFR and TYMS to prevent thymidine synthesis. Given the more robust survival to toxic MTX concentrations conferred by

DHFR^{FS} and TYMS^{SS}, we pursued experiments with MTX as a proof-of-concept to demonstrate anti-folate and AThy resistance. Based on the findings above, it is anticipated improved survival in the presence of MTX will translate into improved survival in the presence of Pem. TYMS^{SS} with DHFR^{FS} were tested in human cells by electroporation into human PBMC. The day following electroporation, cells were stimulated with an OKT3-loaded AaPC capable of polyclonal T cell propagation.[94] The propagation schematic is shown in **Figure 12A**. Two days after AaPC stimulation, the co-cultures received 0.1 µM MTX, 5 µM 5-FU, or 1.4 mM G418 until day 14, as designated in **Figure 12**. The co-cultures were re-stimulated with AaPC at a 1: 1 ratio and given 50 IU / mL IL-2 every 7 days from day 1 to 35. Phenotypic changes in transgene expression were tracked during drug administration for the first 14 days and for the 21 days after drug administration had ended. The weekly changes in transgene expression can be noted in **Figure 12B-I, C-I, D-I**.

Initial testing of DHFR^{FS}, TYMS^{SS}, and NeoR co-expressed with fluorescent proteins demonstrated rapid and persistent selection to nearly complete selection for expression of DHFR^{FS} with MTX and NeoR with G418 (**Figure 12B-I**). Survival and propagation of AThyR+ T cells (TAThyR) compared to No DNA T cells on day 21 showed that the presence of AThyR or NeoR transgene was crucial to T cell survival and growth (**Figure 13A**). On day 35, total inferred cell count for T cells expressing AThyR and NeoR transgenes were compared to untreated No DNA T cells, and NeoR^{+} T cells were the only T cells with significantly inferior growth at Day35 (**Figure 13B-I**). In opposition to experiments in Jurkat, TYMS^{SS} demonstrated selection within the population of surviving T cells on Day 21 in the presence of

Figure 12

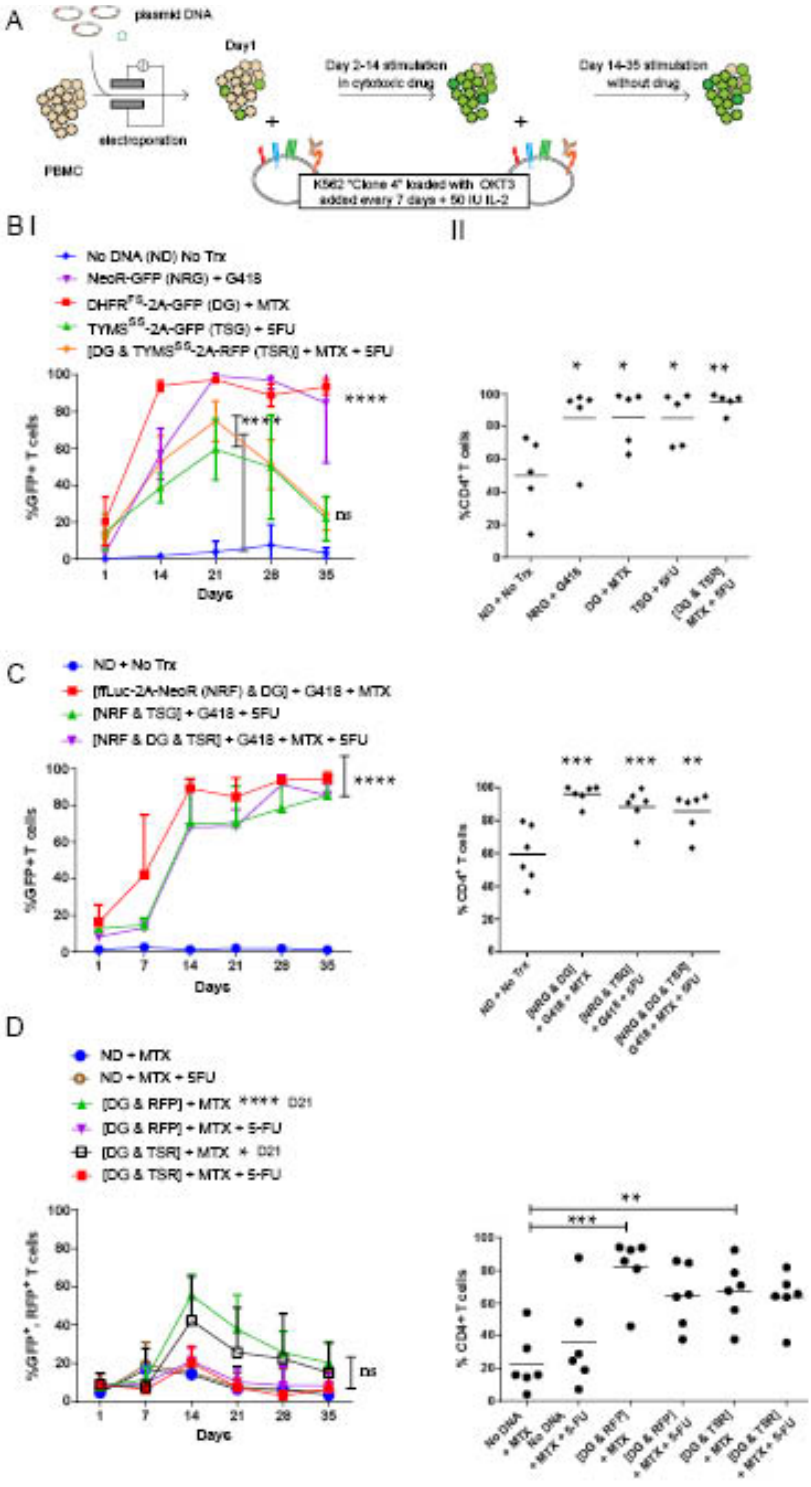

A
plasmid DNA
Day1
electroporation
PBMC
Day 2-14 stimulation in cytotoxic drug
Day 14-35 stimulation without drug
K562 "Clone 4" loaded with OKT3 added every 7 days + 50 IU IL-2
B I
II
No DNA (ND) No Trx
NeoR-GFP (NRG) + G418
DHFR^DG-2A-GFP (DG) + MTX
TYMS^SG-2A-GFP (TSG) + 5FU
[DG & TYMS^SG-2A-RFP (TSR)] + MTX + 5FU
%GFP+ T cells
Days
%CD4+ T cells
ND + No Trx
NRG + G418
DG + MTX
TSG + 5FU
[DG & TSR] MTX + 5FU
C
ND + No Trx
[fLuc-2A-NeoR (NRF) & DG] + G418 + MTX
[NRF & TSG] + G418 + 5FU
[NRF & DG & TSR] + G418 + MTX + 5FU
%GFP+ T cells
Days
% CD4+ T cells
ND + No Trx
[NRG & DG] + G418 + MTX
[NRG & TSG] + G418 + 5FU
[NRG & DG & TSR] G418 + MTX + 5FU
D
ND + MTX
ND + MTX + 5FU
[DG & RFP] + MTX **** D21
[DG & RFP] + MTX + 5-FU
[DG & TSR] + MTX * D21
[DG & TSR] + MTX + 5-FU
%GFP+, RFP+ T cells
Days
% CD4+ T cells
No DNA + MTX
No DNA + MTX + 5-FU
[DG & RFP] + MTX
[DG & RFP] + MTX + 5-FU
[DG & TSR] + MTX
[DG & TSR] + MTX + 5-FU

Figure 12 Selective propagation of primary human T cells resistant to MTX and 5-FU. (**A**) Testing for selection of AThyR transgenes in primary T cells was accomplished by co-electroporation of AThyR transposon and SB transposase DNA plasmids into PBMC. The following day electroporated PBMC were stimulated with OKT3-loaded activating and propagating cells (AaPC) at a 1:1 ratio and 50 IU/ mL IL-2. Drug was added on day 2 and maintained at the same concentration until day 14. Every 7 days, fresh AaPC and IL-2 were added at the same concentration until day 35. (**B-I**) After electroporation, T cells were tracked for expression of AThyRs $DHFR^{FS}$ - DG, $TYMS^{SS}$ - TG, both [DG & TSR], and NeoR -NRG in the presence (day 2-14) then absence (day 14-35) of appropriate selection drug. (**C-I**) Myc-ffLuc-2A-NeoR (NRF) was combined with each AThyR transgene; [DG & NRF], [TSG & NRF], and [DG & TSR & NRF] in order to improve selection for AThyRs selected by 5-FU. Selection occurred under the same condition with the exception that 100 IU IL-2/ mL was added to promote outgrowth of cells treated with G418. (**D-I**) To elucidate the influence of 5-FU and $TYMS^{SS}$ on the selection of $DHFR^{FS}$, RFP or $TYMS^{SS}$-RFP (TSR) were co-electroporated into T cells with $DHFR^{FS}$. There were no experimental differences from the outline noted in (**A**). The percentage of T cells expressing co-receptor CD4 is shown to the right of each corresponding experiment on Day 35 for **B-II**, **C-II**, and **D-II**. All experiments contain 5-6 biological replicates with each experiment independently repeated two times. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

5-FU. However, the selected $TYMS^{SS}$ expressing T cells did not persist to Day 35, and the lack of persistence was also noted when [$DHFR^{FS}$ & $TYMS^{SS}$] were selected using MTX and 5-FU. One possible explanation for this is that thymidine synthesis is restored by $TYMS^{SS}$ and thymidine transporters then make thymine available to un-transformed cells. This is likely mediated by an equilibrative nucleoside transporter as the same transporter that permits 5-FU entry also mediates equilibrative transport of thymine.[100] As $TYMS^{SS}$ restores thymidine synthesis in the presence of methotrexate, $DHFR^{FS}$ is no longer able to select for T cells expressing $DHFR^{FS}$ & $TYMS^{SS}$ as noted in **Figure 12B – I**.

In order to achieve complete selection of $TYMS^{SS}$ for possible use in combination therapies, NeoR was co-electroporated into primary T cells with $DHFR^{FS}$, $TYMS^{SS}$, and [$DHFR^{FS}$ & $TYMS^{SS}$]. The only change made to the propagation method was the addition of 100 IU/ mL IL-2 rather than 50 IU/ mL from days 14-35 to supplement the poor outgrowth already noted in G418 selected T cells. The higher doses of IL-2 were insufficient to rescue poor outgrowth when G418 and 5-FU were combined for T cell selection (**Figure 13B-II**). With the co-transfection of NeoR into $DHFR^{FS}$ and/or $TYMS^{SS}$ expressing T cells, nearly 100% transgenes selection was observed with the same transgene selection kinetics among all groups (**Figure 12C-I**).

Figure 13

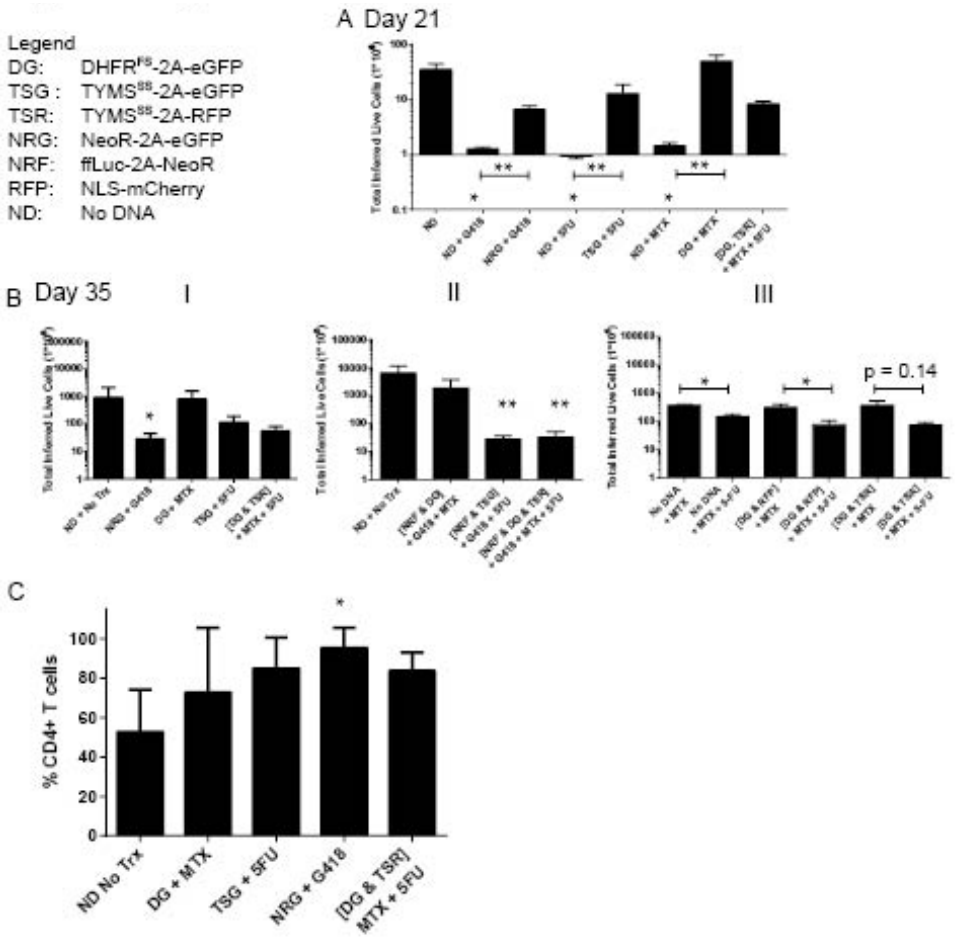


Figure 13 Propagation characteristics of AThyR+ T cells in the presence or absence of MTX, 5-FU, and/ or G418. (**A**) AThyR and NeoR electroporated primary T cells are compared on Day 21 to mock-electroporated T cells treated with the same conditions. The continued propagation of the same experiment is shown on day 35 (**B-I**). This can be compared to day 35 changes in outgrowth potential for primary T cells when NeoR is combined with $DHFR^{FS}$ and/or $TYMS^{SS}$ (**B-II**). (**B-III**) Subsequently, the influence of 5-FU on preserving outgrowth potential for primary T cells on day 35 was tested. Each experiment was independently repeated at least twice with 5-6 replicates. * = $p < 0.05$, ** = $p < 0.01$.

Figure 14

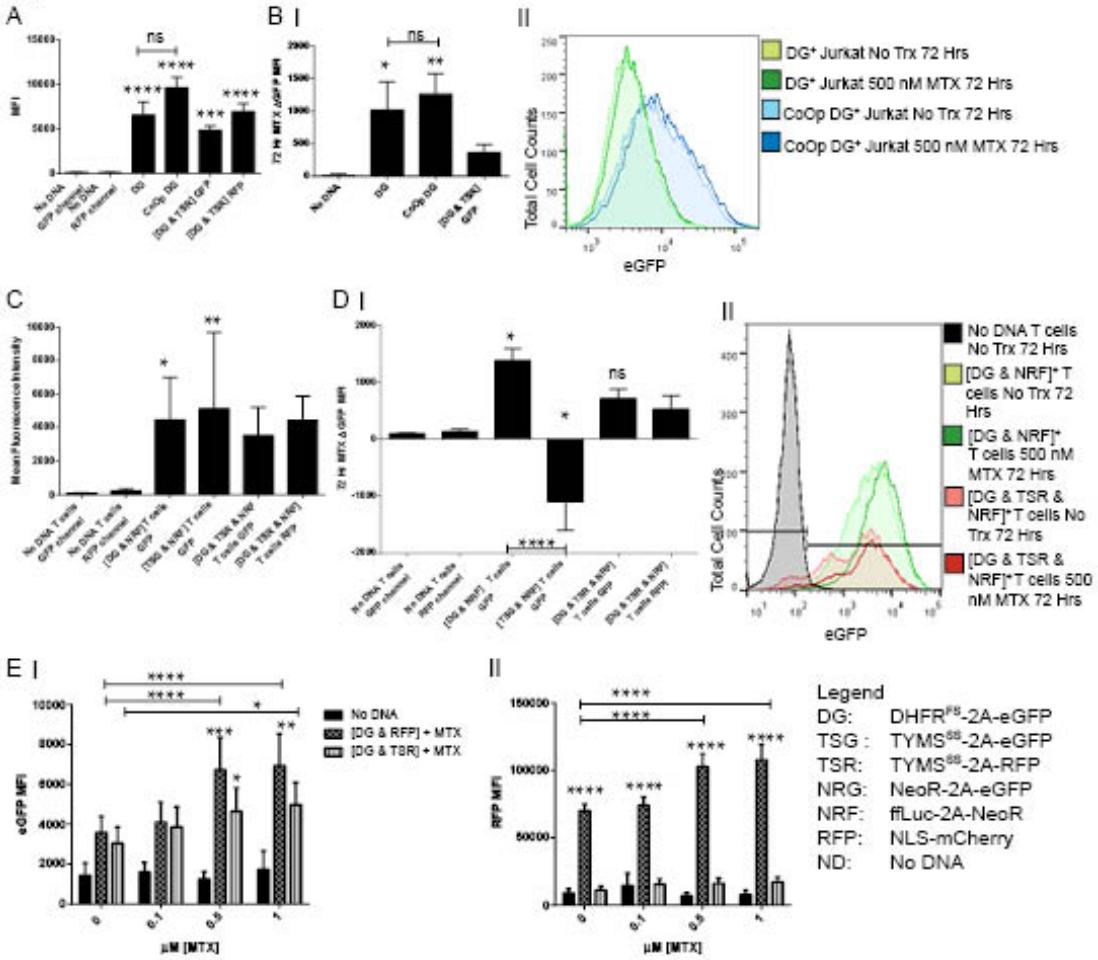
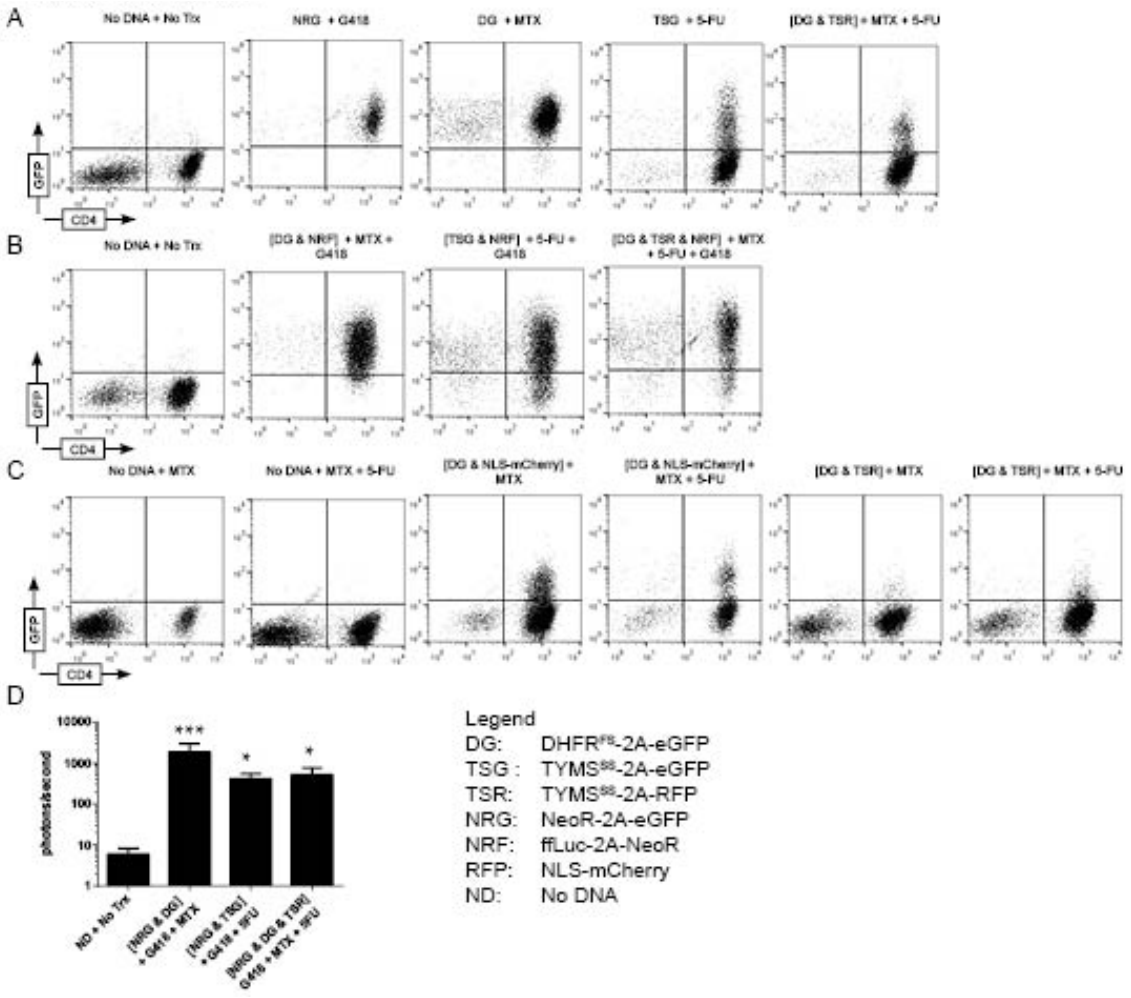

A
B I
II
DG+ Jurkat No Trx 72 Hrs
DG+ Jurkat 500 nM MTX 72 Hrs
CoOp DG+ Jurkat No Trx 72 Hrs
CoOp DG+ Jurkat 500 nM MTX 72 Hrs
Total Cell Counts
eGFP
C
D I
II
No DNA T cells No Trx 72 Hrs
[DG & NRF]+ T cells No Trx 72 Hrs
[DG & NRF]+ T cells 500 nM MTX 72 Hrs
[DG & TSR & NRF]+T cells No Trx 72 Hrs
[DG & TSR & NRF]+T cells 500 nM MTX 72 Hrs
E I
II
No DNA
[DG & RFP] + MTX
[DG & TSR] + MTX
µM [MTX]
Legend
DG: DHFR^FS-2A-eGFP
TSG : TYMS^SS-2A-eGFP
TSR: TYMS^SS-2A-RFP
NRG: NeoR-2A-eGFP
NRF: ffLuc-2A-NeoR
RFP: NLS-mCherry
ND: No DNA

Figure 14 MTX alters expression of *cis*-transgenes co-expressed with AThyRs (**A**) Jurkat transformed with $DHFR^{FS}$ - DG, CoOp $DHFR^{FS}$ - CoOp DG, and [DG & $TYMS^{SS}$ - TSR] were cultured with 1 µM MTX for 2 weeks before culturing without MTX for 3-5 weeks. The stable fluorescent protein expression, in the absence of MTX, is depicted by MFI. (**B-I**) AThyR+ Jurkat were treated for 72 hours with 0.5 µM MTX or no treatment. The Δ MFI difference (Δ = eGFP MFI MTX treated – eGFP MFI untreated) is depicted. (**B-II**) A representative histogram demonstrates the MTX induced change in eGFP MFI for $DHFR^{FS}$ and CoOp $DHFR^{FS}$ in Jurkat. In primary T cells from the experiment noted by **Figure 2C,** day 35 T cells were stimulated with anti-CD3, anti-CD28 antibodies, and 50 IU/ mL IL-2 in the absence of MTX. The fluorescent protein MFI is shown in (**C**), and (**D-I**) depicts the Δ MFI after 72 hours of treatment with 0.5 µM MTX in comparison to no treatment. (**D-II**) A representative histogram demonstrates the observed shift in eGFP fluorescence for $DHFR^{FS+}$ T cells in the presence or absence of MTX. Kruskall-Wallis test was used to determine significant differences. (**E**) Further experiments in primary T cells assessed the influence of $TYMS^{SS}$ on blunting the MTX-induced increase in eGFP co-expressed with $DHFR^{FS}$. This was performed 72 hours after treatment on day 35 with anti-CD3, anti-CD28 antibodies, 50 IU/ mL IL-2, and varying concentrations of MTX. The MTX induced change in eGFP MFI for $DHFR^{FS}$ is shown in (**I**), while the influence of MTX on RFP and RFP co-expressed with $TYMS^{SS}$ (TSR) is shown in (**II**). Jurkat and primary T cell experiments included 6 replicates independently repeated twice; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Figure 15



Legend

DG:	$DHFR^{FS}$-2A-eGFP
TSG :	$TYMS^{SS}$-2A-eGFP
TSR:	$TYMS^{SS}$-2A-RFP
NRG:	NeoR-2A-eGFP
NRF:	ffLuc-2A-NeoR
RFP:	NLS-mCherry
ND:	No DNA

Figure 15 Flow plots of transgene expression for AThyR experiments on day 35. Flow plots of CD4 and GFP expression depict day 35 of a series of experiments designed to characterize the selection and maintenance of transgene expression in donor T cells. T cells grown for 35 days with days 2-14 in the presence of cytotoxic drugs MTX, 5-FU, G418, or a combination, as noted above the flow plot, are shown; (**A**) corresponds to the experiment described for **Figure 2B**, (**B**) corresponds to the experiment described for **Figure 2C**, (**C**) corresponds to the experiment described for **Figure 2D**. (**D**) The presence of ffLuc-2A-NeoR – NRF - on day 35 for experiment noted in (**B**) is demonstrated using D-luciferin to induce T cell chemiluminescence. Each experiment was independently repeated at least twice with 6 replicates. Representative flow plots are depicted. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

The influence of $TYMS^{SS}$ on $DHFR^{FS}$ selection in T cells subjected to MTX was tested. Plasmids expressing $DHFR^{FS}$ were co-electroporated into T cells along with either $TYMS^{SS}$ co-expressing RFP or a vector expressing RFP alone. This experiment followed the same strategy as described for **Figure 12B**. Due to technical limitations, the total amount of $DHFR^{FS}$ expressing plasmid DNA electroporated into the same number of T cells was decreased. Consequently, fewer T cells initially expressed $DHFR^{FS}$ at the beginning of the experiment and $DHFR^{FS}$ was incompletely selected by the addition of MTX within a 14 day time period (**Figure 12D - I**). The progressive loss of $DHFR^{FS}$ after day 14 is reminiscent of $TYMS^{SS}$ expression in **Figure 12B – I.** This demonstrates that AThyR transgenes must select for nearly the entire T cell population to maintain stable expression within the population. With regards to the influence of $TYMS^{SS}$ on the selection of $DHFR^{FS}$, it appears that $TYMS^{SS}$ blunts $DHFR^{FS}$ selection in T cells as selection of [$DHFR^{FS}$ & RFP] expressing T cells was more robust than selection of [$DHFR^{FS}$ & $TYMS^{SS}$] expressing T cells. This is attributed to the restoration of thymidine synthesis in the presence of $TYMS^{SS}$ (**Figure 12D - I**). The presence of 5-FU prevents selection of $DHFR^{FS}$ with or without $TYMS^{SS}$, and this is attributed to the $TYMS^{SS}$ independent inhibition of mRNA and rRNA synthesis previously mentioned.[85, 99]

It was also noted that transgenic selection tended to increase the population of $CD4^+$ T cells by day 35 in all T cell experiments, which was not seen with un-modified T cell cultures. This was noted in any experiment involving one or more transgenes selected in the presence of cytotoxic drug (**Figure 12B – II, 12C – II, 12D – II,** respective flow plots seen in **Supplemental Figure 15A, 15B, and 15C**). The experiment in **Figure 12D - II** demonstrates that it is not

caused by cytotoxic drug, rather, the presence of transgene in combinations with cytotoxic drug leads to $CD4^+$ T cell predominance by day 35. The selection towards $CD4^+$ T cell predominance was not noted 7 days after initial drug selection for AThyR+ T cells (**Figure 13C**), which is consistent with previously published findings using $DHFR^{FS}$ T cells.[82] The longer period of follow-up than prior experiments demonstrated a previously unknown phenomenon that $CD8^+$ T cells are unable to persist for long periods of time following cytotoxic insult, or are selectively outgrown by $CD4^+$ T cells. The mechanism underlying this phenomenon is not known.

MTX increases cis-transgene expression in $DHFR^{FS+}$ T cells

MTX mediated changes in transgene expression could be used in animal models and humans as a tool for *in vivo* control of transgene expression. To our knowledge, a system utilizing clinically available drugs to mediate transgene expression either up or down in T cells has not been described. Hence, we targeted the known drug-inducible regulation of DHFR by MTX [98] for use in T cells. The MTX-inducible expression of transgenes co-expressed with mutein $DHFR^{FS}$ is unknown, and the point mutations may have ablated the MTX-inducible expression mechanism. In order to examine whether this phenomenon persists, $DHFR^{FS}$, CoOp $DHFR^{FS}$, and [$DHFR^{FS}$ & $TYMS^{SS}$] expressed in Jurkat were selected in 1 µM MTX for 2 weeks and rested for 3-5 weeks before testing MTX mediated regulation of $DHFR^{FS}$ expression. By this time, expression of eGFP in each cell was uniformly positive, but the expression level of eGFP, as signified by the MFI, varied based on the choice of AThyR co-expressing eGFP (**Figure 14A**). $DHFR^{FS}$, CoOp $DHFR^{FS}$, and [$DHFR^{FS}$ & $TYMS^{SS}$] expressing

Jurkat were subjected to 72 hours incubation in 0.5 μM MTX, a concentration all $DHFR^{FS+}$ cells were expected to survive. Cells treated with MTX showed a significant increase in eGFP MFI for both $DHFR^{FS}$ and CoOp $DHFR^{FS}$ in comparison to the same cells untreated (**Figure 14B-I** and histogram **Figure 14B-II**)**.** The increase in eGFP MFI was equivalent between $DHFR^{FS}$ and CoOp $DHFR^{FS}$ after 72 hours, suggesting that the up-regulation of eGFP is independent of the mRNA binding mechanism previously thought to mediate this phenomenon.[92, 101] An alternative regulatory mechanism based on the presence of thymidine is suggested here. As previously shown, $TYMS^{SS}$ restores the synthesis of thymidine in MTX treated Jurkat. In this experiment, the co-expression of $TYMS^{SS}$ with $DHFR^{FS}$ in Jurkat blunts the MTX induced increase in eGFP MFI (**Figure 12B-I**). Thus, $DHFR^{FS}$ maintains MTX-inducible expression of *cis*-transgenes which is dependent on MTX mediated inhibition of TYMS.

This phenomenon was next tested in primary T cells. In order to uniformly select each AThyR in primary T cells, AThyRs were co-electroporated with a NeoR selection plasmid as described for **Figure 12C-II**. By day 35, all AThyR+ T cell groups uniformly expressed transgene, and the fluorescent protein MFI from each T cell group may be noted in **Figure 14C**. On day 35, primary T cells were subjected to 72 hours of 0.5 μM MTX following anti-CD3/ CD28 mediated stimulation. The T cells were tested on day 38, and MTX significantly increased eGFP MFI for $DHFR^{FS+}$ cells, as expected. However, upon testing $TYMS^{SS}$ in primary T cells, it was found that MTX mediates a decrease in eGFP MFI for $TYMS^{SS}$ expressing T cells (**Figure 14D-I**). The decrease in eGFP expression demonstrated for $TYMS^{SS+}$ T cells in the presence of MTX was consistent across donors and ablated by the restoration of

dihydrofolate reduction to tetrahydrofolate by $DHFR^{FS}$. Prior reports demonstrated that dihydrofolate reversed or prevented TYMS binding to TYMS mRNA.[91] Thus, MTX mediated decreases in dihydrofolate likely re-establishes TYMS binding to $TYMS^{SS}$ mRNA preventing expression of eGFP. This phenomenon has not been reported to our knowledge and may be of scientific and clinical value. Furthermore, $TYMS^{SS}$ continued to blunt the MTX-mediated increase in eGFP MFI co-expressed with $DHFR^{FS}$, as was previously noted in Jurkat. The histogram in **Figure 14D-II** depicts the MTX-mediated changes in *cis*-expressed eGFP for $DHFR^{FS}$ and [$DHFR^{FS}$ & $TYMS^{SS}$] expressing T cells.

T cells from the experiment shown in **Figure 12D** were also subjected to varying concentrations of MTX. On day 35, T cells received anti-CD3/ CD28 stimulation and were subjected to a range of MTX from 0 to 1 µM for 72 hours. On day 35, no T cell group significantly expressed $DHFR^{FS}$, as indicated by co-expressed eGFP, above background (**Figure 12D – I**). However, $DHFR^{FS+}$ T cells selected with MTX alone persisted enough to significantly improve survival when MTX was re-introduced at concentrations up to 0.5 µM MTX (**Figure 16B**). Flow plots in **Figure 16A** demonstrate MTX-dependent increases in transgene expression and improved survival for transgene expressing T cells for one donor. It should be noted that the addition of $TYMS^{SS}$ in [$DHFR^{FS+}$ & $TYMS^{SS}$]$^{+}$ T cells permitted the survival of transgene negative cells at 1 µM MTX, which was not seen in $TYMS^{SS\ neg}$ T cells subjected to MTX (**Figure 16C**). Presumably, $TYMS^{SS}$ re-established thymidine synthesis in the presence of MTX and equilibrative thymidine transport permitted transgene negative T cells to restore DNA synthesis and survive. MTX-inducible up-

Figure 16

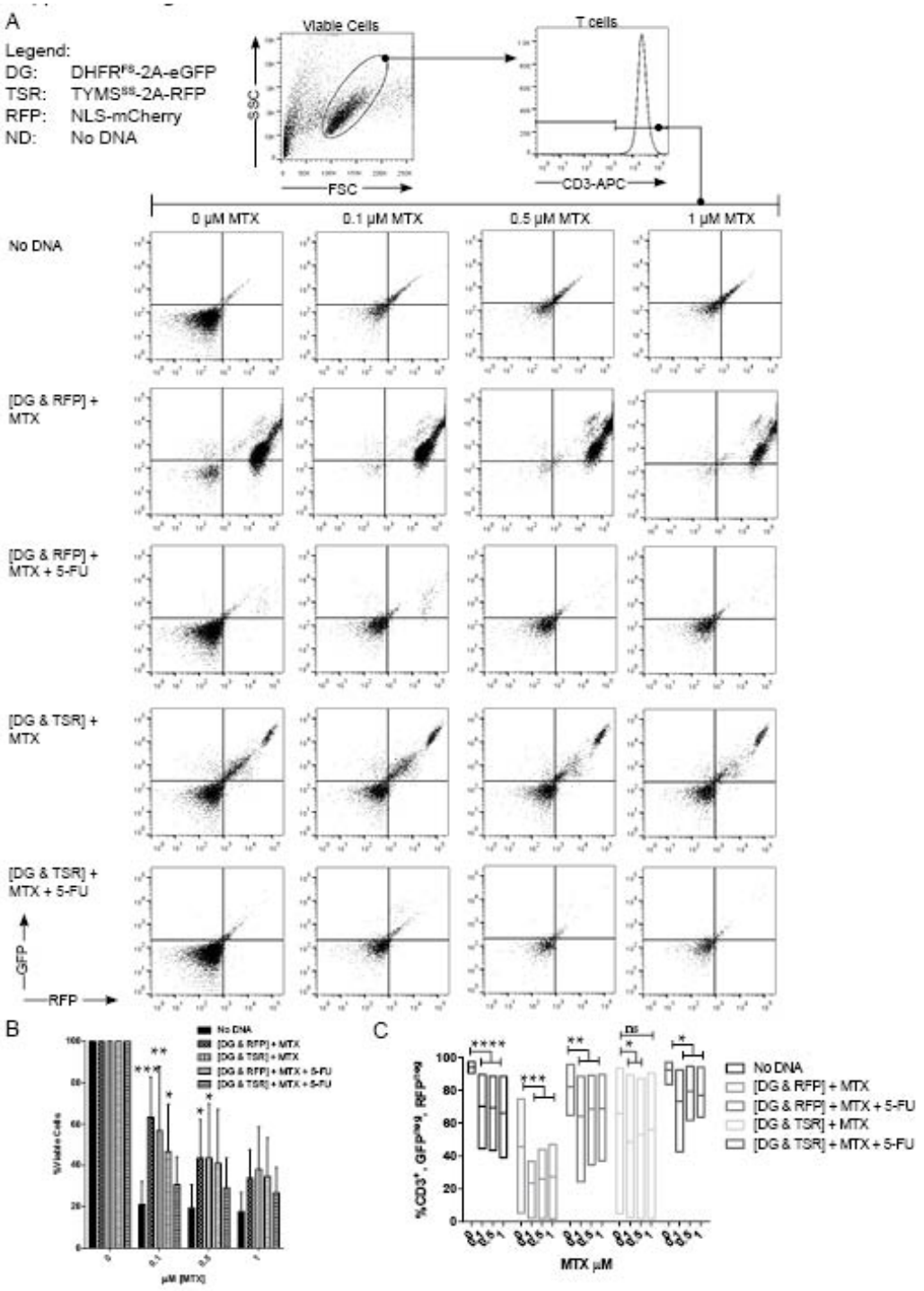

A
Legend:
DG: DHFR^FS-2A-eGFP
TSR: TYMS^SS-2A-RFP
RFP: NLS-mCherry
ND: No DNA
Viable Cells
SSC
FSC
T cells
CD3-APC
0 µM MTX
0.1 µM MTX
0.5 µM MTX
1 µM MTX
No DNA
[DG & RFP] + MTX
[DG & RFP] + MTX + 5-FU
[DG & TSR] + MTX
[DG & TSR] + MTX + 5-FU
GFP
RFP
B
No DNA
[DG & RFP] + MTX
[DG & TSR] + MTX
[DG & RFP] + MTX + 5-FU
[DG & TSR] + MTX + 5-FU
% Viable Cells
µM [MTX]
C
%CD3+, GFP^FS, RFP^SS
MTX µM
No DNA
[DG & RFP] + MTX
[DG & RFP] + MTX + 5-FU
[DG & TSR] + MTX
[DG & TSR] + MTX + 5-FU

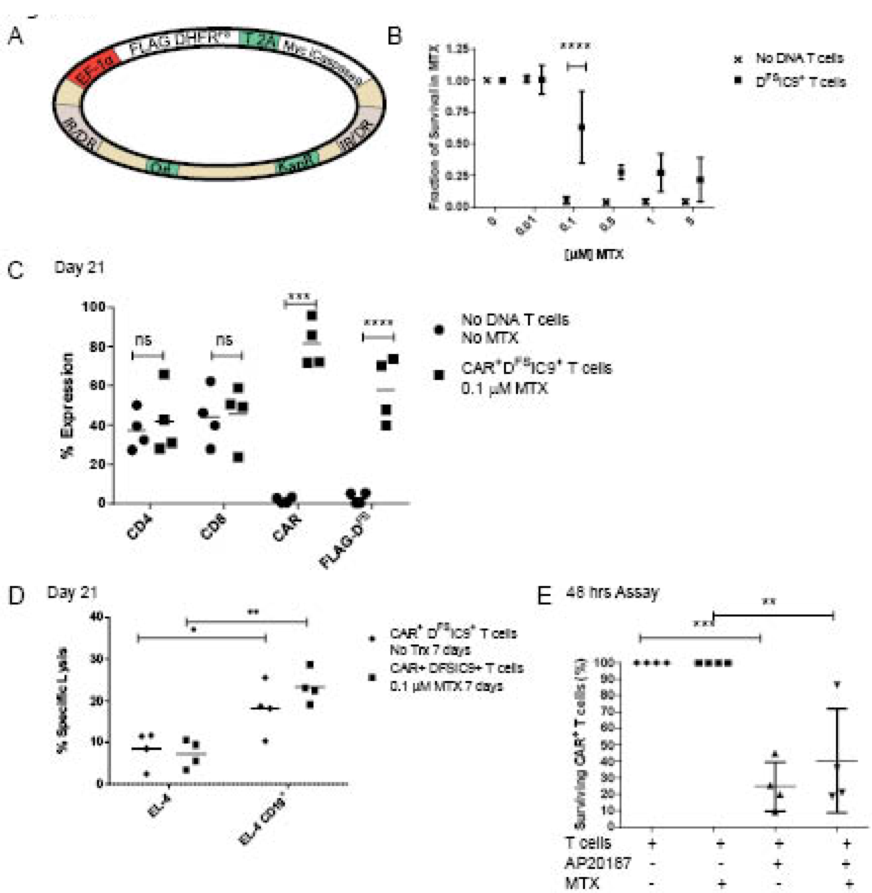
Figure 16 AThyR rescue of AThyR^{+} and AThyR^{neg} T cells following 72 hours treatment in MTX. T cells from the experiment described for **Figure 12D** were stimulated on day 35 with anti-CD3, anti-CD28, and IL-2 along with varying doses of MTX [0, 0.1, 0.5, 1 µM] for 72 hours. The gating strategy and representative flow plots are shown in (**A**). While enhanced viability of AThyR+ T cell cultures is shown in (**B**). In (**C**), Viable, CD3^{+}, GFP^{neg}, RFP^{neg} T cells (AThyR^{neg}) were assessed for survival. Each experiment was independently repeated at least twice with 6 biologic replicates total. Representative flow plots from one are depicted; ns = no significance; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; **** = $p < 0.0001$.

regulation of eGFP co-expressed by $DHFR^{FS}$ and blunting of this eGFP up-regulation by $TYMS^{SS}$ was again observed (**Figure 14E – I**). Of note, the RFP co-electroporated with $DHFR^{FS}$ up-regulated in increasing concentrations of MTX, whereas RFP co-expressed by $TYMS^{SS}$ did not (**Figure 14E – II**). This further indicates that it is the absence of thymidine which leads to increases in co-expressed transgene.

AThyR permits independent selection for transgenes of interest.

AThyRs are human proteins and therefore have lower immunogenicity in humans than NeoR or similar drug resistance transgenes, typically originating from bacteria.[45] Thus, using AThyRs to select transgenes of interest is desirable due to lower immunogenicity, and ease of use *in vitro*. As a proof-of-principle, the suicide gene inducible caspase 9 (iC9) was selected by co-expressing iC9 with $DHFR^{FS}$ in a construct designated D^{FS}iC9 **(Figure 17A)**. Current methods to select iC9 utilize surface-expressed antigen and isolation by magnetic beads.[35] However, this method of selection is more labor intensive than adding drug and does not add the functionality of AThy resistance. The D^{FS}iC9 plasmid significantly selected for survival in T cells after 7 days of AaPC based stimulation including days 2 - 7 days in 0.1 μM MTX (**Figure 17B**). Next, D^{FS}iC9 was co-electroporated with CAR to express in T cells. The CAR was specifically selected by a CAR exodomain binding ligand $(CARL)^{+}$ K562 AaPC,[95] while D^{FS}iC9 was selected using 0.1 μM MTX. After days 2 - 14 in 0.1 μM MTX, CAR^{+} $D^{FS}iC9^{+}$ T cells were rested from MTX or selected for another 7 days in 0.1 μM MTX. T cells selected in 0.1 μM MTX from day 2 - 21 are shown in **Figure 17C** compared to mock-electroporated T cells. As before, there is no selection towards $CD4^{+}$ T cell predominance following MTX selection by day 21.

Figure 17



A
EF-1α
FLAG DHFR
T 2A
Myc iCasp9
IR/DR
Ori
KanR
B
Fraction of Survival in MTX
[μM] MTX
No DNA T cells
D^{FS}IC9+ T cells
C
Day 21
% Expression
ns
CD4
CD8
CAR
FLAG-D^{FS}
No DNA T cells
No MTX
CAR+ D^{FS}IC9+ T cells
0.1 μM MTX
D
Day 21
% Specific Lysis
EL-4
EL4 CD19+
CAR+ D^{FS}IC9+ T cells
No Trx 7 days
CAR+ DFSIC9+ T cells
0.1 μM MTX 7 days
E
48 hrs Assay
Surviving CAR+ T cells (%)
T cells
AP20187
MTX

Figure 17 AThyRs select for transgenes of interest. The superior selection of $DHFR^{FS}$ is desirable for difficult to isolate genes of interest such as suicide genes. Suicide gene inducible caspase 9 (iC9) was designed to express with $DHFR^{FS}$ in the plasmid $D^{FS}iC9$ shown in (**A**). (**B**) This construct was tested in PBMC of 3 healthy donors stimulated with a 1: 1 ratio of OKT3-loaded AaPC and treated with MTX from day 2 until day 7 when survival is shown. (**C**) T cells were electroporated with CD19-specific chimeric antigen receptor (CAR), $D^{FS}iC9$, and SB transposase and expanded on $CARL^{+}$ K562 in the presence of MTX for 21 days to select for each transgene. The expression of costimulatory T cell receptors CD4, CD8, and transgenes CAR and $DHFR^{FS}$ are shown in 21 day CARL expanded transgenic T cells in comparison to mock electroporated T cells expanded on OKT3-loaded AaPC clone.4. (**D**) The effect of MTX on cytotoxicity in $DHFR^{FS+}$ CAR^{+} T cells was tested by stimulating CAR^{+} T cells in the presence or absence of MTX for 7 days after stimulation on day 14. Cytotoxicity was assessed by CRA on Day 21 using CD19 positive or CD19 negative murine lymphoma EL-4 cells. T cells were co-incubated with EL-4 at a 1 target: 5 effector ratio. (**E**) The functionality of iC9 was assessed on day 21 by resting T cells for 48 hours in 10 nM AP20187. T cells had previously been stimulated for 7 days in the presence or absence of MTX. Comparison of surviving CAR^{+} T cells is made to matched, un-treated cells. Experiments in **C-E** were performed with 4 normal donors and repeated twice. Significance for each comparison was initially determined by Two-Way ANOVA followed by Sidak's post-hoc analysis; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

These cells also demonstrated cytotoxicity at the levels expected for the given 5 : 1 target to effector ratio (**Figure 17D**).[95] These findings agree with that of Jonnalagadda *et al.* who linked $DHFR^{FS}$ to CAR expression and noted cytotoxicity independent of MTX treatment.[82] Co-expressing $DHFR^{FS}$ with iC9 rather than CAR added the potential to ablate T cells through the addition of iC9 chemical inducer of dimerization AP20187 (**Figure 17E**). The addition of AP20187 significantly depleted resting CAR^{+} T cells independent of MTX. This demonstrates that D^{FS}iC9 can effectively select for iC9 expression and deplete genetically-modified T cells as necessary. The use of $DHFR^{FS}$ has the advantage of selecting transgene expression in T cells independent of antigen-specificity and antigen expression, making $DHFR^{FS}$ a more portable tool for use in a variety of T cell studies.

DISCUSSION:

Our work establishes 3 findings relevant to investigators designing genetically-modified T cell studies; 1) AThyRs rescue T cells from AThy toxicity mediated by 5-FU and anti-folates targeting DHFR and TYMS. 2) $DHFR^{FS}$ permits MTX-inducible increase in transgene expression that is thymidine dependent, and $TYMS^{SS}$ permits MTX-inducible decrease in transgene expression that is dihydrofolate dependent. 3) AThyRs can be used to positively select for transgenes of interest without the use of immunogenic genes or magnetic selection.

To the first point, we demonstrate that combining AThyRs $DHFR^{FS}$ and $TYMS^{SS}$ leads to significant survival advantages for T cells treated with toxic concentrations of AThys: MTX, Pem, or 5-FU. These AThy drugs are regularly used to treat lung and colon cancer among

other common cancers, [85, 86] and our findings indicate that AThyRs can survive toxic AThy concentrations. It has been shown that myeloid derived suppressor cells, which suppress T cell targeting of cancer cells, are selectively depleted by 5-FU.[102] Likewise, in a mouse model combining 5-FU with an immunotherapeutic strategy, it was shown that 5-FU increases Fas expression on cancer cells and leads to improved targeting of cancer cells when the two therapies were combined, but not when either strategy was used alone.[103] Thus, combining the immunomodulatory effects of chemotherapy like 5-FU with T cells resistant to the cytotoxic effects of 5-FU could substantially improve the anti-cancer response of the patient above that of either therapeutic used alone. This supports further *in vivo* study of an autologous tumor and T cell system where the tumor is concurrently treated with AThys and AThyR+ T cells. An *in vivo* model should resolve the issue of whether $CD4^+$ T cell predominance in AThyR+ T cells persists and if it leads to any detrimental consequences of tumor clearance. As for the use of AThyRs to prevent anti-folate toxicity from MTX or Pem, we establish that MTX is more toxic to T cells than Pem and find that MTX susceptibility to < 1 μM MTX could be completely abrogated by the codon optimization of $DHFR^{FS}$ or by the addition of $TYMS^{SS}$ to $DHFR^{FS}$ in T cells. Concentrations of up to 1 μM MTX are achieved during the treatment of rheumatoid arthritis.[104] Higher doses of MTX are achieved in cancer chemotherapy (> 1mM MTX) with the use of leucovorin.[105] Leucovorin rescues thymidine synthesis through the same pathway as combination $DHFR^{FS}$ and $TYMS^{SS}$. Thus, $[DHFR^{FS}$ & $TYMS^{SS}]^+$ T cells will likely resist cytotoxicity induced by the range of MTX experienced for both immune suppression and cancer treatment, but this remains to be tested in *in vivo* models.

Figure 18

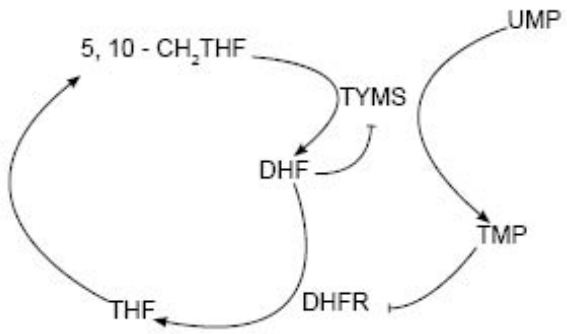


Figure 18 Post-transcriptional regulation of thymidine synthesis locks expression of DHFR to TYMS. MTX-induced increases in DHFR expression were inhibited by restoration of thymidine synthesis (TMP – thymidine monophosphate from UMP - uridine monophosphate). Likewise, MTX-induced decreases in TYMS expression were restored to normal levels by the restoration of DHFR activity reducing DHF – dihydrofolate to THF - tetrahydrofolate. These findings link expression of transgenic DHFR with transgenic TYMS. 5, 10 – methylenetetrahydrofolate (5,10 CH_2THF)

With regard to the second finding, we establish that MTX can be used to regulate transgene expression either to higher or lower expression levels for a transgene expressed *cis* to $DHFR^{FS}$ or $TYMS^{SS}$, but not [$DHFR^{FS}$ & $TYMS^{SS}$]. We note that the increased expression of eGFP expressed *cis* to $DHFR^{FS}$ was blunted by the restoration of thymidine synthesis by $TYMS^{SS}$. Likewise, we found that the decreased expression of eGFP expressed *cis* to $TYMS^{SS}$ was lost when $DHFR^{FS}$ permitted the reduction of dihydrofolate. **Figure 18** depicts this relationship of the metabolite of one enzyme to the expression of the other. From this interrelationship it becomes clear that DHFR and TYMS expression is linked. Thus, there is evidence that the correlated expression of $DHFR^{FS}$ and $TYMS^{SS}$, noted in **Figure 11C,** is biologically linked. The influence of TYMS activity on DHFR expression[92] and the influence of DHFR activity on TYMS expression[96] are known, but the functional linkage in expression between DHFR and TYMS has not been examined to our knowledge. Clinical utility of the MTX-inducible increase or decrease of transgenes co-expressed *cis* to AThyR transgene is unknown. However, earlier studies in mice utilized transgenic tumor cells containing native DHFR linked to HSV-TK1 and found a 1.5- 4 fold increase of HSV-TK1 functionality with the addition of MTX.[98] Therefore, MTX-inducible positive or negative modulation of *cis*-transgenes could lead to clinically useful methods where MTX is used to modulate the spatial and temporal expression of dangerous but necessary transgenes, such as certain CAR or cytokine.[25] The correlated expression of $DHFR^{FS}$ with *trans* expressed $TYMS^{SS}$ may also have clinical utility in expressing proteins such as TCR α and β that need to be expressed at nearly equivalent

amounts and where the use of 2A mediated cleavage sites may adversely affect protein structure and function.[106]

For the purpose of selecting genes of interest, AThyRs were compared to one of the earliest drug resistance transgenes – NeoR.[107] This was also the first drug resistance transgene used in humans.[43] It was found that $DHFR^{FS}$ is superior to NeoR in promoting survival, selection, and drug-dependent increases of expression for eGFP. Notably, $DHFR^{FS}$ and $TYMS^{SS}$ have lower immunogenicity as human proteins, and MTX can be used both *in vitro* and *in vivo* [46] to improve transgene selection whereas G418 cannot.[108-110] Our findings that $DHFR^{FS}$ can select for cells expressing important transgenes such as the suicide gene iC9 makes $DHFR^{FS}$ and [$DHFR^{FS}$ & $TYMS^{SS}$] a reasonable alternative to magnetic beads for selecting a gene or genes of interest. In fact, the potential to select for AThyR+ T cells *in vivo* using MTX suggests that transgene selection could be performed within the patient rather than *ex vivo*.

In conclusion, the use of AThyR transgenes $DHFR^{FS}$ and $TYMS^{SS}$ alone or in combination provides a unique capacity to select for transgene expression within the bulk population, modulate the expression of *cis* as well as *trans* transgenes, and promote survival in toxic concentrations of AThys. Our findings broaden the potential use of $DHFR^{FS}$ in combination with $TYMS^{SS}$ to cancers such as lung, colon, breast, and pancreas that are in dire need of new therapeutic options. In a future application we seek to combine AThyRs with pancreatic cancer specific T cells to enhance anti-tumor efficacy.

CHAPTER 4: Development of pharmacological approaches to select or deplete T_{regs}

INTRODUCTION:

Regulatory T cells (T_{reg}) are a subset of T cells vital to the balance of the adaptive immune response. If T_{reg} are deficient or defective, then autoimmunity develops.[111] If T_{reg} are dominant within an environment, then immune-tolerance permits the outgrowth of tumors.[112] Thus, control of T_{reg} is desirable for the treatment of a large number of human diseases falling under the category of autoimmunity or cancer. However, the rarity of this T cell subset[11] and the challenging nature of selecting for these cells, which are characterized by the expression of an intracellular transcription factor –FoxP3- in addition to other markers,[111, 113] makes studying how these cells are selectively expanded or depleted a difficult task.

Selection of T_{reg} is desirable for the treatment of autoimmunity and occurs with several therapeutics by differing mechanisms of action such as irradiation,[114] MTX, 5- fluorouracil (5-FU),[115] 5-azacytidine,[116] and cilostamide.[117] These findings were described *in vitro* and *in vivo* using rodent models.[116-120] In humans, an increase in T_{reg} was noted following the administration of multi-drug regimens to treat cancer.[121] A shared mechanism of action for preferential selection of T_{reg} is not clear, but MTX appears to be a strong candidate for understanding how T_{reg} selection is occurring. MTX has a known role in inhibiting purine metabolism [86] similar to cilostamide, thymidine synthesis similar to 5-FU, [86] and DNA methylation[122] similar to 5-azacytidine, each of which selects for T_{reg} as noted. MTX inhibits *de novo* purine synthesis upstream at Glycinamide ribonucleotide transformylase (GART),

downstream at 5-aminoimidazole-4-carboxamide riboside (AICAR) transformylase (AICARtf), *de novo* thymidine synthesis at thymidylate synthase (TYMS), and single carbon metabolism, which includes methylation, at dihydrofolate reductase (DHFR) (See **Figure 19A**).[86] Since MTX is first-line therapy in the treatment of severe rheumatoid arthritis[123] and GvHD prophylaxis,[124] discerning how MTX influences T_{reg} selection is of clear importance.

Depletion or blocking of T_{reg} is currently most effective using biological agents in the treatment of cancer.[112] This is exemplified by anti-CTLA-4 therapy, [125] which depends in part upon the inhibition of T_{reg}.[126] Among non-biological agents, cyclophosphamide is thought to deplete T_{reg} [112, 127, 128] in the treatment of cancer.[129, 130] However, this finding may be dose and time dependent[128][131] The mechanism of cyclophosphamide induced T_{reg} depletion may be related to adenosine monophosphate (AMP) Kinase (AMPK) as cyclophosphamide is known to deplete levels of ATP within $T_{reg,}$[127] and low levels of ATP are known to activate AMPK.[132] Recent findings of T_{reg} depleting gene targets 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase [133] and p300 [134] are also AMPK targets – further implicating AMPK in a pathway of T_{reg} survival. In this study, we demonstrate AMPK signaling is enhanced in activated T_{reg}, and we further demonstrate that AMPK target ribosomal translation via eukaryotic elongation factor – 2 (eEF2).[135][136] Interestingly, MTX potentiates activation of AMPK via the inhibition of AICARtf.[137] This suggests that an AMPK – eEF2 axis may be involved in the selection or depletion of T_{reg}.

To determine the influence of MTX on T_{reg} selection, we utilize $DHFR^{FS}$ and $TYMS^{SS}$ capable of resisting MTX and 5-FU targeting of native DHFR or TYMS, respectively. We find

that alleviating thymidine blockade of MTX promotes T_{reg} expansion, likely through inhibition of purine synthesis. Further analysis of $T_{CD4,\ FoxP3}$ selection by MTX and toxic agents targeting similar pathways suggests that inhibited purine synthesis is not the primary mechanism of T_{reg} selection. Rather, inhibition of T_{reg} replication by inhibited ribosomal protein translation appears to mediate selection in chemotherapy. Multiple drugs are known to inhibit ribosomal synthesis and translation,[99, 136] and rapamycin, a drug commonly used in T_{reg} selection,[138] also inhibits translational initiation and ribosomal synthesis.[139] Our study elucidates a common pathway for the selection and depletion of T_{reg}, and consequently develops a novel method to transgenically improve selection of T_{reg} as well as a biochemical method to deplete T_{reg}.

MATERIALS AND METHODS:

Cells and culture conditions:

Cells: Healthy donor derived peripheral blood from MDACC Blood Bank, Houston, Texas, was subjected to density gradient centrifugation to isolate mononuclear cells which were either rested in complete media (CM) or frozen as previously outlined. The use of rested or frozen peripheral blood derived mononuclear cells (PBMC) is outlined in each experiment. T cells from PBMC were stimulated using thawed OKT3 antibody-loaded K562 clone #4, an activating and propagating cell (AaPC).[94] The presence of mycoplasma was tested in AaPC before stimulation of T cells. Cell counting was accomplished by 0.1 % Trypan Blue (Sigma-Aldrich, T8154) exclusion using automated cell counting (Nexcelcom, Lawrence, MA). Cell Isolation was accomplished using magnetic bead based sorting with the $CD4^+$, $CD25^+$ Regulatory T Cell

Isolation Kit following the manufacturer's instructions (Miltenyi Biotec, San Diego, CA, 130-091-301). Briefly, $CD4^+$ T cells were negatively selected before sorting one time with anti-CD25 beads was used to differentiate between effector T cells ($CD25^{neg}$) and T_{reg} ($CD25^{pos}$).

***Culture Conditions*:** Acellular stimulation was accomplished as previously described using soluble anti-CD3 – 30 ng/ mL, anti-CD28 – 100 ng/ mL, and human IL-2 – 50 IU/ mL, as previously described . When indicated, the following drugs were used: 5-FU, MTX, cisplatin (CDDP), pemetrexed, raltitrexed, G418, hygromycin B, zeocin, rapamycin, metformin, AICARtf / inosine monophosphate (IMP) cyclohydrolase (ATIC) dimerization inhibitor (iATIC) (**Table 8**). Acellular stimulation experiments received addition of toxic drug or treatment on the same day as stimulation.

DNA expression plasmids:

Selection vectors: FLAG-$DHFR^{FS}$-2A-eGFP pSBSO (noted as $DHFR^{FS}$-GFP (DG)), FLAG-$TYMS^{SS}$-2A-eGFP pSBSO (noted as $TYMS^{SS}$-GFP (TSG)), NLS-mCherry pSBSO (RFP), FLAG-$TYMS^{SS}$-2A-NLS-mCherry pSBSO (noted as $TYMS^{SS}$-RFP (TRG)), Neomycin Resistance (NeoR)-2A-eGFP pSBSO (noted as NeoR-GFP (NRG)), and Myc-ffLuc-NeoR pSBSO (NRF), were designed constructed and utilized as previously described . Sleeping Beauty (SB) indirect/ direct repeat (IR/DR) sites were present in each construct to induce genomic integration with SB transposase. Each transgene was expressed by elongation factor 1 alpha (EF1α) promoter.

Table 8 Chemical Agents

Agent	Manufacturer	ID No.
5-fluorouracil	APP Pharmaceuticals, Schaumburg, IL	NDC 63323-117-10
Methotrexate	Hospira, Lake Forest, IL	NDC 61703-350-38
CDDP	Pfizer, New York, NY	NDC 0069-0084-07
Pemetrexed	Lilly, Indianapolis, IN	NDC 0002-7640-01
Raltitrexed	Abcam Biochemicals, Cambridge, MA	Ab142974
iATIC	EMD Millipore	118490
G418	Invivogen, San Diego, CA	Ant-gn-1
Hygromycin	Invivogen	Ant-hg-1
Zeocin	Invivogen	Anti-zn-1
Rapamycin	Wyeth, Philadelphia, PA	NDC 0008-1030-04

Genetic Transformation and Propagation of Cells:

The Amaxa Nucleofector® II was utilized to transform human PBMC, where 1-2*10^7 thawed PBMC were electroporated in Amaxa T cell Nucleofector solution using program U14, as previously described . The next day, PBMC were stimulated with CM with AaPC at a ratio of 1: 1 including 50 IU/ mL IL-2. The co-culture of T cells and AaPC was maintained at 1*10^6 cells/ mL with each subsequent stimulation. Outgrowth of T cells was promoted by re-stimulated of co-cultures every 7 days with IL-2 and AaPC at the concentrations noted. Fresh IL-2 was added when media was changed between stimulations. During transgenic experiments, drugs were added 48 hours after co-culture initiation and maintained at the given concentration until day 14. After day 14, no drugs were added to T cell cultures.

Western blot:

When noted, T cells were removed from cultures for western blot by centrifugation of 1*10^6 T cells, and rapid freezing of the cell pellet in liquid nitrogen. T cell pellets were lysed and prepared with 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 1mM phenylmethylsulfonyl fluoride, 150 mM p-nitrophenyl phosphate and 0.3 μM Aprotinin, pH 7.4. SDS-PAGE separated proteins and primary antibodies noted in **Table 9** were used to detect the presence of protein via chemiluminescence.

Table 9 Western Blot Antibodies

Antibody	Manufacturer	Cat. No.	Dilution
AMPKα	Cell Signaling Technology (CST), Danvers, MA	2603S	1: 1000
p-AMPKα (T172)	CST	2535S	1: 1000
S6	CST	2317S	1: 1000
p-S6 (S235/236)	CST	3945S	1:1500
Actin	Sigma	A2228	1:10000
Hsp-70	Santa Cruz Biotechnology, Dallas, TX	SC-24	1:5000
eEF2 p-eEF2 (T56)	LifeSpan Biosciences, Seattle, WA LifeSpan Biosciences	LS-B8940 LS-C198899	

Flow cytometry:

Cultured T cells were washed in FACS staining solution[95] before surface antibody staining was performed in FACS staining solution with fluorochrome-conjugated antibodies at 4º C for at least 30 minutes. Intracellular transcription factor and cytokine staining utilized the FoxP3 / transcription factor staining buffer set manufacturer's protocol (eBioscience, 00-5523-00), and was performed following surface staining. The BD FACSCalibur (BD Biosciences) analyzed most samples expressing FoxP3. Antibody targets, concentrations, and manufacturers are listed in **Table 10**. Flow cytometry data analysis utilized FlowJo v 10.0.5 (Tree Star Inc., Ashland, OR). Flow cytometric imaging of cells stained for phosphorylated antigens was accomplished using the ImageStream^X Mark II (Amnis, Seattle, WA) with the following protocol; after surface staining, samples were fixed in 100% methanol (Sigma) for 1 hour at 4º C before washing and staining in FoxP3 / transcription factor staining buffer set wash buffer as outlined by the manufacturer's protocol. Analysis of image cytometry data utilized Amnis IDEAS v 6.0.

Thymidine Incorporation Assay:

A thymidine incorporation assay was performed with anti-CD3/ CD28 and IL-2 used to stimulate each well containing 2 * 10^5 viable cells. Varying ratios of effector T cells (T_{eff}) to T_{reg} were combined in each well and all wells were run in triplicate in U-bottom 96 well plates. At 48 hours 1 µCi [^{3}H] Thymidine (Perkin-Elmer, Waltham, MA) was added to each well, and 24 hours later the cells were assessed for radioactivity on a Top Count NXT (Perkin-Elmer). T_{reg}

mediated suppression of growth was determined by the following equation: (No Treatment T_{eff} [cpm] - (T_{reg} & No Treatment T_{eff} [cpm])) / No Treatment T_{eff} [cpm].

Statistical Analysis:

Graphical representation and statistical analysis of data was performed with Prism v6.0 (Graph Pad Software Inc., La Jolla, Ca). One-Way ANOVA was used when appropriate with Tukey's or Dunnett's multiple comparison tests as applicable, non-Gaussian distributions were assessed by the Kruskall-Wallis test followed by Dunn's multiple comparison test. Total cell counts and expression data involving $T_{CD4,\ FoxP3}$ tended to be non-Gaussian in distribution. Single variable tests (experimental vs. control) were made using the Mann-Whitney test. Statistical significance was designated as $\alpha < 0.05$.

Table 10 Flow Cytometry Antibodies

Antibody	Manufacturer	Cat. No.	Dilution
CD3-APC	BD Pharmingen	340661	1:33
CD3-PerCP-Cy5.5	BD Pharmingen	340949	1:33
CD4 FITC	BD Pharmingen	340133	1:33
CD4 -PE	BD Pharmingen	347327	1:33
CD4-PerCP-Cy5.5	BD Pharmingen	341645	1:33
CD8-APC	BD Pharmingen	340659	1:33
CD25-APC	BD Pharmingen	555434	1:33
CD39-APC	BD Pharmingen	560239	1:33
CD45RO-APC	BD Pharmingen	559865	1:33
CD152-APC	BD Pharmingen	555855	1:33
KI-67-AF647	BD Pharmingen	561126	1:50
Annexin V	BD Pharmingen	556422	1:20
7-AAD	BD Pharmingen	559925	1:20
Propidium Iodide	BD Pharmingen	556463	
FoxP3-PE	eBiosciences	12-4777-42	1:20
Helios-APC	Biolegend	137222	1:05
LAP-APC	Biolegend	349608	1:20
IFN-g-APC	Biolegend	502516	1:20

IL-2-APC	Biolegend	500315	1:20
p-eEF2 (T56)	LifeSpan Biosciences	LS-C198899	1:20
p-AMPKα (T172)	AbCam	Ab133448	1:20
CD4-Pacific Blue	BD Pharmingen	558116	1:33
p-S6 (S244) – AF647	BD Pharmingen	560465	1:20
Goat anti-Rabbit – AF488	Life Technologies	A-11034	1:100

RESULTS:

Drug selection of $T_{CD4, FoxP3}$ by MTX occurs in part through toxicity.

In order to determine how MTX contributes to the selection of $T_{CD4, FoxP3}$, freshly derived PBMC were stimulated with anti-CD3/ CD28 antibodies and IL-2 in the presence of cytotoxic drugs or lethal γ-irradiation. After 7 days there was a significant difference in survival markers Annexin V and 7-AAD in stimulated T cells receiving any cytotoxic insult with stimulation (**Figure 19B-I**). The selection of $T_{CD4, FoxP3}$ was not as consistent as cytotoxicity. Following 7 days of stimulation, 2 Grey γ-irradiation significantly increased the amount of $T_{CD4, FoxP3}$ in the surviving population (**Figure 19B-II**). This lethal treatment did not target a common pathway being considered, nor did cisplatin, yet both increased $T_{CD4, FoxP3}$. However, the $T_{CD4, FoxP3}$ increase induced by cisplatin is insignificant. The only significant increases derived from drugs in this experiment were 5-FU and MTX, recapitulating earlier findings.[115] With the exception of ribosomal elongation inhibitor G418,[140] each cytotoxic treatment appeared to increase the percentage of surviving $T_{CD4, FoxP3}$. This pattern of increasing $T_{CD4, FoxP3}$ percentage in the face of varied cytotoxic insult suggests a common pathway that can be enhanced by certain drugs. This pathway is likely related to the slower proliferation rate of T_{reg}, [141] and appears to be ribosomally mediated as G418 can inhibit this general trend of increasing $T_{CD4, FoxP3}$ percentage.

Figure 19

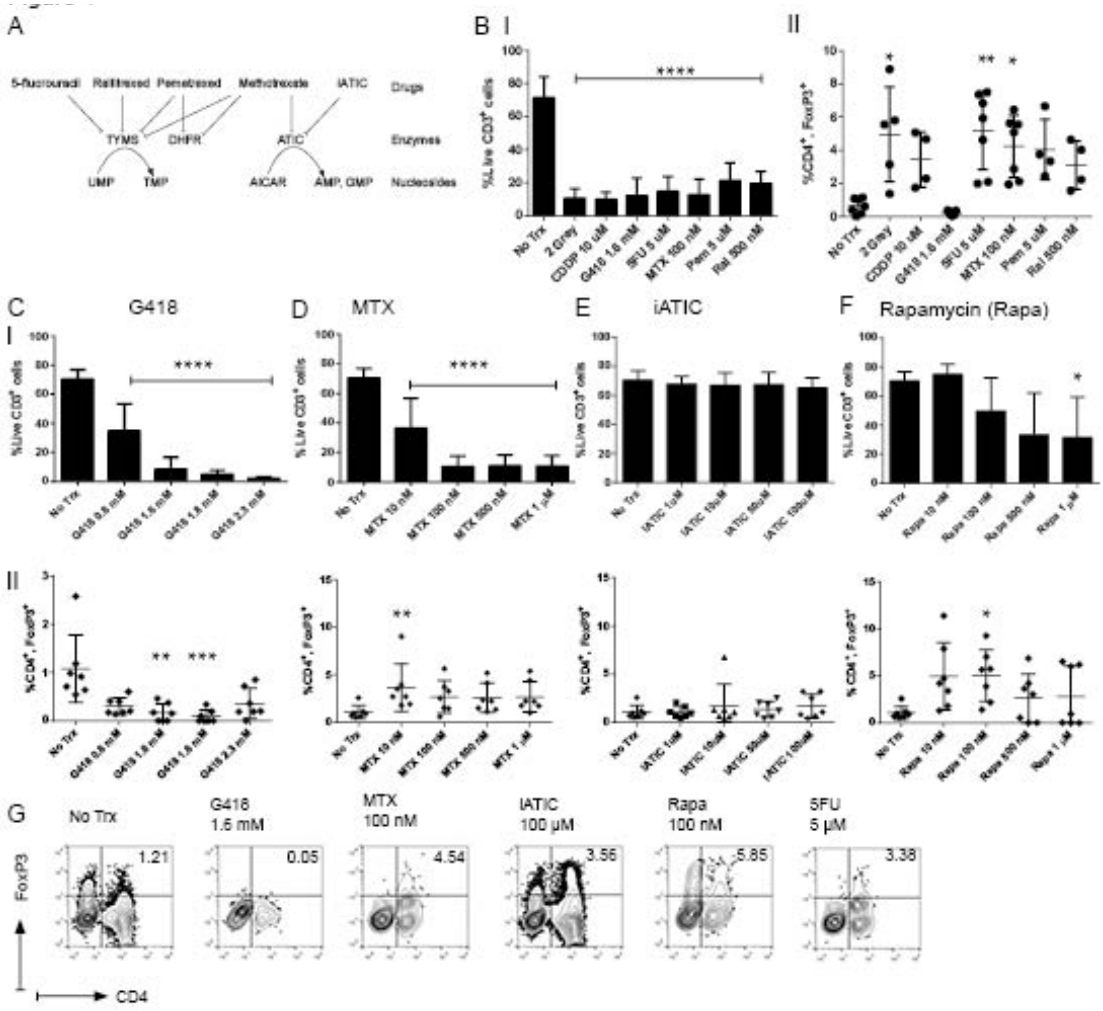

A
5-fluorouracil
Raltitrexed
Pemetrexed
Methotrexate
IATIC
Drugs
TYMS
DHFR
ATIC
Enzymes
UMP
TMP
AICAR
AMP, GMP
Nucleosides
B I
%Live CD3+ cells
****
No Trx
2 Grey
CDDP 10 uM
G418 1.6 mM
5FU 5 uM
MTX 100 nM
Pem 5 uM
Ral 500 nM
II
%CD4+ FoxP3+
C G418
D MTX
E iATIC
F Rapamycin (Rapa)
G
No Trx
G418 1.6 mM
MTX 100 nM
IATIC 100 µM
Rapa 100 nM
5FU 5 µM
1.21
0.05
4.54
3.56
5.85
3.38
FoxP3
CD4

Figure 19 Drug selection of $T_{CD4,\ FoxP3}$ by MTX occurs in part through toxicity. The known selection of $T_{CD4,\ FoxP3}$ by MTX was analyzed by targeting enzymes that contribute to the action of MTX. As $T_{CD4,\ FoxP3}$ are a rare component of PBMC, drug based inhibition was originally sought to analyze the phenomenon. Multiple drugs with actions similar to MTX were used to assay for the selection of $T_{CD4,\ FoxP3}$. In this case, γ-irradiation, G418, and cisplatin (CDDP) were used for controls as none of those treatments act on the known enzymatic targets of MTX. The association of each drug to the enzyme targets of MTX is shown in **A**. PBMC stimulated with anti-CD3/ CD28 and soluble human IL-2 were given lethal doses of each treatment and assayed after 7 days for viability **B-I**. These treatments resulted in variable selection for $T_{CD4,\ FoxP3}$ on day 7 as seen in **B-II**. The inability of folate analogs targeting DHFR, TYMS, or GARFT to significantly select for $T_{CD4,\ FoxP3}$ suggested that inhibition of ATIC contributes to this selection. A dose dependence study followed analyzing the contribution of ATIC inhibitor in the selection of $T_{CD4,\ FoxP3}$. The study in **B-II** noted that G418 depleted $T_{CD4,\ FoxP3}$, thus, this was used as a negative control while the known selection of $T_{CD4,\ FoxP3}$ by rapamycin (Rapa) was a positive control. A non-folate analog known to inhibit ATIC (iATIC) was used as a specific inhibitor of ATIC. The cytotoxicity of G418 **C-I**, MTX **D-I**, iATIC **E-I**, and Rapa **F-I** is shown in the top panel while the selection for $T_{CD4,\ FoxP3}$ is shown in the bottom panel for G418 **C-II**, MTX **D-II**, iATIC **E-II**, and Rapa **F-II**. The drug iATIC did not select for $T_{CD4,\ FoxP3}$, ruling out this pathway as the primary means of MTX selection. However, visual inspection of the flow plots for CD4 and FoxP3 expression in **G**, represented by one donor, demonstrate that FoxP3 expression was enhanced by iATIC similar to the action of Rapa, suggesting that MTX

selection relies in part on cytotoxicity and in part by inhibition of ATIC to enhance selection of $T_{CD4, FoxP3}$. All assays used 4-7 donors independently repeated 2-3 times. Statistical significance was assessed using One-Way ANOVA for viability and Kruskall-Wallis test for percentage of $T_{CD4, FoxP3}$; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

The findings of T_{reg} depletion with G418 and T_{reg} selection by MTX were further evaluated for dose dependence by stimulating thawed PBMC with anti-CD3/CD28 + IL-2 for 7 days, as before. G418 was significantly cytotoxic at all doses tested, but significantly depleted $T_{CD4,\ FoxP3}$ at two moderate drug doses (**Figure 19C**). MTX was also cytotoxic at all doses tested, but had significant elevation of $T_{CD4,\ FoxP3}$ at lower doses (**Figure 19D**). Rapamycin (Rapa) was used as a T_{reg} selection control[138] and showed similar $T_{CD4,\ FoxP3}$ selection at a moderate drug concentration independent of cytotoxicity, which only occurred at the highest doses (**Figure 19F**). The selection for or against T_{reg} at moderate drug doses rather than higher doses suggests that T_{reg} have a narrow therapeutic window for drug induced selection or depletion. A specific inhibitor of ATIC [142] was used to test whether MTX mediates selection of $T_{CD4,\ FoxP3}$ through inhibition of ATIC. Inhibition of AICARtf or the heterodimeric complex ATIC, in which AICARtf is found, increases AICAR. AICAR is known to activate AMPK,[142] and it is known that activated AMPK inhibits ribosomal activity through inhibition of mTORC1[136] and eukaryotic elongation factor 2 (eEF2).[139] **Figure 19E** demonstrates that ATIC inhibition alone was neither cytotoxic nor selective for $T_{CD4,\ FoxP3}$. Further analysis of flow plots represented by the same donor in **Figure 19G** show expression of CD4 and FoxP3 for several of the drugs used. Use of iATIC characteristically mediated increased expression of FoxP3 in $CD4^+$ T cells similar to that of Rapa, but did not inhibit proliferation of $FoxP3^{neg}$ T cells as MTX, G418, or Rapa. Thus, iATIC enhanced FoxP3 expression in $CD4^+$ T cells but diluted these cells by permitting proliferation of $FoxP3^{neg}$ T cells. It appears that MTX mediated selection of $T_{CD4,\ FoxP3}$ occurs by depletion of rapidly proliferating effector T cells and enhancement of FoxP3

expression via a pathway similar to Rapa that includes ribosomal inhibition. The increased susceptibility of T_{regs} to ribosomal inhibitor G418 solidifies this relationship between enhanced FoxP3 expression and increased susceptibility to ribosomal inhibition.

T_{regs} are preferentially expanded in primary T cells resistant to the anti-folate and anti-thymidine actions of MTX.

Prior studies by Jonnalagadda *et al.* have demonstrated the potential of $DHFR^{FS}$ to promote T cell survival in the presence of MTX.[46, 82] We previously performed selection studies of $DHFR^{FS}$ transgene in bulk human PBMC using MTX and found $CD4^+$ T cells predominance. The preference of multiple drug selection vectors to selectively propagate $CD4^+$ T cells in the same way suggested a common phenomenon. It was hypothesized that regulatory T cells were inhibiting $CD8^+$ T cells proliferation following drug selection. To test this hypothesis, drug resistant T cells were derived by transformation with $DHFR^{FS}$, $TYMS^{SS}$, NeoR, or a combination, and numerically expanded as previously described. Briefly, transformed T cells were selected in the presence of 0.1 μM MTX, 5 μM 5-FU, or 1.6 mM G418 as designated from day 2 to 14 while stimulation with OKT3-loaded AaPC and 50 IU / mL IL-2 occurred every 7 days until day 35.[94]

Initial testing for T_{regs} by elevated expression of FoxP3 in the $CD4^+$ T cell population demonstrated there was a significant $T_{CD4,\ FoxP3}$ percentage increase in $DHFR^{FS}$ expressing T cells. Selection using MTX in comparison to mock-electroporated (No DNA) T cells on Day 21 showed this increase (**Figure 20A**), and this increase persisted to Day 35 when 5-FU was combined with MTX during selection (**Figure 21A**). The transgenic T cells were almost entirely

$CD4^+$ in each experimental population after selection, but the predominance of T_{regs} appeared to often exceed the 5-10% typically found in the un-manipulated $CD4^+$ T cell compartment.[141] Markers of T_{reg} function were also assessed. Low IL-2 expression [113] is a known trait of T_{regs} and is assessed with FoxP3 expression. The percentage of the T cell population with a $FoxP3^{pos}$, $IL\text{-}2^{neg}$ expression pattern is shown in **Figure 21B**. Expression of latency associated peptide (LAP) - a part of the TGF-β complex [143] and strongly associated with activated $T_{reg,}$ and is seen in **Figure 21C**. The protein CTLA-4[113] mediates intercellular inhibition of effector T cell responses and is shown by $FoxP3^+$, $CTLA4^+$ expression in **Figure 21D**. These findings help to corroborate whether the $T_{CD4,\ FoxP3}$ in each experiment were T_{reg} as donor to donor variability and low $T_{CD4,\ FoxP3}$ numbers made suppression assays an ineffective T_{reg} measure.

The transgenes $DHFR^{FS}$ and $TYMS^{SS}$ were compared individually and in combination to the control selection vector NeoR and un-treated No DNA T cells. Selection towards T_{reg} in this experiment may be noted in **Figure 21A, B, C –I**. This experiment demonstrated that [$DHFR^{FS}$-GFP (DG) & $TYMS^{SS}$-RFP (TSR)]$^+$ T cells selected in MTX + 5-FU had an increased population of cells characteristic of T_{reg} when compared to mock-transformed T cells. To further elucidate the contribution of $DHFR^{FS}$ and $TYMS^{SS}$ to T_{reg} selection, NeoR was co-electroporated with $DHFR^{FS}$, $TYMS^{SS}$, or the combination. The addition of NeoR permitted equivalent selection of $DHFR^{FS}$, $TYMS^{SS}$, and the combination in all T cell populations. With un-transformed T cells removed, it became clear that $DHFR^{FS}$ alone, but not $TYMS^{SS}$ alone could select for cells characteristic of T_{regs} (**Figure 21A, B, and C –II**). [DG & TSR]$^+$ T cells continued to select for

Figure 20

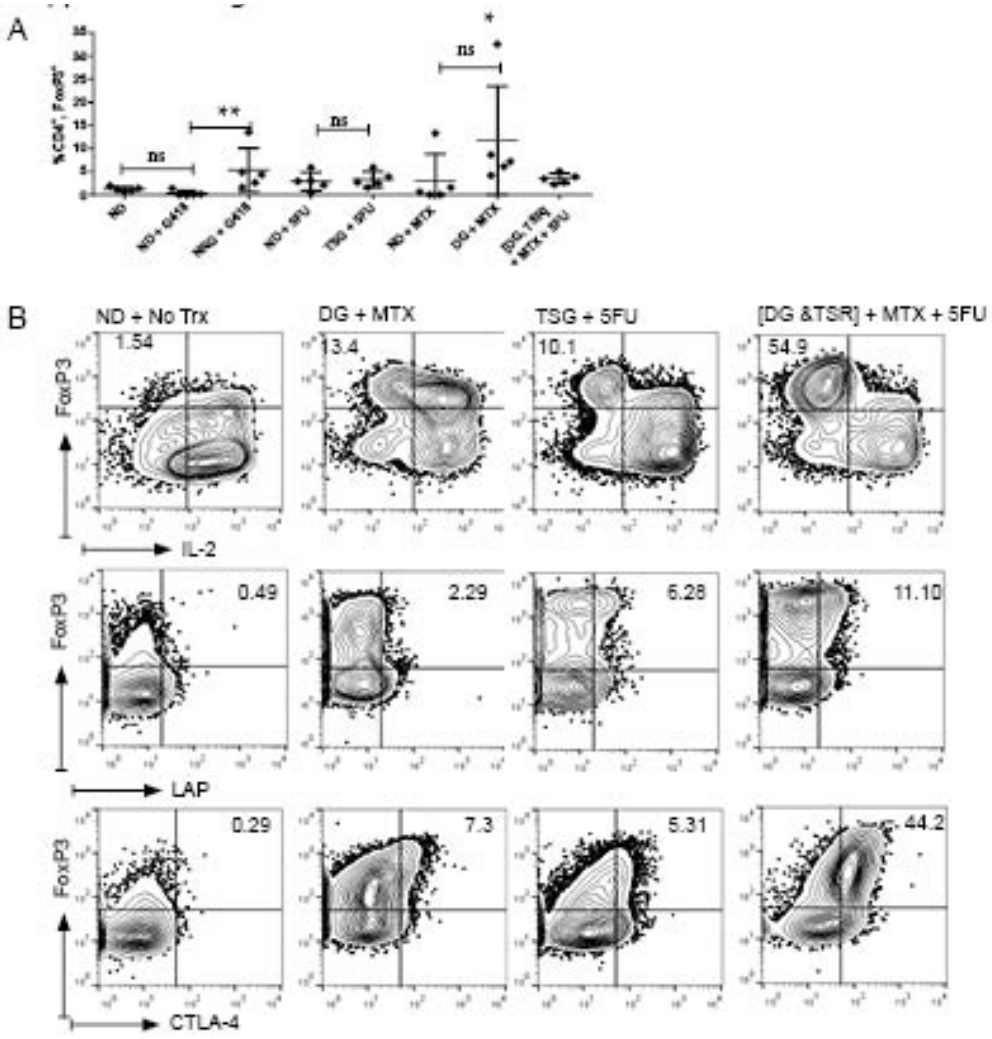

A
ns
**
ns
ns
*
B
ND + No Trx
DG + MTX
TSG + 5FU
[DG &TSR] + MTX + 5FU
1.54
13.4
10.1
54.9
FoxP3
IL-2
0.49
2.29
6.28
11.10
LAP
0.29
7.3
5.31
44.2
CTLA-4

Figure 20 Correlative findings in the selection of T_{regs} from primary T cells through resistance to the anti-DHFR and anti-TYMS actions of MTX. Selection of $T_{CD4,\ FoxP3}$ was assessed at day 21 in each experiment. The selection of $T_{CD4,\ FoxP3}$ in the experiment corresponding to column **I** of **Figure 21** is shown in **A**. It is notable for the rescue of $T_{CD4,\ FoxP3}$ with NeoR and early selection of $T_{CD4,\ FoxP3}$ with MTX selection of $DHFR^{FS}$. Flow plots in **B** show co-expression of FoxP3 with IL-2 **top row**, LAP **middle row**, or CTLA-4 bottom row for the same experiment after stimulation on Day 35. This experiment utilized 5 donors and was independently repeated twice. Significance was assessed by Two-Way ANOVA and Sidak's post-hoc; * = $p < 0.05$, ** = $p < 0.01$.

Figure 21

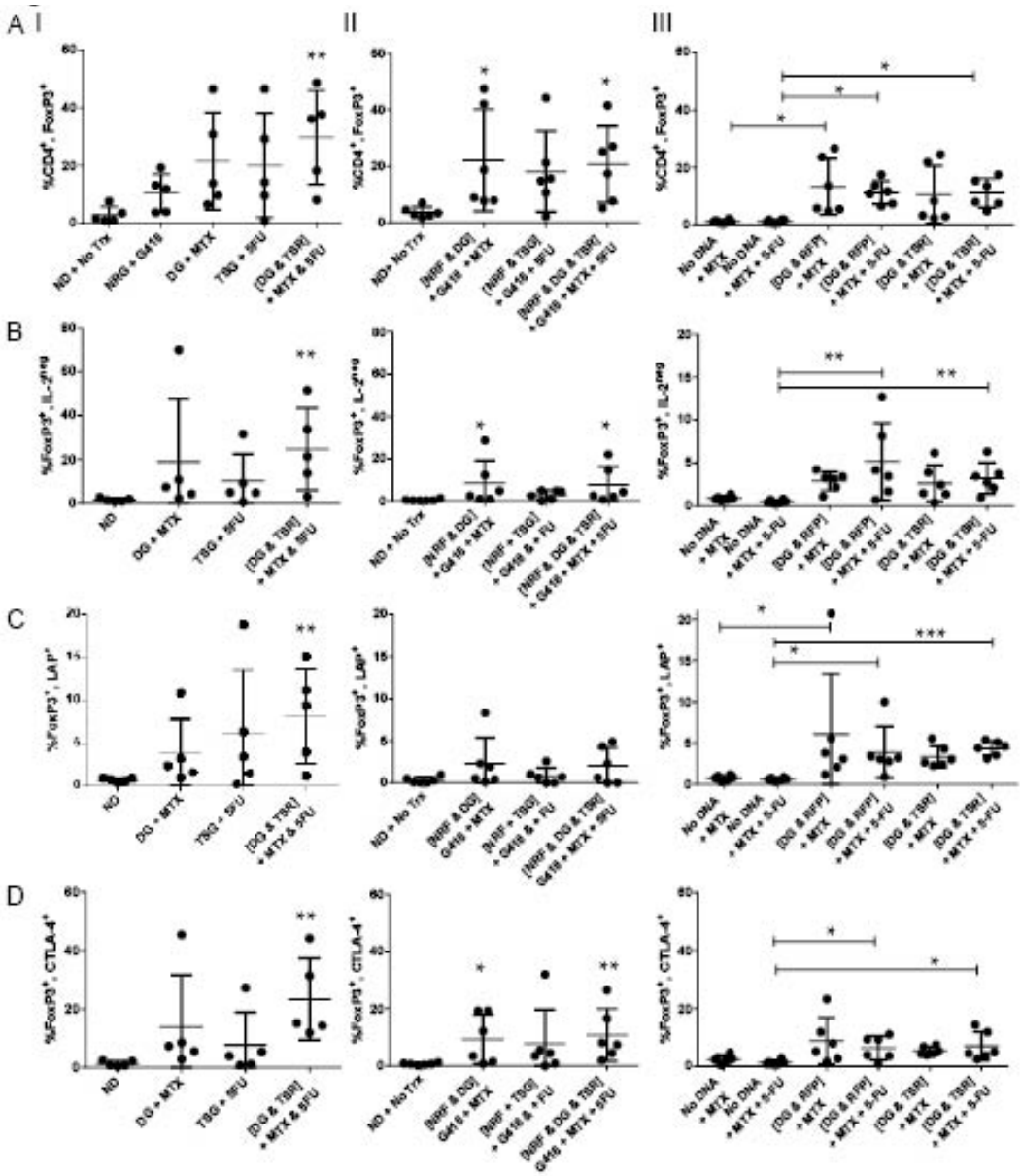

A
I
II
III
%CD4+, FoxP3+
ND + No Trx
NRG + G418
DG + MTX
TSG + 5FU
[DG & TSR] + MTX & 5FU
ND> No Trx
[NRF & DG] + G418 + MTX
[NRF & TSG] + G418 + 5FU
[NRF & DG & TSR] + G418 + MTX + 5FU
No DNA + MTX
No DNA + MTX + 5-FU
[DG & RFP] + MTX
[DG & RFP] + MTX + 5-FU
[DG & TSR] + MTX
[DG & TSR] + MTX + 5-FU
B
%FoxP3+, IL-2+/-
ND
C
%FoxP3+, LAP+
%FoxP3+, LAP-
%FoxP3+, LAP+
D
%FoxP3+, CTLA-4+

Figure 21 Primary T cells resistant to the anti-DHFR and anti-TYMS actions of MTX preferentially expand T_{regs}. Primary T cells were electroporated with $DHFR^{FS}$ and $TYMS^{SS}$ transgenes resistant to the anti-DHFR and anti-TYMS actions of MTX, respectively, in order to assess the contribution of each pathway to the selection of $T_{CD4,\ FoxP3}$. T cells were electroporated with plasmids expressing drug resistant transgenes and stimulated with artificial antigen presenting cells (AaPCs) weekly at a 1: 1 ratio. T cells were selected for 2 weeks in the combined with $TYMS^{SS}$-2A-RFP (TSR) and selected using both MTX and 5FU, or control selection vector NeoR-2A-GFP (NRG) selected with G418. Selection of $TYMS^{SS}$ by 5-FU was incomplete. Thus, ffLuc-2A-NeoR (NRF) vector was included with the MTX resistant transgenes DG, TSG, or [DG & TSR] to remove untransformed T cells in the experiments shown in column **II**. Equivalent selection for each transgene showed that MTX enhanced selected for T_{reg} in the presence of MTX resistant DHFR. It was still uncertain whether the enzymatic activity of TYMS or 5-FU played a part in the selection of T_{reg}. Therefore, the experiment shown in column **III** was performed to test the influence of TYMS inhibition in the selection of T_{reg}. Selection of T_{reg} phenotype was found to be associated with 5-FU, but independent of TYMS activity. The Kruskall-Wallis test was used to assess differences between groups for 5-6 biologic replicates and tests were independently repeated twice; * = $p < 0.05$, ** = $p < 0.01$.

cells with T_{reg} features. Finally, the contribution of $TYMS^{SS}$ to the selection of T_{reg} by $DHFR^{FS}$ was assessed by co-electroporation of TSR or a control vector- RFP. The characteristics of T_{regs} from this experiment are shown in **Figure 21A, B, and C–III**. This experiment demonstrates that selection of $DHFR^{FS}$ with MTX can enhance outgrowth of T_{reg} and that 5-FU enhances this selection independent of $TYMS^{SS}$. Selection of T_{reg} required folate rescue by $DHFR^{FS}$. This is expected as folate is known to be crucial to T_{reg} survival.[144] Surprisingly, selection of T_{reg} did not require *de novo* thymidine synthesis as $TYMS^{SS}$, which alleviates MTX and 5-FU inhibition of TYMS, was dispensable.

Previous findings showed survival and toxicity of 5-FU in PBMC is mediated by TYMS and an alternative mechanism.[145] RNA inhibition, [85] including inhibition of ribosomal synthesis, [99] is a known alternative mechanism. Combining the known mechanisms of T_{reg} selecting drugs MTX, 5-FU, and rapamycin yielded the diagram in **Figure 22,** which details how each drug interacts with ribosomal function. It was noted in an experiment depicted in **Figure 20A** that Neomycin resistance gene rescued $T_{CD4,\ FoxP3}$ from the treatment of G418. This finding suggests that a specific action of G418 is responsible for $T_{CD4,\ FoxP3}$ depletion, and this phenomenon was further explored.

Figure 22

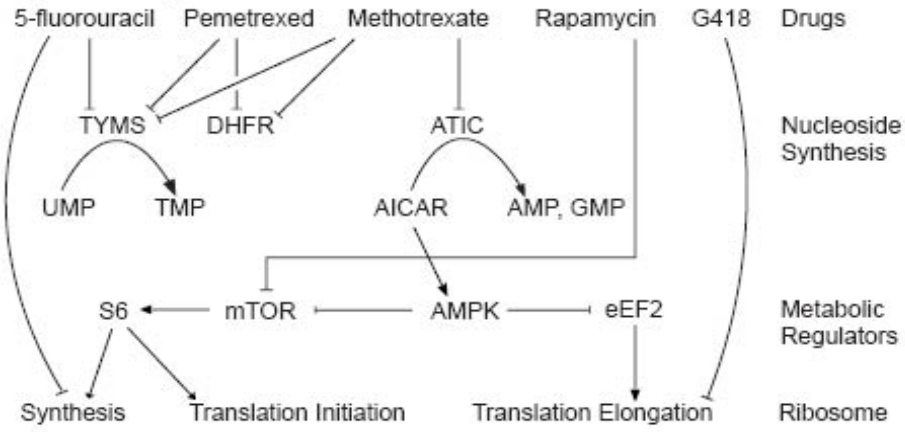


Figure 22 Diagrammatic representation of biochemical and protein interactions thought to influence selection of T_{reg}

Ribosomal Inhibition by aminoglycoside G418 selectively depletes replicating $T_{CD4, FoxP3}$.

Thawed PBMC were activated with anti-CD3/CD28 + IL-2 for 7 days in the presence of alternative doses of G418, Hygromycin B - a different aminoglycoside,[146] Zeocin – a DNA targeting antibiotic, and Rapa to assess the dose dependent selection or depletion of $T_{CD4, FoxP3}$ by aminoglycosides (**Figure 23A**). Depletion of $T_{CD4, FoxP3}$ is again noted in the presence of aminoglycoside G418. The alternative aminoglycoside - hygromycin – developed an insignificant increase in $T_{CD4, FoxP3}$ at 0.2 mM hygromycin. This increase significantly decreased with higher doses of hygromycin - 1.5 and 2.3 mM. Hygromycin showed no significant depletion of $T_{CD4, FoxP3}$ from untreated control.

This dose dependent depletion of $T_{CD4, FoxP3}$ is consistent with that seen for G418, and was not noted with increasing doses Zeocin or Rapa. An increase of $T_{CD4, FoxP3}$ was noted with increasing doses of Zeocin, yet this was insignificant, similar to that seen for other cytotoxic drugs in **Figure 19B-II**. A representative flow plot of CD4 and FoxP3 expression from the same donor can be seen in **Figure 23B.** Here, the trends can be visualized.

It was considered that polyclonal stimulation may play some part in the G418 depletion of $T_{CD4, FoxP3}$. To test this, PBMC were rested in CM for 9 days after thawing +/- G418 and tested for the presence of $T_{CD4, FoxP3}$. Significant depletion of $T_{CD4, FoxP3}$ by G418 persisted under resting conditions (**Figure 23C – left panel**). This was replication dependent as $CD4^+$,$FoxP3^+$,Ki-67^+ cells showed significant G418 mediated depletion while $CD4^+$,$FoxP3^+$,Ki-67^{neg} cells were not significantly depleted by the same post-Hoc measure (**Figure 23C – right**

Figure 23

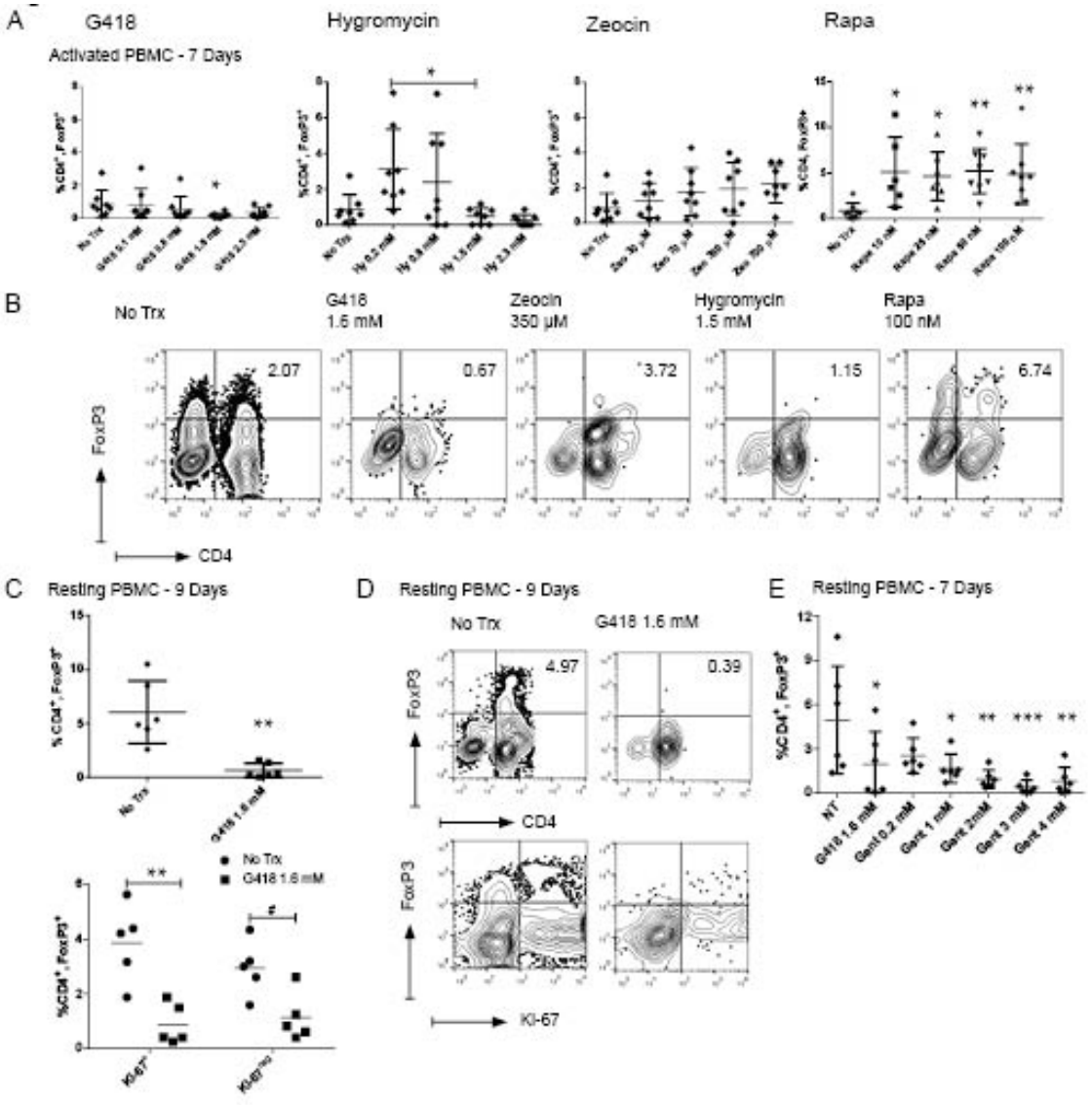

A
G418
Hygromycin
Zeocin
Rapa
Activated PBMC - 7 Days
B
No Trx
G418 1.6 mM
Zeocin 350 µM
Hygromycin 1.5 mM
Rapa 100 nM
2.07
0.67
3.72
1.15
6.74
FoxP3
CD4
C
Resting PBMC - 9 Days
No Trx
G418 1.6 mM
Ki-67+
Ki-67neg
D
Resting PBMC - 9 Days
No Trx
G418 1.6 mM
4.97
0.39
FoxP3
CD4
Ki-67
E
Resting PBMC - 7 Days
NT
G418 1.6 mM
Geni 0.2 mM
Geni 1 mM
Geni 2mM
Geni 3 mM
Geni 4 mM

Figure 23 Ribosomal Inhibition by aminoglycosides selectively depletes replicating $T_{CD4, FoxP3}$. Prior experiments demonstrated that G418 depleted $T_{CD4, FoxP3}$ in stimulated bulk PBMC. It was then tested whether this action of G418 included other drugs within the aminoglycoside class. G418 and hygromycin are aminoglycoside antibiotics known to inhibit translational elongation. Zeocin is an antibiotic that does not target the ribosome. Thawed PBMC were stimulated with anti- CD3/ CD28 and IL-2 in the presence of increasing concentrations of G418, hygromycin, zeocin, or rapamycin for 7 days and the selection for $T_{CD4, FoxP3}$ is shown in **A**. Flow plots of FoxP3 and CD4 expression in **B** show the representative trends for one donor following the use of each drug. The loss of $T_{CD4, FoxP3}$ was tested in un-stimulated, thawed PBMC over the course of 9 days with or without G418 as shown in **C - top panel** while **C - bottom panel** shows the effects of G418 on proliferating and non-proliferating $T_{CD4, FoxP3}$ as indicated by Ki-67. Representative flow plots for one donor demonstrate the effect of G418 on CD4 and FoxP3 expression in **D - top panel** while FoxP3 and Ki-67 expression are shown in **D - bottom panel**. Gentamicin is an FDA approved aminoglycoside antibiotic and was subsequently tested in comparison to G418 for depletion of $T_{CD4, FoxP3}$ over a 7 day period. All experiments were performed with 6 normal donors and repeated independently twice. Kruskall-Wallis was used for **A**, Mann-Whitney used for **C – top panel**, and Two-Way ANOVA with Sidak's post-hoc or post-hoc Mann-Whitney *t*-tests in **C - bottom panel**, One-Way ANOVA with Sidak's post-hoc in **E**; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ # = $p < 0.05$ for post-hoc *t*-test.

panel). Representative flow diagrams of resting PBMC in **Figure 23D – upper panel** show the loss in expression of FoxP3 for $CD4^+$ T cells after treatment with G418. An alternative view of Ki-67 and FoxP3 expression in **Figure 23D – lower panel** demonstrates that $FoxP3^{neg}$ T cells continue to proliferate in the presence of G418, further supporting the selective targeting of G418 to $T_{CD4,\ FoxP3}$ at this concentration. Thus, proliferating $T_{CD4,\ FoxP3}$ are depleted following treatment with aminoglycoside G418.

As G418 and hygromycin are considered toxic to live animals, gentamicin, an aminoglycoside well known for its use in humans and animal models,[147] was tested for selective $T_{CD4,\ FoxP3}$ depletion. **Figure 23E** depicts this depletion of $T_{CD4,\ FoxP3}$ in resting PBMC after 7 days and demonstrates the consistent action of aminoglycosides in depleting $T_{CD4,\ FoxP3}$. It was next tested whether depletion of $T_{CD4,\ FoxP3}$ corresponded with a loss of T_{reg} marker expression or selective T_{reg} toxicity.

Sorted Treg differentiate the effects of MTX, 5-FU, and G418 on selection in bulk PBMC.

Magnetic sorting for CD4 and CD25 expressing PBMC yielded a $CD4^+$ $CD25^+$ population that is widely considered to contain T_{reg}, and a $CD25^{neg}$ population of effector T cells (T_{eff}).[113] These populations were treated with the same concentrations of MTX, 5-FU, G418, or no treatment, as above, for the first 7 days of co-culture with AaPC. After this period of time, co-culture continued without drug by stimulating with AaPC every 7 days until Day 21. Cells were assayed at this time for expression of CD25, CTLA-4, LAP, and IL-2, as before. The experimental outline can be seen in **Figure 24A**. A [^{3}H] thymidine incorporation assay was also performed to determine the effect of each drug on the functionality of propagated T_{reg}.

Figure 24

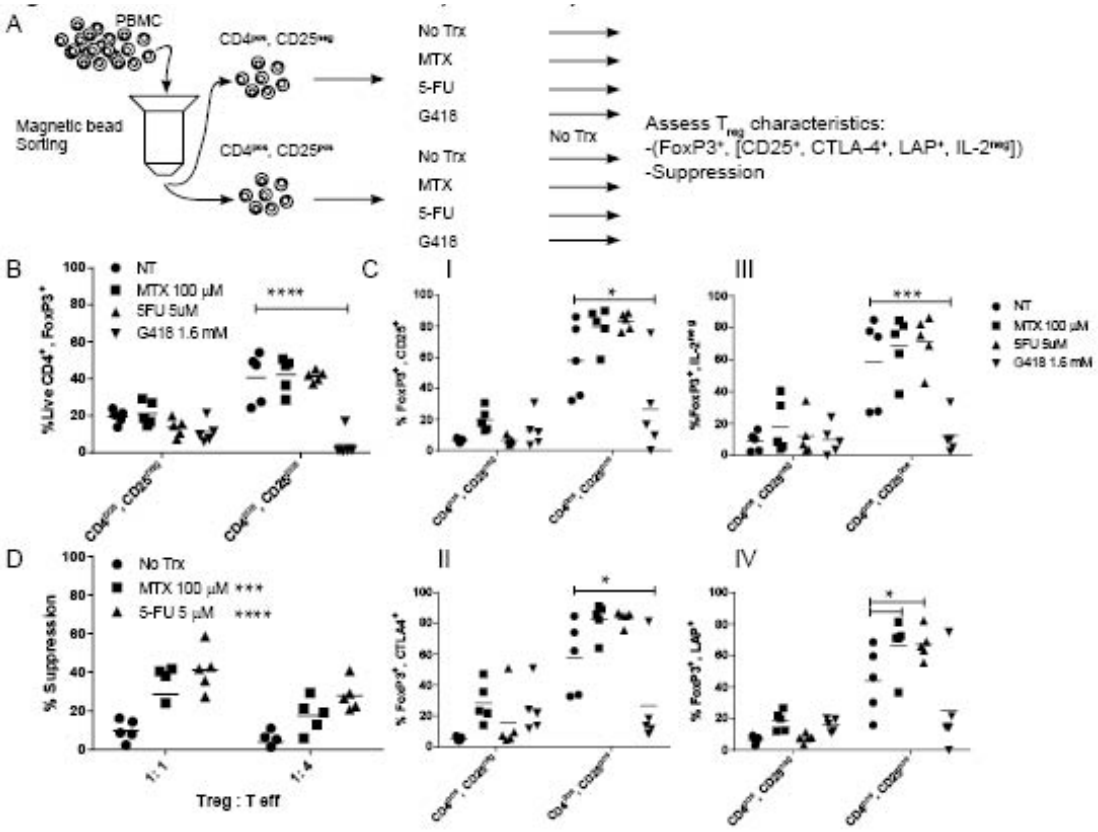

A
PBMC
Magnetic bead Sorting
No Trx
MTX
5-FU
G418
No Trx
MTX
5-FU
G418
Assess T reg characteristics:
-(FoxP3+, [CD25+, CTLA-4+, LAP+, IL-2neg])
-Suppression
B
NT
MTX 100 µM
5FU 5uM
G418 1.6 mM
C
I
III
II
IV
D
No Trx
MTX 100 µM
5-FU 5 µM
% Suppression
Treg : T eff
1:1
1:4

Figure 24 The effects of MTX, 5-FU, and G418 in sorted T_{reg}. PBMC were depleted of $CD4^{neg}$ cells and the remaining $CD4^{+}$ cells were magnetically separated by CD25 with the $CD25^{+}$ cells cultured as T_{reg} and $CD25^{neg}$ cells cultured as T_{eff}. Each group of T cells was co-cultured as before with AaPC at a 1:1 ratio and stimulated weekly for 3 weeks. The T_{reg} and T_{eff} were treated with MTX, 5-FU, or G418 as before for 7 days before stimulating without drug for the remaining 2 weeks of the experiment, which is diagrammatically shown in **A**. On Day 21, markers and activity of T_{reg} were assessed to determine the contribution of each drug to selection or depletion of T_{reg}, and the live $T_{CD4,\ FoxP3}$ on Day 21 are shown in **B**. Each group of T cells was then stimulated to assess expression of known T_{reg} markers. After stimulating with soluble anti-CD3/ CD28 and IL-2 for 48 hours T cells were assessed for co-expression of FoxP3 with CD25 in **C – I,** FoxP3 with CTLA-4 in **C- II**, and FoxP3 with LAP in **C – IV**. Six hours of stimulation with PMA/ ionomycin was used to assess loss of IL-2 secretion in FoxP3 expressing T cells, **C – III**. A 72 hour suppression assay was performed by mixing treated T_{reg} with untreated T_{eff} and looking at uptake of [^{3}H] Thymidine at two separate concentrations, shown in **D**. This experiment was performed with 5 normal donors and repeated twice. All experiments were assessed with Two-Way ANOVA and significance was determined by Sidak's post-hoc analysis; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

When the surviving $CD4^+$ cells were assayed on day 21 it was found that no drug significantly selected for $T_{CD4,\ FoxP3}$ in the T_{eff} compartment, nor did MTX and 5-FU improve selection for $T_{CD4,\ FoxP3}$ in the T_{reg} compartment (**Figure 24B**). The most consistent finding was that G418 persistently decreased surviving T_{reg} following drug treatment. This was demonstrated by loss of surviving $T_{CD4,\ FoxP3}$ (**Figure 24B**). T_{reg} markers such as CD25 (**Figure 24C-I**), CTLA-4 (**Figure 24C – II**), decreased IL-2 expression (**Figure 24C – III**), or LAP (**Figure 24C – IV**), in combination with FoxP3 expression was also decreased following stimulation on day 21. Thus, T_{reg} are lost, likely due to toxicity of G418, rather than inhibited as 2 weeks of growth promoting co-culture conditions could not sufficiently restore T_{regs} following G418 treatment.

The T_{reg} promoting properties of MTX and 5-FU appeared to depend in part upon the presence of T_{eff}, as the enhanced selection of $T_{CD4,\ FoxP3}$ was no longer noticeable after T_{eff} were removed from the culture system (**Figure 24B**). The improved selection towards T_{reg} phenotypes may have been accomplished by depletion of T_{eff} which are known to contaminate T_{reg} sorting.[113] It is likely that the ability of T_{reg} to survive the cytotoxic insult of MTX or 5-FU in comparison to T_{eff} was a primary component of the enhanced selection. Although there was a trend towards improved selection of T_{reg} phenotypes (**Figure 24C – I, II, III**) when MTX or 5-FU was used, there was no significant difference for expression of CD25, CTLA-4, or loss of IL-2. However, the T_{reg}- specific marker LAP was significantly increased by early treatment with MTX or 5-FU (**Figure 24C – IV**). As LAP was the only increased marker of those assayed, it is likely that LAP and the associated expression of TGF-β [143] was the probable cause for improved

suppression of MTX and 5-FU treated T_{reg} above untreated T_{reg} (**Figure 24D**). Thus, MTX and 5-FU appear to have two components in enhancing selection of T_{reg}: 1) T_{eff} are selectively depleted by MTX and 5-FU, and 2) MTX and 5-FU increase the expression of LAP weeks after treatment.

Stimulation of $T_{CD4,\ FoxP3}$ enhances AMPK activation and leads to inhibition of eEF2 – a factor essential to translational elongation.

AMPK is hypothesized to play a role in selection of $T_{CD4,\ FoxP3}$, as noted above (**Figure 22**). Furthermore, we hypothesized that enhanced activation of AMPK leads to inhibition of eEF2 in $T_{CD4,\ FoxP3}$.[135] Preferential inhibition of translational elongation could explain selection for $T_{CD4,\ FoxP3}$ in the presence of many cytotoxic drugs and depletion of $T_{CD4,\ FoxP3}$ in the presence of inhibitors of translational elongation. We tested this hypothesis by assessing phosphorylation of AMPK 24 hours after activation of PBMC using flow cytometry (**Figure 25A & B**) and imaging cytometry (**Figure 25C**). The phosphorylation of AMPK on T172 indicates activation,[136] and was enhanced in stimulated over unstimulated $T_{CD4,\ FoxP3.}$ This enhanced activation of AMPK was increased in $CD4^+$, $FoxP3^{neg}$ T cells (**Figure 25A – upper panel**) as well, but the significant increase ($p = 0.03$ by *t*-test) did not persist following post-hoc analysis. Likewise, flow plots of activated AMPK with FoxP3 show this enhancement of AMPK activation is much more noticeable in the FoxP3-expressing subset (**Figure 25B – upper panel**).[148] A marker of translational initiation –S6 – is susceptible to mTOR regulation, and is phosphorylated when active.[139] Phosphorylation of S6 (p-S6) was significantly enhanced in $T_{CD4,\ FoxP3}$ following stimulation (**Figure 25A – lower panel**), which was previously shown by

Cabone *et al.*[149] While p-S6 increased in the FoxP3^{neg} T cells (p = 0.01 by *t*-test), this increase was not significant following post-hoc analysis. The enhancement of p-S6 is observable in the representative flow plot for **Figure 25B – lower panel**. The activation of metabolic regulators AMPK and S6 was enhanced in both FoxP3^{+} and FoxP3^{neg} CD4^{+} T cells following activation, but the increase was only significant in $T_{CD4,\ FoxP3}$ in a Two-Way ANOVA with post-hoc Sidak's test. The increased activation of AMPK and S6 following activation of $T_{CD4,\ FoxP3}$ can be seen with image cytometry profiles shown in **Figure 25D** before – **top panel** – and after stimulation with anti-CD3/ CD28 and IL-2– **bottom panel**. The same compensation and visualization were applied to each panel making the top and bottom panels comparable.

Figure 25

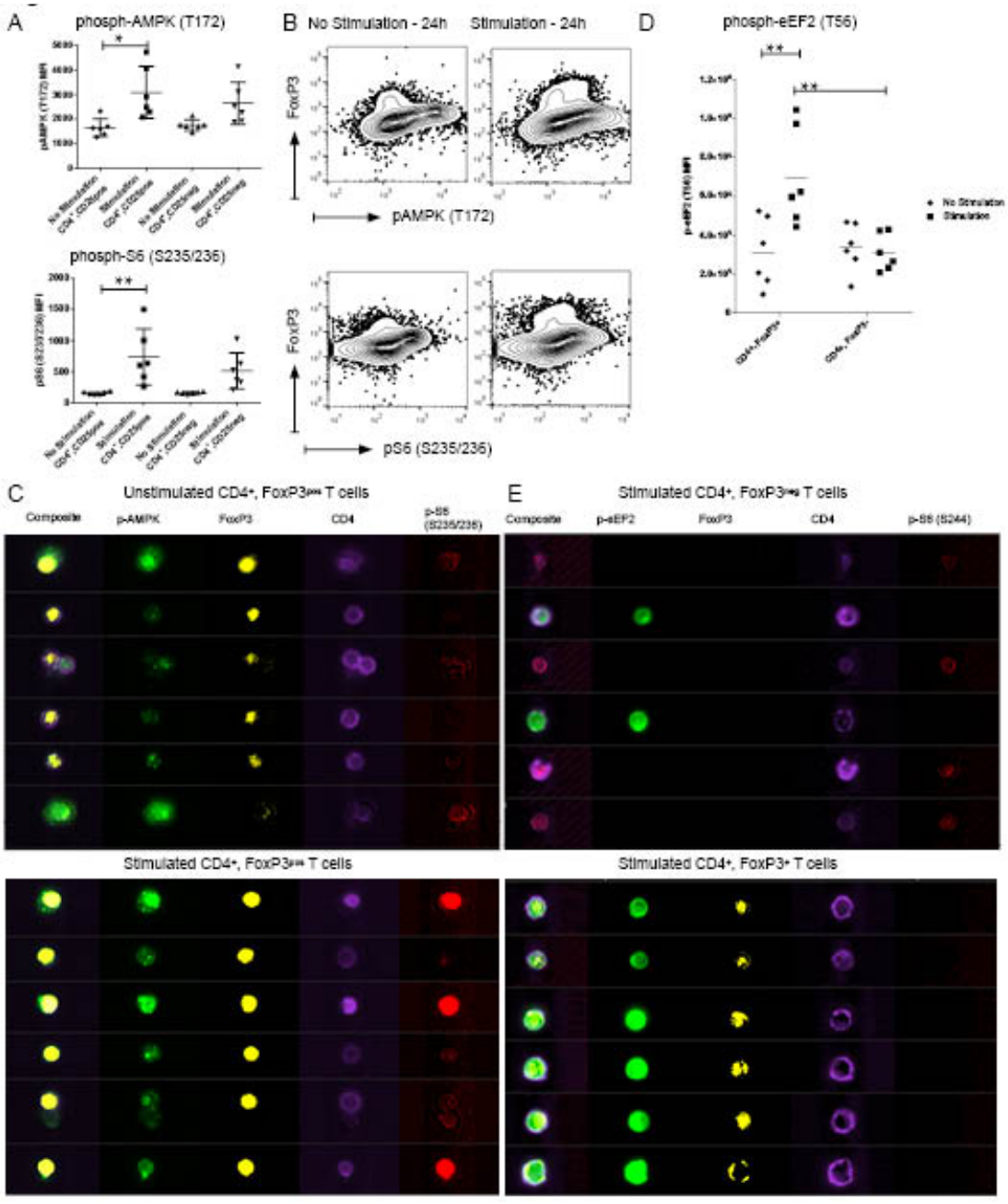

A
phosph-AMPK (T172)
B
No Stimulation - 24h
Stimulation - 24h
FoxP3
pAMPK (T172)
phosph-S6 (S235/236)
pS6 (S235/236)
D
phosph-eEF2 (T56)
No Stimulation
Stimulation
C
Unstimulated CD4+, FoxP3low T cells
Composite
p-AMPK
FoxP3
CD4
p-S6 (S235/236)
E
Stimulated CD4+, FoxP3neg T cells
Composite
p-eEF2
FoxP3
CD4
p-S6 (S244)
Stimulated CD4+, FoxP3low T cells
Stimulated CD4+, FoxP3+ T cells

Figure 25 Stimulation of $T_{CD4,\ FoxP3}$ enhances AMPK activation and leads to inhibition of translational elongation factor eEF2. AMPK was hypothesized to mediate selection of $T_{CD4,\ FoxP3}$ under cytotoxic conditions. PBMC were stimulated with or without anti- CD3/ CD28 and IL-2 for 24 hours and flow cytometry was used to assess phosphorylation status of AMPK in $T_{CD4,\ FoxP3}$. Differentiation of $T_{CD4,\ FoxP3}$ from $CD4^+$ $CD25^{neg}$ T cells was accomplished by gating in the stimulated and unstimulated experiments. **A – top panel**, depicts the mean fluorescence intensity (MFI) of AMPK activated by phosphorylation at T172 after stimulation while **A – lower panel** depicts the MFI of activated S6 by phosphorylation at sites S235/ S236. A flow plot depicting the changes in phosphorylation for $T_{CD4,\ FoxP3}$ and $CD4^+$ $CD25^{neg}$ T cells is seen in **B – upper panel** for AMPK and **B – lower panel** for S6 with respect to FoxP3 expression in gated $CD4^+$ cells. An image cytometry gallery in **C** shows fluorescent and morphologic changes in $T_{CD4,\ FoxP3}$ following stimulation. Activation of AMPK was found to be more robust in $T_{CD4,\ FoxP3}$ and it was further hypothesized that this activation led to preferential inhibition of translation and cell cycle progression by inactivation of translational elongation through eEF2. Inhibitory phosphorylation of eEF2 at T56 was assessed following 24 hours of stimulation, as before. An image cytometer was used to analyze p-eEF2 T56 MFI and depicts an increase unique to activate $T_{CD4,\ FoxP3}$ in **D**. This difference from $CD4^+$ $FoxP3^{neg}$ T cells is shown with an image cytometry gallery in **E**. All experiments were repeated independently at least twice with 6 biological replicates. Two-Way ANOVA with Sidak's post-hoc used in all analyses; * = $p < 0.05$, ** = $p < 0.01$.

Enhanced activation of AMPK in $T_{CD4,\ FoxP3}$ suggests translational elongation may be inhibited by phosphorylation of eEF2 and could account for the increased survival of $T_{CD4,\ FoxP3}$ in the presence of cytotoxic drugs and susceptibility to inhibitors of translational elongation, like aminoglycosides. We performed the same experiment as in **Figure 25 A-C** to assess the inactivation of eEF2 by phosphorylation at T56.[135] Image cytometry was used to quantify and visualize all events. **Figure 25D** demonstrates a significant increase in phosphorylation of eEF2 in the same subset of T cells - $T_{CD4,\ FoxP3}$ - following stimulation. Also, inhibitory phosphorylation of eEF2 was significantly increased above stimulated $FoxP3^{neg}$ T cells, which was not noted with AMPK or S6 phosphorylation. The increased phosphorylation of eEF2 only in stimulated $T_{CD4,\ FoxP3}$ suggests that $T_{CD4,\ FoxP3}$ would have decreased replicative capacity upon stimulation, as shown by Cao *et al.*[141] As decreased levels of active eEF2 inhibit progression through the cell cycle, [150] it also suggests that increased phosphorylation of eEF2 may account for the survival of $T_{CD4,\ FoxP3}$ in cytotoxic environments, which was noted in **Figure 19**. Similarly, decreased translational capacity would make $T_{CD4,\ FoxP3}$ increasingly susceptible to inhibitors of translational elongation, as was shown with aminoglycosides in **Figure 23**. Therefore, the activity of eEF2 may be the primary factor influencing both selection and depletion of T_{reg} in these studies.

DISCUSSION:

In this study, we further the understanding of $T_{CD4,\ FoxP3}$ selection by MTX and 5-FU in humans, and determine that these cells constitute T_{reg} by functional expression parameters of CD25, CTLA-4, LAP, and loss of IL-2. Prior studies by Tohyama *et al.,* [115] in human cells

described this phenomenon but had not determined how these drugs enhanced selection of $T_{CD4,\ FoxP3}$. Using recently described transgenes resistant to the actions of MTX and 5-FU on DHFR and TYMS, we developed an understanding that each drug operates independently of folate and thymidine synthesis to select for T_{reg}. While the action of MTX on DHFR and the action or 5-FU on TYMS are the commonly understood mechanisms for anti-cancer efficacy, both drugs have alternative targets that promote tumor killing.[85, 86] Our study demonstrates that these alternative actions of each drug promote a T_{reg} phenotype in surviving cells. Prior findings show MTX enhances AMPK activation through the inhibition of AICARtf and accumulation of AICAR which activates AMPK.[137] This action of MTX was hypothesized to promote T_{regs}, [123] but we found that specific inhibition of AICAR synthesis was neither toxic to T cells nor selective for $T_{CD4,\ FoxP3}$. This essentially ruled out AICARtf inhibition as the primary means of T_{reg} selection. However, FoxP3 expression in $T_{CD4,\ FoxP3}$ was enhanced by the specific action of AICARtf inhibition, suggesting some action of AMPK may improve T_{reg} phenotype. Isolated T_{reg} studies resolved this issue by determining that the action of MTX was twofold. 1) Selection of T_{reg} is dependent on the depletion of T_{eff}, as removal of T_{eff} prevents the selective increase of T_{reg} following MTX treatment. 2) The action of MTX does enhance T_{reg} functional activity in some regard as LAP expression and suppression of T_{eff} proliferation were increased above untreated T_{reg}. The activation of AMPK in the absence of folate depletion by MTX was achieved in the transgenic T cell experiments and increased the percent of T cells with a functional T_{reg} phenotype. Thus, MTX depletes T_{eff} and promotes an immunosuppressive T_{reg} phenotype.

Use of MTX consistently depleted $CD8^+$ T cells in transgenic experiments to leave primarily $CD4^+$ T cells even when MTX toxicity was rescued by resistant $DHFR^{FS}$ and/ or $TYMS^{SS}$. This suggests that $CD8^+$ T cells may be more susceptible to AMPK signaling. A higher replication rate could exceed the capacity of the transgene to rescue transgenic cells. This is more likely the case as both 5-FU and G418 also resulted in $CD4^+$ T cell predominance following transgenic rescue, although neither drug is known to signal through AMPK. A decreased replicative rate of T_{reg} is also supported by the finding of increased AMPK activation and increased eEF2 inactivation. Translational elongation factor eEF2 is the primary means of ribosomal elongation, [135] and inhibition would significantly impede translation and progress towards cell division.[150] Therefore, activated T_{reg} grow slower and take longer to succumb to any genotoxic insult, even one which will eventually kill all cells. This is consistent with findings in this study and others, where pan-cytotoxic γ-irradiation or chemotherapy selected for T_{reg}.[114] The selective inhibition of eEF2 in T_{reg} has clear biological implications in slowing suppressive mechanisms during early poly-clonal activation to permit the development of a sufficient immune response.

While the action of MTX in selecting for T_{reg} appears to be well delineated by our experiments, the action of 5-FU in T_{reg} selection is not as clear. It was determined that depletion of T_{eff} by 5-FU is required to improve selection for T_{reg} in culture, as with MTX. It was also found that 5-FU acted independently of TYMS to select T_{reg} by increasing LAP expression and suppression of T_{eff} proliferation. Both actions of 5-FU were similar to MTX but independent of thymidine synthesis. In the pathway diagram in **Figure 22**, we hypothesize that 5-FU

inhibition of ribosomal processing[85, 99] independently led to inhibition of eEF2, which is known to occur during ribosomal stress.[151] This hypothesis has not yet been tested, but would support our findings that MTX and 5-FU in combination enhance the selection of T_{reg} independent of thymidine synthesis and that the addition of 5-FU improves T_{reg} selection above MTX alone.

A novel finding of our study was that aminoglycosides deplete T_{reg} at high doses. This finding was supported by rescue of T_{reg} from G418 mediated depletion when Neomycin resistance gene, which prevents G418 toxicity,[45] was present. Furthermore, multiple aminoglycosides were able to decrease the percent of $T_{CD4,\ FoxP3}$, and studies in isolated T_{reg} demonstrated that this treatment was specifically toxic to T_{reg}. The mechanisms through which aminoglycosides mediate toxicity are diverse,[147] and were not addressed in these studies. We hypothesize that inhibition of mammalian translational elongation eEF2 was the factor determining aminoglycoside susceptibility. G418 and gentamicin have been shown to inhibit the human and mitochondrial ribosomes at the concentrations used, [152] and could logically combine with inhibition of translational elongation in T_{reg} to mediate cell death. While aminoglycosides have been in use for several decades the capacity of this drug to deplete T_{reg} has not been described. The most likely explanation for this is that the drug is used at much lower doses *in vivo* than those used to deplete T_{reg} *in vitro*, and is often discontinued for toxicity to multiple tissues.[147] Also, due to toxicity, aminoglycosides are often second-line antibiotic therapeutics limited to critically-ill patients with systemic infection[153] where gross immune dysregulation is confounded by the mechanisms known to perpetuate sepsis.[154] We are in the process of testing whether aminoglycosides effectively deplete T_{reg} *in vivo*, and if this is the

case, then pretreatment with aminoglycosides may become an effective means of enhancing anti-tumor immunity. This finding needs to be further developed *in vivo* before any conclusions can be made.

In conclusion , this study develops a mechanistic understanding of how multiple chemotherapeutics select or deplete T_{reg} through AMPK activation and decreased eEF2 activity. Understanding how T_{reg} are selected and depleted can inform drug design and drug combination choices in the clinic. Here, we develop a better understanding of MTX, a first-line anti-inflammatory drug, and demonstrate that selection of T_{reg} may be improved by combination with low-dose 5-FU or occasional addition of leucovorin to rescue slowly replicating T_{regs} from inhibition of folate and thymidine synthesis. These findings open up new understandings in the methods to target and treat disease as well as possible mechanisms of dysregulation in autoimmune disease.

CHAPTER 5 Discussion and Future Directions

SYNOPSIS

These studies were developed with the intention of improving T cell therapeutics. Each aim is intended to expand the repertoire of *in vitro* ASIS methods and enhance the selection of desired T cells. While the objective of each study was achieved, the studies are limited to *in vitro* work. No *in vivo* data has been included in these studies to suggest that any of the phenotypic findings extend beyond *in vitro* observations. This is a clear caveat of this system. However, working with human cells has benefits and limitations, and an example will help in understanding this statement; a limitation might be that the depletion of T_{reg} by aminoglycosides is not reproducible in a murine model, yet aminoglycosides could still target T_{reg} in human cells. In fact, human T_{reg} are well known to differ from murine T_{reg} by phenotypic markers.[113] As aminoglycosides are a full class of FDA approved drugs with well-known pharmacokinetics and side effects,[153] the depletion of T_{reg} could more easily be tested in humans in small scale clinical trials. This brings into question the necessity of an animal model for studies originally designed for human cells, as animal models have different biological backgrounds.

There is another limitation of animal models in that they do not recapitulate the natural cellular environment. Consider $CARL^+$ K562 as one example; $CARL^+$ K562 could be compared with standard AaPC (clone.4) to assess differences in outcomes for patients receiving CAR^+ T cell therapy. An animal model designed to assess the *in vivo* functionality of CAR grown on differing AaPC would utilize immunocompromised mice with xenograft tumor and xenograft T cells. With a few exceptions, these models do not recapitulate many aspects of the human

body or an intact immune system.[26] In the standard NSG mouse model there are no endogenous B cells or T cells, and a dysregulation in macrophages, this would clearly alter the environment experienced by CAR^+ T cells and the immune response induced by the reaction of these cells towards the tumor.[155] It is a reasonable argument that an animal model assessing the differences between AaPC would miss potential differences between the two AaPC that could lead to gross differences in tumor eradication and patient survival. Thus, it might be more prudent to test AaPC differences directly in humans. In a clinical trial, a product with equivalent CAR expression on T cells could be compared for differences induced by growth on differing T cells whether propagated on $CARL^+$ K562 or clone.4 K562. A clinical trial, while more expensive and complicated, would yield much more relevant data than a mouse model. Therefore, certain changes to methodology, such as novel *in vitro* ASIS methods might be better assessed in humans rather than mice.

Notwithstanding, a murine model would still be quite useful in assessing whether certain experimental findings can be repeated in an *in vivo* model. As an example, $DHFR^{FS}$ selected with MTX and grown on clone.4 AaPC yielded an almost uniform increase in the $CD4^+$ T cell population in multiple experiments, while $DHFR^{FS}$ selected by MTX and grown on $CARL^+$ K562 through CAR did not change the $CD4^+$ T cell predominance. This finding was difficult to explain, even though it was consistent with the published findings of others.[82] Based on our subsequent studies with MTX, it is likely that MTX was inducing activation of AMPK in our $DHFR^{FS+}$ CAR^+ T cells as well as the $DHFR^{FS+}$ CAR^+ T cells published by Jonnalagadda *et al.*[82] The long-term effects of AMPK activation is uncertain for CAR^+ T cells and could be

beneficial[156] or detrimental[157] based on available data. An animal model would be ideal for discriminating the true outcome of this alteration in signaling.

Overall, the data presented here is limited to *in vitro* studies and some aspects of the work remain to be resolved with further *in vitro* and *in vivo* studies. However, we believe that the aims and objectives were sufficiently achieved within our system to discuss the implications of this work on future preclinical and clinical studies. We believe that the following primary findings are not unique to our system and have biological and clinical implications:

1) Genetically-modified T cells can be selected and propagated independent of antigen-specificity based on the use of a CAR containing a conserved domain that is recognized and ligated by $CARL^{+}$ AaPC.
2) Genetically-modified T cells can be selected and propagated independent of antigen-specificity utilizing AThyR transgenes $DHFR^{FS}$ and $TYMS^{SS}$ individually or together.
3) AThyR transgenes $DHFR^{FS}$ and $TYMS^{SS}$ confer special properties to T cells in the presence of MTX:
    a. *Cis*-transgene co-expressed with $DHFR^{FS}$ can be titrated or up-regulated by applying varying concentrations of MTX
    b. Co-expression of $TYMS^{SS}$ with $DHFR^{FS}$ blunts the findings noted in a.
    c. Co-expression of $TYMS^{SS}$ with $DHFR^{FS}$ confers improved resistance to MTX
    d. Application of MTX in $DHFR^{FS}$ expressing T cells appears to perturb signaling pathways.

i. This is likely achieved through the AMPK pathway, but this remains to be proven

4) Consistent selection of AThyR transgene $TYMS^{SS}$ depends upon the depletion of 5-FU resistant T cells.

5) High-dose aminoglycosides selectively deplete T_{reg} in culture

   i. This is likely mediated by eEF2 inhibition, but remains to be proven

6) MTX and 5-FU enhance selection of T_{reg} in culture

   a. Through selective depletion of T_{eff}

   b. Through enhanced expression of LAP, associated with TGF-β

These findings were briefly discussed above. Here the biological context will be discussed with clinical implications discussed in the following section.

The objective to establish novel, transgenic, *in vitro* ASIS methods for T cell therapeutics has been achieved. We believe these technical improvements will make T cell therapy a safer and more reliable treatment option. Transgenic modifications to T cell products have been attempting to improve safety and reliability for decades.[43, 158] Unfortunately, these advances have suffered from technical issues and the immunogenicity of the transgenes chosen.[41, 159] As an example: The earliest reports of gene modification in human cells used drug resistance to select for transformed cells,[160] and drug resistance was the first reported gene modification in human T cells.[161] Drug resistance in T cells, [161] including resistance to MTX in T cells, [46, 82] has been under-utilized due to technical limitations regarding the transgenes chosen. That being transgenes originating from bacteria and viruses are

immunogenic[41, 159] while the non-immunogenic transgene resistant to MTX is not useful in the context of most cancers where higher doses of MTX and MTX analogs with higher anti-thymidylate activity make transgenic resistance to MTX obsolete *in vivo*.[86] The technological advance reported here include the use of transgenes to confer a broader spectrum of chemotherapeutic drug resistance to common anti-thymidylate drugs, and the capacity to utilize these transgenes to modify expression of *cis* expressed transgenes for *in vivo* use. Likewise, it is possible that these drug resistance transgenes can alter signaling pathways responsible for T cell selection and survival when combined with MTX. Thus, the findings here expand the number of transgenic options for *in vitro* ASIS while increasing the *in vivo* utility of choosing these transgenes. For this reason we suspect that these transgenes will be integrated into strategies to select and deplete T cell therapeutics *in vitro* and *in* vivo achieving the overall goal of improving safety and efficacy in T cell therapeutics.

As mentioned, MTX-dependent alterations in transgene expression and intracellular signaling could be useful to manipulate T cell phenotype *in vitro* or *in vivo* towards a desired outcome. In transgenic T cell studies, phenotypic alterations tend to modulate survival and growth or apoptosis and cell death. An example of selection for survival is the transgenic modification of T cells to over-express Bcl-2, an inhibitor of apoptosis, to prevent T cell death.[162] A study by a different group utilized a constitutively active form of AKT to enhance T cell activity and survival.[163] These modifications, however, were independent of drug induction. Drug-dependent modifications to T cell phenotype are best exemplified by suicide genes. Suicide genes such as herpes simplex virus – thymidine kinase (HSV-TK) [158] and inducible

caspase 9 (iC9) [36] which induce T cell death when drug is added. However, prior to the addition of drug, these genes are not believed to substantially alter the phenotype of the T cell.[35]

The drug-inducible modification of T cell phenotypes has broadened as several groups begin to explore the chimerization of cytokine signaling. The signaling of cytokine IL-4, which signals towards a Th2 phenotype in T cells and away from a cytotoxic Th1 phenotype[5] was chimerically modified to provide a proliferative Th1 signal to T cells similar to that of IL-2 or IL-15. Although this signal utilized an endogenously produced IL-4, the protein could be used *ex vivo* to expand T cells by selectively altering biochemical signaling pathways.[42] This concept was later used by another group to transduce the extracellular presence of IL-4 into the intracellular signal of IL-7 in genetically-modified T cells. Ultimately the modified T cells were used *in vivo* to enhance clearance of IL-4 secreting tumors in a xenogeneic mouse model.[164] The use of endogenous proteins, such as IL-4, limits the capacity to selectively activate biochemical pathways in transgene expressing T cells. That is because systemic administration of IL-4 to a patient, while not as toxic as IL-2, and more selective towards transgenic T cells than IL-7 or IL-15, still has off-target effects on other immune cells and the entire body.[165, 166] In another study, a non-endogenous protein was used to activate cytokine signaling. A common protein was chemically conjugated with multiple small molecules. A scFv-specific to the small molecule was chimerized by the addition of the modified protein and led to intracellular signaling in murine B cells. In this way, a drug with little direct physiological effects was used to cross-link and initiate biochemical signaling within the transgenically modified cells.[167] The use

of chimeric proteins to alter cytokine signaling of endogenous human proteins will likely continue. A recent abstract presentations detailed the chimerization of a TGF-β receptor with TLR4 signaling endodomains to remove the physiologic suppression of TGF-β on T cells.[168] Thus, the use of drug-susceptible transgenes to augment T cell phenotypes rather than ablate transgenic T cells is in a nascent stage. Above are the few reported transgenes targeting biochemical pathways. Here we suggest a novel transgene to target a biochemical signaling pathways. Also, we propose a different biochemical pathway than the cytokine signaling pathways typically targeted.

Here we propose a novel method to selectively activate AMPK in the presence of MTX. This developed from the observation that MTX, in combination with $DHFR^{FS}$, resulted in different T cell phenotypes depending on the context (reference **Ch. 3 Figure 12 & 17, Ch. 4 Figure 19 & 21**). In Chapter 4, it was outlined how AMPK contributed partly to T_{reg} outgrowth, and that $DHFR^{FS}$ when combined with MTX appears to stimulate the AMPK pathway towards enhanced T_{reg} outgrowth. This was noted in strong stimulatory conditions where polyclonal CD3 crosslinking was combined with strong costimulatory signaling. In these experiments, MTX treatment of $DHFR^{FS}$-expressing cells resulted in $CD4^+$ T cells predominance compared to un-transformed T cells (**Ch. 3 Figure 12**). This should be contrasted with experiments where CAR^+ T cells received stimulation solely through the stimulatory and costimulatory domains of CAR (**Ch. 3 Figure 17**). In the $DHFR^{FS+}$, CAR^+ T cell experiment, there was no difference from un-manipulated T cells in $CD4^+$ predominance. This isolated experiment, while consistent on repetition, appears to conflict with $DHFR^{FS+}$ T cell experiments not using CAR.

A resolution to this conflict may be understood by making note of initial experiments with $DHFR^{FS}$ not presented here. Initial constructs of $DHFR^{FS}$ were designed to co-express with CD19-specific CAR as $DHFR^{FS}$-2A-CAR on T cells. Attempts to grow T cells expressing $DHFR^{FS}$-2A-CAR on the strongly stimulatory AaPC clone.4 expressing CD19, CD86, and CD137L, resulted in multiple failed experiments where CAR^+ T cells did not selectively propagate in the presence of MTX (data not shown). Consequently, the development of an AaPC with no costimulation, $CARL^+$ K562, permitted the propagation of $DHFR^{FS}$-2A-CAR and the construct was easily developed, tested, and reported.[169] Therefore, CD3 and costimulatory signaling clearly play a role in toxic drug mediated depletion of $CD8^+$ T cells.

These experiments reported above were not designed for direct comparison and do not explicitly comment on the capacity of AMPK to select for one phenotype over another in the context of costimulation. With that noted, there have been a number of studies on AMPK signaling in the context of T cell activation that will assist in understanding this phenomenon: AMPK is considered a negative regulator of T cell activity.[170] T cell activation leads to a metabolic switch to glycolysis that is required to sustain the rapid proliferation and biosynthetic pathways of an inflammatory T cell.[171] AMPK mediates an antagonistic signal against glycolysis and towards lipolysis, inhibiting biosynthetic pathways, and inhibiting proliferation, at least in part through inhibition of mTOR.[136] T cells deficient in AMPK lead to excessive inflammation and poorer outcomes in an animal model of disease such as experimental autoimmune encephalomyelitis (EAE).[157] Activation of AMPK also decreases lymphocyte involvement and disease severity in an experimental asthma model.[172] Loss of AMPK in T

cells results in excessive activation of $CD8^+$ T cells towards an inflammatory phenotype of increased IFN-γ secretion.[148] The un-opposed switch towards glycolysis in $CD8^+$ T cells prevents $CD8^+$ T cell memory development, and AMPK deficient T cells fail to survive after the initial response to antigen.[156] The excessive secretion of cytokines seen in AMPK deficient $CD8^+$ T cells was not noted in AMPK deficient $CD4^+$ T cells.[148] On the opposite end of the spectrum, activation of AMPK during the experimental asthma model did decrease $CD4^+$ T cell numbers and promoted T_{reg} outgrowth.[172]

The above studies, all performed in mice, confer with our findings in human cells; AMPK activation in $CD4^+$ predominant T cells activated by TCR and costimulation leads to increases in T_{regs}. As mentioned the AMPK activation leads to mTOR inhibition,[136] and T cell specific mTOR knockouts in mice resulted in increases in the T_{reg} population [173] similar to that seen with enhanced AMPK activation via metformin.[172] This points to the hypothesis in $CD8^+$ T cells that rescue from drug depletion by CAR-mediated expansion will permit AMPK inducible T cell memory formation in $DHFR^{FS}$, CAR^+ T cells. An important component of this hypothesis is that administration of MTX to $DHFR^{FS+}$ T cells will lead to AMPK activation in $CD8^+$ T cells. It has been shown that inhibition of mTOR in $CD8^+$ T cells following activation results in improved T cell memory upon repeat presentation of antigen.[174] Thus, the use of CAR with $DHFR^{FS}$ could potentially be used to selectively activate $CD8^+$ T cell memory in transgenic T cells with the addition of MTX. This would constitute an attractive and novel method of selectively activating biochemical pathways to promote a desired T cell phenotype.

In conclusion, our studies start by developing simple *in vitro* ASIS methods for transgenic T cells, but lead to new strategies for understanding and manipulating the expression of T cell phenotypes. There was no expectation that developing tools for T cell selection and propagation would lead to a better understanding of T cell biology and novel transgenic techniques. However, the development of new tools often precedes discovery. It was after the development of AThyRs that we serendipitously found a method to selectively grow T_{reg}, and based on this finding we subsequently developed a novel method for the depletion of T_{reg}. The above work advocates for critical analysis of well controlled science. Had a simple screening of $CD4^+$ and $CD8^+$ T cell subset predominance been neglected, a whole avenue of T_{reg} biology would have been missed. While this work remains incompletely understood, proceeding with clinical and preclinical work is merited based on the findings.

CLINICAL IMPLICATIONS

Few cell therapies and no cell-based gene therapies are currently approved by the FDA.[20] This fact does not preclude the use of some transgenic techniques reported here to be used in Phase I – III clinical trials. $CARL^+$ AaPC could easily be integrated into open and ongoing clinical trials involving CAR^+ T cells. However, few groups in the United States outside of MD Anderson utilize CAR-mediated *ex vivo* expansion. The use of CARL may be quite limited as it depends on the development of a novel CAR without a validated AaPC. Our group has published three different CARs, one of which is CD19-specific CAR currently in clinical trials with a validated clone.4 AaPC.[175] Another targeting fungal antigens has all of the preclinical data performed using the same clone.4 AaPC as CD19-specific CAR but loaded with

an activating antibody.[176] The third was grown on CARL$^+$ K562,[95] but has no plans for clinical development. Consequently, there is uncertainty with respect to further development of CARL$^+$ AaPC, and for that reason the development and validation of a clinical-grade AaPC expressing CARL has not yet begun. It is unlikely CARL will have any clinical impact without the development of a clinical-grade AaPC.

In regard to the AThyR transgenes DHFR^{FS} and TYMS^{SS}, a combination therapy of AThyR+ T cells with AThy therapies is, *a priori,* a straight-forward means to improve anti-tumor immunity. However, our *in vitro* studies demonstrated that CD8$^+$ T cells, the primary effectors of tumor immunity,[177] are selectively inhibited from outgrowth during polyclonal stimulation. *In vivo* studies would be necessary to compare this approach to re-infusion of activated T cells following chemotherapy. Re-infusion of activated T cells following chemotherapy would be much easier to perform than the extensive manipulation necessary to generate AThyR+ T cells. Expressing AThyRs may be advantageous as discussed above for activating biochemical pathways of interest or ensuring the inclusion of a suicide gene in CAR$^+$ T cells. CAR-mediated expansion techniques would also maintain the cytotoxic CD8$^+$ T cell population. Consequently, AThyRs seem to be more reasonable for use in CAR$^+$ T cells, but the proposed uses of AThyR+ CAR$^+$ T cells need to be validated *in vivo*.

The findings of selective increase or decrease in the T_{reg} population using FDA approved drugs have the most immediate potential for clinical impact. Aminoglycosides, such as gentamicin, could be used in clinical trials targeting easily localized chronic infections and cancers. While we suspect that long-term systemic administration of aminoglycosides will

selectively deplete T_{reg} in preference to other T cells, we also believe that the properties that lead to selective depletion of T_{reg} are found in other tissues, such as the kidneys and nervous system.[153] Based on this understanding, we seek to target diseases of a local nature, and we propose the following clinical trials: 1) intra-lesional injection of gentamicin or penicillin (a non-aminoglycoside antibiotic) into treatment resistant verrucae (a human papilloma virus infection)[178] with punch and excisional biopsies for assessment of inflammatory response and T_{reg} persistence. 2) Ultrasound guided intratumoral injection of gentamicin or penicillin in newly diagnosed breast cancers with post-resection pathological assessment of tumor mass reduction, inflammatory response in tumor and lymph nodes, and T_{reg} persistence. If the above studies are consistent with T_{reg} depletion and reduction or loss of tumor, then the following is proposed: 3) A randomized control trial treating metastatic melanoma and squamous cell carcinoma with intra-tumoral injections of gentamicin or penicillin followed by checkpoint blockade therapy involving either anti-CTLA-4[179] or PD-1 blockade.[180] We believe that this series of clinical trials will inform clinicians on the utility of depleting T_{regs} in chronic infection and cancer. We also believe this approach will avoid excessive risk to critical organs such as brain, heart, or lung.

Finally, we found that MTX and 5-FU both increase T_{reg} selection and improve LAP expression in *ex vivo* expanded T_{reg}. The methods described here provide a protocol for improved *ex vivo* expansion of T_{reg}. Further refinement of the drug duration and drug combinations is necessary to develop a highly enriched and suppressive T_{reg} population that is of clinical-grade. The combination of MTX with 5-FU may enhance suppressor cell outgrowth

when folate synthesis is rescued. It was seen in our *in vitro* model, and recapitulating the finding *in vivo* could lead to a new drug combination for the treatment of inflammatory disorders. A rodent model of an inflammatory disorder commonly treated by MTX such as rheumatoid arthritis (RA)[104] could be tested in conjunction with low-dose 5-FU and folinic acid replacement. Folinic acid, also called leucovorin, is commonly used to rescue folate synthesis during the treatment of high dose MTX.[181] If the animal model is consistent, a clinical trial in RA could assess the additional benefit versus toxicity of MTX plus low-dose 5-FU with folinic acid rescue in comparison to MTX alone.

FUTURE DIRECTIONS

The potential of $CARL^+$ K562 to expand CAR^+ T cells independent of antigen served as a simple tool to decrease the time required to thoroughly test and validate new specificities of CAR. We also noted CARL provides a unique opportunity to understand the influence of CAR signaling differences from typical T cell activation by TCR and co-stimulation. To further develop CARL as a tool in dissecting the optimal configuration of CAR, improvements in the CAR specificity towards CARL may be necessary. In studies (not shown here) defining the specificity of CARL towards CAR, it was determined that the likely binding region of CARL on CAR is in the CH_3 domain of the IgG4 Fc stalk expressed on CAR. This is advantageous as all immunoglobulin Fc receptors bind IgG in the CH_2 domain.[182] Fc receptors on the AaPC, such as CD32 or CD64, can ligate and activate CAR^+ T cells containing the native CH_2-CH_3 IgG4 with intact Fc binding domain (not shown here). While ligation via the Fc receptor may seem a simpler method to activate and expand CAR *in vitro*, CAR binding Fc receptors could lead

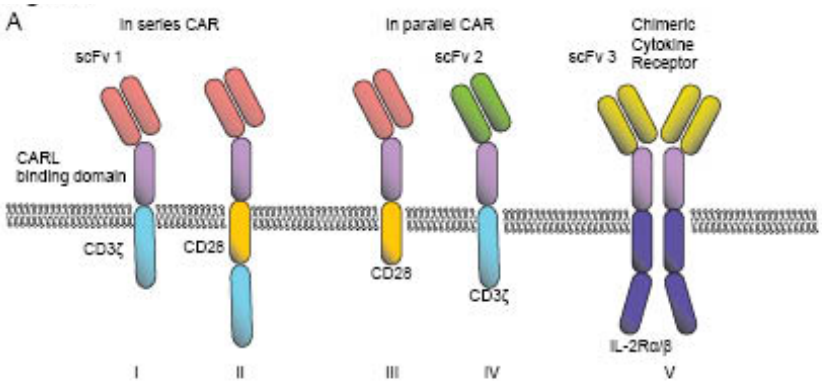
researchers to frivolous experiments and wasted time trying to explain inconsistent cytotoxicity of CAR$^+$ T cells which is due to Fc binding rather than antigen specificity. The potential to bind Fc receptors *in vivo* also remains an issue. This has been observed both *in vitro* and *in vivo* by Jonnalagadda *et al.*, where unmodified CAR Fc led to deleterious Fc binding *in vitro* and removal by myeloid cells *in vivo.* A mutation to the Fc binding sites of CAR IgG4 domain or removal of the CH_2 domain abrogated these deleterious findings and led to improved survival in a xenograft mouse model. [183] To avoid this issue, we tested CAR containing mutations to the Fc binding regions of IgG4, as was done by Jonnalagadda *et al.*, and observed no changes in the activation and propagation of CAR$^+$ T cells by CARL (not shown here). Thus, CAR with the appropriate Fc mutations, or quite possibly only containing the CH_3 domain, will be able to expand on CARL$^+$ AaPC free from deleterious binding. The ability to ligate any specificity of CAR on the surface of a T cell without the risk of off-target activation opens up the potential to refine CAR$^+$ T cell specificity and phenotype.

CARL$^+$ AaPC could be used to define the optimal signaling and antigen specificity requirements of CAR$^+$ T cells in comparison to equivalent activation by TCR and costimulation. Currently, the field is trying to determine the optimal antigenic targets, scFv specificities, and endodomain signaling strengths to develop a CAR$^+$ T cell that strongly targets and kills tumor. At the same time, the development of a proficient memory subset of T cells is sought to respond to resurgence of tumor associated antigen.[25] A philosophical divide is developing over what CAR design will best meet these needs;[25] a CAR with antigen specificity and costimulatory signaling occurring in series, or multiple antigen specificities with costimulatory

signaling occurring in parallel (See **Figure 26A**). The CAR protein that has already been described uses in-series activation, where each signaling domain is attached in series to the next signaling domain. Second-generation CAR signaling is in-series and a single scFv mediates a combination of TCR signal via CD3ζ and a costimulatory signal. The choice of costimulation is also contentious, but tends to be either CD28 or CD137.[24] Costimulation is not limited to one or two costimulatory signals and some authors have included multiples of costimulatory endodomains.[72] In parallel signaling uses several different specificity scFvs each promoting a different stimulatory or costimulatory signal to activate T cells when all antigens are present (See **Figure 26A**). This in parallel system was best demonstrated by Kloss *et al.,* who used one CAR targeting CD19 with stimulatory domains to provide suboptimal activation while a second CAR targeting PSMA was also expressed on the T cell to provide costimulation. Sufficient signal for fully activating CAR^+ T cells was possible when both CD19 and PSMA were present on the same T cell, and activation was not possible if only one antigen was present on a target cell.[184] This study was a proof of concept with *in vivo* demonstration of efficacy. The idea was not new, and had been attempted in various permutations in the preceding 3 years without *in vivo* testing.[185, 186] Developing in-parallel CAR^+ T cells has proven to be a technical challenge difficult to repeat among the various interested groups. This is likely due to the challenge of testing various antigen-specific scFvs with various signal strength combinations. Using viral vectors and no selective propagation, this is a feat difficult to comparatively test. Viral transformation of T cells with multiple transgenes requires selection or multiple stages of genetic transformation to develop cells sufficient for testing. $CARL^+$ K562 presents a simple

solution to this problem as activation of CARs by CARL will propagate even a small percentage of T cells expressing the right combination of CARs to a uniform population. That is because CARL will only activate and propagate CAR^+ T cells deriving sufficient signal strength from CAR. Therefore, CAR^+ T cell propagation on $CARL^+$ K562 is an independent test of sufficient signal strength for CAR combinations. This approach is not currently possible using any other system, and allows for testing of even more difficult questions challenging the field. One of these questions is whether in-series or in-parallel CARs provide better signal strength for activation and propagation. Another question is how CAR intrinsically differs from endogenous signaling via TCR, costimulation, and cytokines. An experiment proposed in **Figure 26B** demonstrates how un-modified T cells stimulated through TCR, CD28, and IL-2 - signals 1 through 3, could be compared to chimerized receptors providing signals 1 through 3. The objective of these experiments would be to quantify differences between in-series and in-parallel CAR^+ T cell activation by the use of image cytometry. This study as designed would also begin to answer questions of how CAR signaling differs from endogenous TCR signaling by quantifying differences in signal strength across the multiple signaling pathways. Furthermore, these findings will be correlated with high-resolution microscopy to elucidate how localization of various CARs contribute to differences in signaling strength from endogenous TCR. This would be the first reductionist approach towards understanding CAR design for activation and propagation of T cells.

Figure 26



B CARL mediated testing of the structure and function relationship of CAR

	Electroporate	Propagate	Test
	none	αCD3, αCD28+ K562 + IL-2	
	I	CARL, αCD28 + K562 + IL-2	-Pathway activation kinetics (quantitative and image cytometry at multiple time points) -Cytokine secretion profiles -Propagation kinetics -High resolution microscopy of T cell AaPC interactions
	II	CARL+ K562 + IL-2	
	III & IV	CARL+ K562 + IL-2	
	III & IV & V	CARL+ K562	

C DHFR^{FS} mediated induction of memory phenotypes in CAR+ T cells

	Electroporate	Propagate	Test
	CAR	CARL+ K562 + IL-2	
	DHFR^{FS}-2A-CAR	CARL+ K562 + IL-2	-Propagation kinetics -Cytotoxicity -Memory phenotype -Maintenance and suppression of disease in a murine xenograft model +/- MTX
	DHFR^{FS}-2A-CAR	CARL+ K562 + IL-2 + MTX days 1-14	
	DHFR^{FS}-2A-CAR	CARL+ K562 + IL-2 + MTX days 14-21	

Figure 26 Future studies targeting biochemical signaling in T cells. A schematically represents CARs which signal in-series (I & II) or in parallel (III & IV). CAR I represents 1st generation CAR, II represents 2nd generation CAR, III & IV represent in-parallel CARs, and V represents a chimeric cytokine receptor. **B** shows the comparative testing of these constructs to unmodified T cells during the canonical activation of T cells through TCR, CD28, and IL-2. **C** presents an experimental approach to enhancing T cell memory using DHFR^{FS} in combination with MTX.

A separate proposed study would try to optimize another key physiologic parameter of T cells – establishing T cell memory. Here, we proposed that $DHFR^{FS}$ in combination with MTX will activate AMPK, subsequently inhibit mTOR, and promote memory T cell formation in a drug inducible manner. This hypothesis is untested in CAR^+ T cells and must be further studied. **Figure 26C** depicts the proposed future studies designed to test enhanced memory formation in $DHFR^{FS+}$, CAR^+ T cells.

We believe that the proposed studies utilize the tools developed here to address fundamental issues which remain in developing and translating T cell therapeutics. Hopefully, using these tools will effectively answer questions of how to establish and maintain T cells therapies for the eradication of disease in humans.

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VITA

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