ABSTRACT

Title of Document:	LINEAGE REPROGRAMMING OF TUMOR-INFILTRATING CYTOTOXIC T LYMPHOCYTES USING PROTEIN STEM CELL TRANSCRIPTION FACTORS.
	Anjuli BhaduriHauck, Master of Science, 2015
Directed By:	Associate Professor, Dr. Zhengguo Xiao, Department of Animal and Avian Sciences

Adoptive cell transfer therapy (ACT) is one of the most promising immunotherapies against cancer. However, this treatment regimen requires the expansion of a small population of effector cells, known as tumor infiltrating lymphocytes, into the billions in order to overcome the immunosuppressive tumor microenvironment. The cytotoxic T lymphocytes (CTLs) within this invading immune cell population are the most critical components to kill the growing cancer cells. Nonetheless, the rapid expansion of already exhausted tumor-infiltrating cytotoxic T lymphocytes (TICTLs) may further push them to a terminally differentiated state that reduces their proliferative response upon antigen stimulation. Recently, induced pluripotent stem cells (iPSCs) generated from TICTLs have been suggested as a way to create a renewable source of rejuvenated tumor-specific CTLs, but retroviral reprogramming is inefficient, and can lead to an increased chance of tumorigenesis. To improve the expansion of TICTLs, we used transient protein

exposure to SOX2, OCT4, and NANOG (SON) in order to push these exhausted TICTLs to a less differentiated stage, preferably stem cell-like memory CTLs (Tscm). These three transcription factors were transiently delivered using a nuclear protein delivery system. We found only the TICTLs treated with SON (STICTLs) exhibited an increased proliferation rate and extended survivability, independent of additional cytokines and antigen stimulation both *in vitro* and *in vivo*; effector CTLs did not respond to the SON regimen. These highly proliferative STICTLs could be associated with up regulation of certain genes related in cell cycle control, such as cyclin D1. Though these STICTLs still express a T cell receptor (TCR), as well as many critical downstream components, they were unable to elicit a reaction against antigen exposure. Though clearly not iPSCs, it is possible that the SON treatment had pushed the TICTLs into a state similar to an early double negative thymocyte. Our findings indicate that TICTLs are uniquely responsive to protein SON compared to naïve and effector CTLs; suggesting TICTLs may also be sensitive to regulation by other more lineage specific transcription factors, thus present new avenue for cancer immune therapy.

LINEAGE REPROGRAMMING OF TUMOR-INFILTRATING CYTOTOXIC T LYMPHOCYTES USING PROTEIN STEM CELL TRANSCRIPTION FACTORS

By

Anjuli BhaduriHauck

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2015

Advisory Committee:
Dr. Zhengguo Xiao, Chair
Dr. Carol Keefer
Dr. Wenxia Song

Dedication

This thesis is dedicated to my grandfather, Dr. Nirendra Nath Bhaduri.

Acknowledgements

I would like to thank Dr. Zhennguo Xiao, my advisor and research mentor, for all of his support and patience, as well as giving me the opportunity to work in his lab and learn from his expertise. Through his guidance, I have found new perspectives and portals into the world of the immune system. I would also like to thank my master's thesis committee, Dr. Carol Keefer and Dr. Wenxia Song, for their guidance and advice through the formulation and execution of my project.

Next, I must thank all of the past lab members who have been my sounding board and extra pair of hands. Thank you to Elliot Mattson, Emma Varner, Dr. Yingjun Li, and Lei Li for all of your technical support and assistance. A special thank you to Karla Garcia for her contribution to the editing, execution, and general support during my thesis project.

Finally, I must thank my family who has encouraged and inspired me to continue through thick and thin. To my friends, Latisha Judd, Cynthia Scholte, Sarah Margerison, Meaghan Kell, and Rebecca Miller, thank you for your help editing and for all of the emotional support. Lastly and certainly the best, thank you to my amazing boyfriend, Nicholas Prior, who has suffered through this ordeal as much as I have, and has continued to care for me and help me through each step of the way. I don't know what I would have done without all of your help. Thank you all.

Table of Contents

List of Figures

List of Abbreviations

2SI-2signal activation:Antigen (SIINFEKL presented by MHC I) and Costimulation (B7)

3SI-3 signal activation: Antigen (SIINFEKL presented by MHC I), Costimulation (B7), and Cytokine (IL-12)

Ab-Antibody

ACT-Adoptive Cell Transfer Therapy

Ag-Antigen

APC-Antigen-presenting cell

B6-C57BL/6 mouse

B7-Peripheral membrane protein on activated APC cells

BM-Bone marrow

BRAF V600E-Common melanoma antigen targeted by vemurafenib

CCR7-Chemokine receptor 7

CD-Cluster of differentiation

CD62L-L-selectin

CD127-Interleukin-7 receptor-α subunit

CARs-Chimeric antigen receptors

C-MYC-transcription factor regulates proliferation, stem cell pluripotency and cancer

ConA-Concanavalin: A Plant lectin that agglutinates T cells for activation

cTEC-Cortical thymic epithelial cells

CTL-Cytotoxic T lymphocyte

CTLA4-Cytotoxic T Lymphocyte associated protein 4

DC-Dendritic cell

DN-Double negative cells

DP-Double positive cells

dsRNA-Double stranded RNA

EDTA-Ethylenediaminetetraacetic acid

EG7-OVA- T cell lymphoma that transgenically expressed the OVA antigen

EOMES-Eomesodermin

ERK-Extracellular signal-related kinase

FBS-Fetal bovine serum

Flt-3-Fms-related tyrosine kinase 3 (CD135)

GSK3B-Glycogen synthase kinase 3 beta

GZB-Granzyme B

H2-K_B -A classical MHC I haplotype present in B6 mice

IFN-γ-Interferon-gamma

IDO-Indoleamine 2,3-dioxygenase

IL-Interleukin

iPSC-induced Pluripotent Stem Cell

i.v.-Intravenous

JAK-Janus kinase

KLF-Kruppel-like factor

KLRG1-Killer cell lectin-like receptor subfamily G member 1

LCK-Lymphocyte-specific protein tyrosine kinase

LCMV-lymphocytic choriomeningitis virus

LM-OVA-Listeria Monocytogenes expressing OVA

LN-Lymph node

LPS-Lipopolysaccharide

MDSC-Myeloid-derived suppressor cells

MHC-Major histocompatibility complex

NANOG-Master regulatory transcription factor of embryonic stem cells

NFAT-Nuclear factor of activated T-cells

OCT4-POU5F1: transcription factor regulates embryonic stem cell self-renewal

OT-I-Transgenic mouse with $CD8^+$ T cells that express a TCR specific for the SIINFEKL peptide of chicken ovalbumin presented by MHC I

PBL-Peripheral blood

PBMC-Peripheral blood mononuclear cell

PBS-hosphate buffered saline

PCR-Polymerase chain reaction

PDK1-Phosphoinostide-dependent kinase 1

PHA-Phytohemagglutinin is a plant lectin that agglutinates T cells and activates them

PKC-signal transduction enzyme protein kinase C

PMA-phorbol 12-myristate 13 acetate activates PKC

PI3K-Phosphoinositide 3-kinase

PIP-Phosphatidylinositol

piPSC-protein induced pluripotent stem cells

QQ-nuclear localizing reagent

qPCR-quantitative PCR (Real-Time PCR)

RT-PCR-reverse transcriptase PCR

S.C.-Subcutaneous

SCF-Stem cell factor

SIINFEKL-amino acid sequence of OVA antigen

SON-Combination of transcription factors SOX2, OCT4, and NANOG

SOX2-sex determining region Y-box 2 regulator of pluripotency

SP-Single positive

STAT Signal transducer and activator of transcription protein

STICTL-SON treated Tumor-infiltrating Cytotoxic T Lymphocytes

T-bet-T-box transcription factor TBX21

T_{cm} -Central memory T cell

TCR-T cell receptor

T_{em}-Effector memory T cell

TIL-Tumor infiltrating lymphocytes

T-iPSCs-induced pluripotent stem cells originating from $CD8^+$ T cells

TLR-Toll-like receptor

TNFα-Tumor necrosis factor alpha

TNFR-Tumor necrosis factor receptor

T_{reg}-Regulatory T cell

T_{scm}-Stem cell-like memory T cell

TWS119-GSK3B inhibitor

VV-OVA-Recombinant Vaccinia virus expressing $OVA_{257-264}$ peptide from chicken ovabumin

Wnt-signaling pathway activated by Frizzled family receptors

WT-Wild type

ZAP70-Zeta-chain associated protein kinase

Chapter 1: Introduction

Cancer and the immunosuppressive microenvironment

Cancer kills over 500,000 people each year, making it the second most common cause of death in the United States [1, 2]. In 2015 alone, an estimated 1.6 million people will be diagnosed with cancer [2]. Each cancerous cell originates from a normal cell that has mutated to grow indefinitely [3]. As the complex processes of cancer formation and growth are being elucidated, the treatment options are slowly shifting from nonspecific and potentially toxic treatments, such as radiation and chemotherapy, toward immune-based options that specifically target the tumor [4, 5]. Immunotherapy has the potential to provide long lasting remission because of its specificity against the cancer cells, the capacity for memory formation, and a reduction in adverse events [6-9]. In order to develop effective therapies to control the cancer, we must first understand the interactions between the immune system and the tumor cells.

According to the Immunoediting Hypothesis, cancerous cells are only able to develop into a tumor if they are able to evade the attacks from the immune system [7, 9-11]. There are two sides of the immune interface with cancer: anti-tumor and pro-tumor. Although other immune cells, such as natural killer cells, can aid in the battle against cancer, the anti-tumor response is mainly administered by cytotoxic T lymphocytes (CTLs) (Figure 1). CTLs are characterized by their surface expression of CD8 [12-14]. Each CTL possesses a T cell receptor [15] specific for a particular

cancer antigen [12, 16]. Naïve CTLs are antigen-inexperienced T lymphocytes that require activation by an antigen-presenting cell (APC), such as a dendritic cell (DC). Upon presentation of the correct antigen, the naïve CTL will proliferate and differentiate into an army of effector CTLs [12, 16, 17]. Three signals (3SI) must be present in order to fully activate these lymphocytes: antigen, costimulation, and a cytokine signal [18, 19]. On the surface of the antigen presenting cell, the antigen is presented on the major histocompatibility complex (MHC) class I, along with costimulatory surface molecules, like B7.1 and B7.2, providing the first and second activation signals [16, 19]. The third signal of activation is an inflammatory cytokine, such as IL-12 or Type I IFN [18-21]. IL-12 is produced by macrophages, B cells, and dendritic cells in the surrounding environment [22]. Without costimulation or a cytokine signal, the resulting T cells will most likely become tolerant [18, 23]. When CTLs are tolerant of a particular antigen, they no longer mount an effector response to kill their target. However, with proper stimulation from these three signals, the naïve CTL rapidly proliferates and differentiates into effector CTLs. These effector CTLs then infiltrate the tumor, prepared to bind and kill the cancer cells [13, 14, 24, 25].

However, the pro-tumor aspect of immune system is cultivated from within the tumor microenvironment. The cancer takes advantage of previously established immune checkpoints and inhibitory mechanisms to evade and tolerize the immune system [26]. The cancer cells begin by secreting suppressive factors like VEGF, IL-10, TGF-β, and gangliosides [27-31]. These suppressive factors recruit myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), immature

dendritic cells, and regulatory T cells (T_{regs}) to the tumor and then enhance the regulatory component of their normal function [28, 31-34]. Though all of these different cell types are known to contribute to the inhibition of an anti-tumor response, the exact mechanism of how this suppression occurs is still under extensive investigation.

These pro-tumor cells use a variety of strategies both at the site of T cell activation in the secondary lymphoid tissue and within the tumor itself. It begins at the level of antigen presentation. Immature dendritic cells are peripheral immune cells that promote self-tolerance [35, 36]. Under cancer conditions, these immature dendritic cells are fostered within the tumor by the excessive amounts of suppressive factors that inhibits dendritic cell differentiation and maturation [37]. IL -10 nudges the dendritic cells to express the co-inhibitory molecules PD-L1 (B7-H1) and B7-H4 rather that the costimulatory molecules B7.1 and B7.2 [26]. B7-H1 molecules bind to the suppressive receptor PD-1 on T cells, directly suppressing T cell activation [38]. Without the proper signals at activation, the naïve CTLs are unable to adequately differentiate and expand, and may become tolerant.

Already at a disadvantage, the anti-tumor response faces greater immune suppression within the tumor itself. Tumor-associated macrophages (TAMs) promote angiogenesis and these new blood vessels supply a steady amount of oxygen and nutrients to the cancer cells [3, 39]. In addition to this, tumor-associated macrophages also secrete anti-inflammatory signals such as IL-10, TGF-β, and arginase-1 [39, 40]. TGF-β secretion lessens the adaptive immune response in a few different ways [39]. First, TGF-β impairs dendritic cells migration and increases the apoptosis of these

dendritic cells [39]. TGF-β also directly inhibits natural killer cell and CTL anti-tumor activity within the tumor [39]. IL-10 suppresses the production of pro-inflammatory cytokines IFN-γ and IL-12 that encourage T cell differentiation, further reducing CTL anti-tumor activity [39]. Both tumor-associated macrophages and myeloid-derived suppressor cells expressed the inhibitory B7 homologs, PD-L1 (B7-H1) and PD-L2 (B7-DC) as well as the costimulatory versions B7.1 and B7.2. PD-L1 and PD-L2 bind to their receptor, programmed cell death protein 1 (PD-1). The costimulatory B7.1 and B7.2 can bind to cytotoxic T lymphocyte antigen 4 (CTLA4) instead of CD28. Both PD-1 and CTLA4 directly inhibit TCR signaling and thus anti-tumor activity.

Myeloid-derived suppressor cells are a heterogeneous cell population of immature and mature myeloid cells, dendritic cells, activated granulocytes, and macrophages [26, 34, 41, 42]. These myeloid-derived suppressor cells are able contribute to the suppressive environment within the tumor in a variety of ways [26, 34, 41, 42]. In addition to the mechanisms mentioned above, myeloid-derived suppressor cells produce reactive oxygen and reactive nitrogen species using inducible NO synthase and NADPH oxidase [39, 41, 43, 44]. These reactive species further suppress T cell proliferation and limiting their reaction against the tumor [39, 41, 43, 44]. Myeloid-derived suppressor cell's expression of the inhibitory molecule CTLA4 also helps promote differentiation of regulatory T cells [45].

Another strategy these cells use to promote tolerance of the tumor is through the reduction of essential amino acids in the local environment. The myeloid-derived suppressor cell population uses enzymes, like arginase-1, to limit the availability of

amino acids in the local environment [39, 44, 46-49]. The depletion of L-arginine by arginase-1 especially impairs T cell proliferation and cytokine production, thus promoting T cell anergy [47]. Anergy is a state of tolerance T cells acquire after being exposed to their antigen [50]. The upregulated expression of indoleamine 2,3-dioxygenase (IDO) on the surface of myeloid-derived suppressor cells and immature dendritic cells also aids in the depletion of essential amino acids, because IDO is an enzyme that degrades tryptophan [51]. This elevated expression of IDO has been linked to an increased infiltration of regulatory T cells [48].

The regulatory T cells that infiltrate the tumor further incite the immunosuppression [52-54]. Under normal circumstances, regulatory T cells act to maintain tolerance of self-antigens and to reduce inflammatory responses after a challenge. Within the tumor, regulatory T cells use anti-inflammatory cytokines and cell-to-cell contact to reduce the anti-tumor response. The detailed mechanism by which regulatory T cells directly induce cell tolerance is still a major topic of research [55]. Regardless, it is clear that Tregs assist with the suppression of the pro-inflammatory reaction. Like the surrounding tumor cells and tumor-associated macrophages, regulatory T cells produce IL-10 and TGF-β [52, 56]. They also constitutively express CTLA4, the inhibitory receptor that bind to B7 costimulatory molecules expressed on antigen presenting cells, thus impairing activation of anti-tumor CTLs. These attributes contribute to the regulatory T cells ability to inhibit the immune's effector response.

Once the effector CTLs have entered the tumor microenvironment, all of these pro-tumor immunosuppressive signals work to prevent these CTLs from controlling

the tumor. The regulatory T cells can directly bind to the TICTLs, while myeloid-derived suppressor cells, immature DCs, and tumor associated macrophages work with cancer cells to establish the immunosuppressive microenvironment with signals like IL-10, TGF-β (Figure 1). The combined effect of these pro-tumor immune mechanisms prevents the TICTLs from eliciting an effector response sufficient to control tumor growth. These confused tumor-infiltrating CTLs (TICTLs) take on an increasingly exhausted state, and remain within the tumor [57, 58]. The tolerance created in this environment is one of the biggest obstacles for controlling the cancer [10, 32, 54, 58-65].

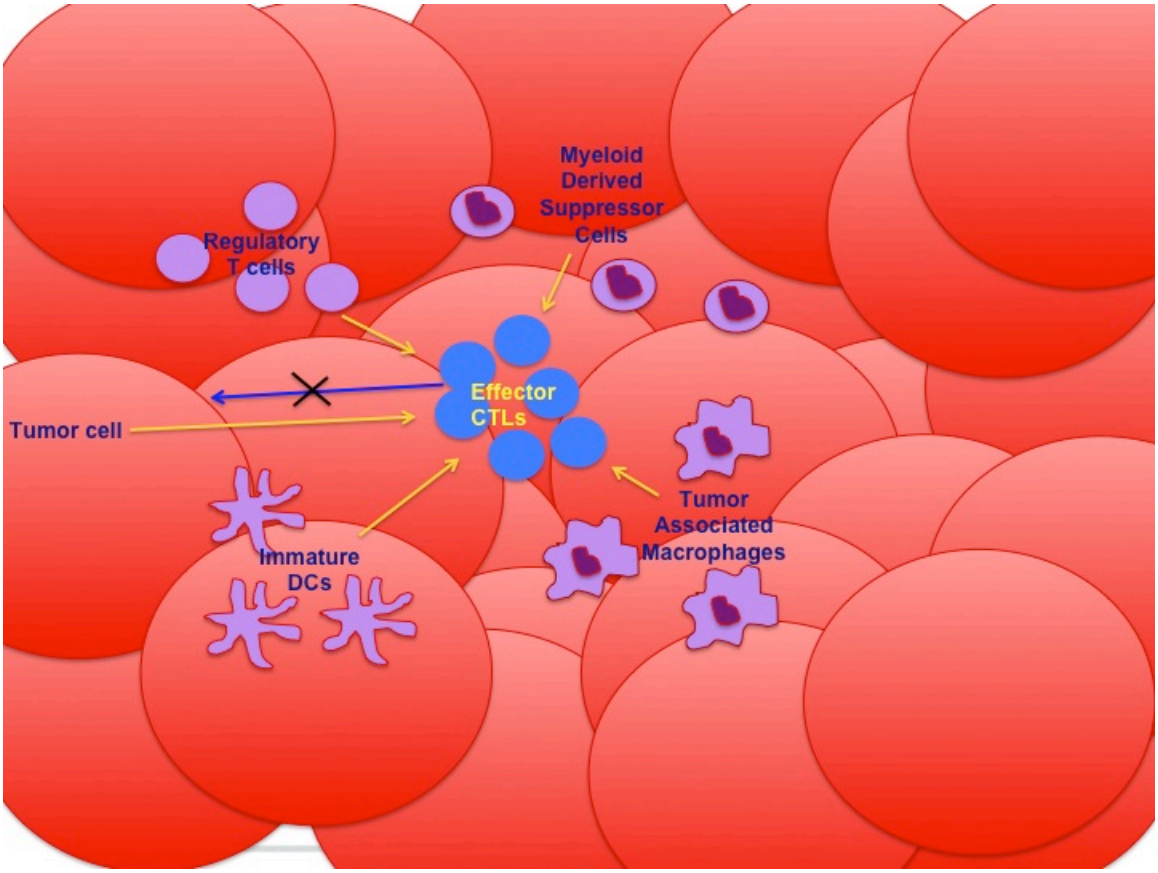


Figure 1: A look inside the tumor. A representation of how the pro-tumor immune cells and cancer cells act on the anti-tumor effector cells to prevent the effector CTLs from killing the tumor cells.

Nonspecific immune therapies

The mimicry of immunological tolerance mechanisms has given new targets to non-specific cancer immunotherapies [10]. Ipilimumab and nivolumab are monoclonal antibodies that bind to CTLA4 and PD-1 respectively [9, 10]. Ipilimumab blocks CTLA4 from being activated thus allows the T cell anti-tumor activity to continue [10, 66-68]. So far ipilimumab has been approved for use against melanoma and is currently in clinical trials against non-small cell lung cancer (NCT00527735), bladder cancer (NCT01524991), and prostate cancer (NCT00323882) [69, 70]. Though melanoma patients treated with ipilimumab showed an increased overall response rate of 10.9% and overall survival rate of 47.3%, the unlimited proliferation of T cells also increased the risk of adverse events related to an overactive immune system [70, 71]. Nivolumab targets PD-1, another inhibitory receptor expressed on the surface of activated T cells [38, 66]. As mentioned above, when PD-1 binds to one of its ligand PD-L1 or PD-L2, the T cells' effector function is down-regulated [72]. Nivolumab competitively inhibits PD-1 and prevents its activation, resulting in an increase in T cell effector function and a clear tumor regression in 31% of patients [38, 73]. The toxicity of this therapy appears to less than that of other immune therapies like IL-2 supplementations and ipilimumab [69]. In addition to melanoma, nivolumab has been approved for lung cancer and is currently in clinical trials for other forms of cancer including leukemia (NCT02420912), renal cell carcinoma (NCT01472081) [74]. Many of these clinical trials are also investigating the use of these inhibitors in conjuncture with other treatment options including each other (NCT01621490). Both of these immunomodulators limit the level of tolerance the

cancer establishes in the CTLs by blocking PD-1 and CTLA4. But the tumor cells can use other immunosuppressive mechanisms to inhibit the anti-tumor response of TICTLs.

The supplementation with certain cytokines can help stimulate the immune response. The cytokine interleukin-2 (IL-2) is a survival signal used to promote the proliferation and cytolytic action of T cells [75, 76]. IL-2, also known as Aldesleukin, was the pioneering immunotherapy to elicit a reduction in tumor size [77]. Approved by the FDA in 1998, IL-2 was able to show durable response rates against metastatic cancers, like melanoma and renal cancer, in about 15-20% of patients when a high dose of either 600,000 or 720,000 Units/kg were administered every 8hrs for 14-15 consecutive doses [76, 77]. Despite this positive tumor response, IL-2 has also been linked to increased regulatory T cell activity, which countermands the pro-inflammatory, anti-tumor response that we hope to incite [58, 75, 78]. In more recent years, IL-2 has been combined with other forms of immunotherapy, like adoptive cell transfer therapy, ipilimumab (NCT01856023), and BCG vaccination, to develop a more effective regimen to deliver long lasting remission [77, 79]. While all of these treatment options have shown to have an effect on the immune response's ability to slow the progression of the cancer, the cancer still continues to evade and grow. More targeted treatment options, such as antigen specific immunotherapies, may prove to be more effective in halting the advancement of the cancer.

Cancer vaccination

Many cancer-specific antigens have been identified in a variety of cancers that are expressed at levels that could be used to elicit an anti-tumor response [80].

However, so far all attempts at developing a cancer-specific vaccine that is capable of producing a clear tumor response on its own has failed. Some of the cancer vaccines made it through phase I and phase II clinical trials before disappointing results, preventing them from continuing into phase III clinical trials. Cancer vaccination approaches are divided into three categories: peptide or protein vaccines, dendritic cell vaccines, and whole tumor cell vaccination. In peptide vaccines, a known cancer antigen, like gp100, is isolated and injected into the patient with an adjuvant to attempt to increase the immune response against that antigen [81]. In the case of the gp100 cancer vaccine, there was not a significant difference in survival or tumor regression in metastatic melanoma patients when the gp100 vaccine was used alone, but there was improved survival when administered with high dose IL-2 [82]. This vaccine is now used as a control for other clinical trials [71, 83-85]

Another option, known as dendritic cell vaccination, loads peptides from melanoma antigens onto CD34+ dendritic cells [82, 86]. After immunization with these loaded dendritic cells, the overall survival increased by 2 years, though there was no change in the tumor [82, 87]. The last common vaccination procedure is whole-tumor cell vaccination [82]. This is the classic method of immunization using whole irradiated tumors allows for exposure to a broader spectrum of the cancer-associated antigens [82]. This method showed an 8.1% clinical response compared to 3.6% response for immunization with synthetic peptides in phase I clinical trial [82, 88]. The problem with fighting cancer is not just antigen exposure. Vaccinations do not take into sufficient account the suppression cancer is able to overlay on the

immune system, and this could be the main reason why a therapeutic vaccination against melanoma has been ineffective so far.

Antibody-based therapies against cancer-specific antigens can be very therapeutically relevant. One example of these specific antibody therapies is Vemurafenib. Vemurafenib is a monoclonal antibody based therapy that targets the mutated B-raf enzyme (BRAF) associated with 40-60% of melanoma [9, 66]. The antibody essentially tags the cells expressing the appropriate antigen, like BRAF, making them easier targets for immune response to destroy [16, 89, 90]. Phase I and Phase II clinical studies have shown that there is a 53-57% response rate in patients with melanoma containing the BRAF V600E mutation [89, 91]. The development of more monoclonal antibodies for tumor specific antigens will be helpful to increase the response against the tumor, but the response against the tumor is fleeting. In the case of BRAF V600E, the cancer can quickly become resistant to this method of treatment. The cancer cells respond to anti-BRAF treatment by bypassing the reliance on BRAF by indirectly activating the same RAS-RAF-MEK-ERK survival pathway using the BRAF alternative, CRAF, to continue to survive [92, 93]. Though further investigation is required to understand exactly what mechanism is responsible for the development of this resistance, it is clear that the intense selective pressure that produces such a strong initial response rate also selects for mutations that allow the cancer to resist in the future [94-96]. Rather than focus on the short-lived humoral approaches, immunotherapies based on the cellular part of the adaptive immune system may be able to directly overcome the suppressive atmosphere within the tumor.

Adoptive cell transfer therapy

Among the more successful treatment options is adoptive cell transfer therapy (ACT). ACT utilizes activated T lymphocytes that have infiltrated the tumor (TILs) to control the cancer by removing TILs from the immune suppressive tumor microenvironment, expanding them *in vitro*, then infusing these cells back into the patient to attack the tumor [97-102]. In both the *in vitro* and *in vivo* aspects of ACT, the survival signal Interleukin-2 (IL-2) is used to promote the proliferation and cytolytic action of T cells [8, 103].

The standard protocol begins with the surgical excision of the tumor [103]. The patient is then given lymphodepleting chemotherapy, while the TILs are then expanded *in vitro* from either a small chunk of the tumor or single cell suspension of digested tumor in a 24-well plate for 1-2weeks [81, 103]. When roughly $1.5x10^6$ cells are present in each well, it was split into 2 daughter wells [81, 103]. The fastest growing cultures undergo the rapid expansion protocol [103]. Under this protocol, these cells were co-cultured with anti-CD3, IL-2, and irradiated peripheral blood monocyte feeder cells for 14 days [100, 103-106]. These irradiated peripheral blood monocytes are non-dividing cells that present tumor antigens to the lymphocytes with which they are co-cultured [103, 104, 107, 108]. The TILs are then infused back into the patient when the expanded cell number is greater than $3.1x10^{10}$ and up to $1x10^{11}$ cells [85, 103, 109, 110]. The patient also is administered a high dose of IL-2 (720,000 IU/kg) every 8 hours after TIL injection fro 2-3 days [8, 104]. This standard treatment of ACT results in a 51% response rate in patients with metastatic melanoma [99].

Limitations of ACT

Despite this promising response rate, ACT has considerable limitations in a clinical setting and in efficiently activating TILs. IL-2 often causes additional clinical complications, such as capillary leak syndrome, hypotension, and pulmonary failure [111, 112]. IL-2 has also been linked to increased regulatory T cell activity and may contribute to further immune suppression of CD8+ T cells by regulatory T cells [63]. While it is recognized that cytolytic T lymphocytes (CD8+ T cells) are the most effective cancer killing cells, the expansion these already exhausted TICTLs must undergo to meet the high cell number requirement ($3.1x10^{10}$-$1x10^{11}$ cells) for transfer could be pushing them to a terminally differentiated state [6, 113]. This push to an older more terminal state impairs proliferative ability, which in turn reduces the TICTL's ability to respond to the cancer [57, 60, 113, 114].

A few clinical trials are underway to overcome many of limitations associated with ACT. Some are looking into the interaction between ACT and other commonly used therapies to see if there is an improved clearance of the cancer. In addition to trials that are attempting to optimize the chemotherapy lymphodepletion regimen (NCT01807182, NCT01993719), other trials are combining drugs that target cancer-related pathways with ACT. An ongoing clinical trial is evaluating the safety of vemurafenib when used in conjuncture with ACT (NCT01585415) [84]. A more effective treatment regimen that reinvigorates the exhausted T cells could be developed to help overcome the tumor immune suppression.

Another approach to over coming ACT's limitations is focused on developing effective chimeric antigen receptors (CARs) to improve T cell activation and cancer

specificity. Ideally, these engineered receptors would target tumor-associated antigens that are express on the surface of all cancer cells, including cancer stem cells, but not on other tissues [115, 116]. Unfortunately, the antigens commonly expressed on the cancer are rarely perfect targets. Many of the antigens cancer expresses are derived from normal genes that have been subject to point mutations that lead to an altered epitope [117]. Costimulatory signals are added to each of the CARs to enhance activation [115, 118, 119]. This method does not require the lymphocyte to be naturally specific for the target antigen. Instead, the lymphocytes used in these trials are often purified from the peripheral blood, not the tumor. These lymphocytes are then retrovirally transduced with the CAR, expanded and injected back into the patient with a high dose of IL-2. Engineering the TCR and inserting it into a harvested lymphocyte can achieve cancer antigen specificity without pushing the cells to their terminally differentiated state.

So far effective CARs for surface cancer antigens MART-1, anti-VEGFR2, anti-CD19, and anti-GD2 are in phase I or phase II clinical trials (NCT00910650, NCT01218867) [120, 121]. There are many other ongoing clinical trials looking at the effectiveness and safety of anti-GD2 (NCT02107963, NCT01822652), and anti-CD19 (NCT02349698) against a variety of different cancer types including melanoma and lymphoma. In some cases, the adoptive transfer of the CAR possessing T cells has increased the response rate to 66% or higher [119]. Even though the number of lymphocytes able to infiltrate the tumor does not limit this method, CAR T cells still are only reactive on one particular cancer antigen. This limits the targeting capability of the effector response. This treatment could be

providing a selective pressure for the cancer to develop without the expression of that antigen. If the TCR diversity of wildtype TICTLs can be preserved and the cell number of this heterogeneous population increased without exhausting these cells, then these cells may be able to kill cancer more efficiently.

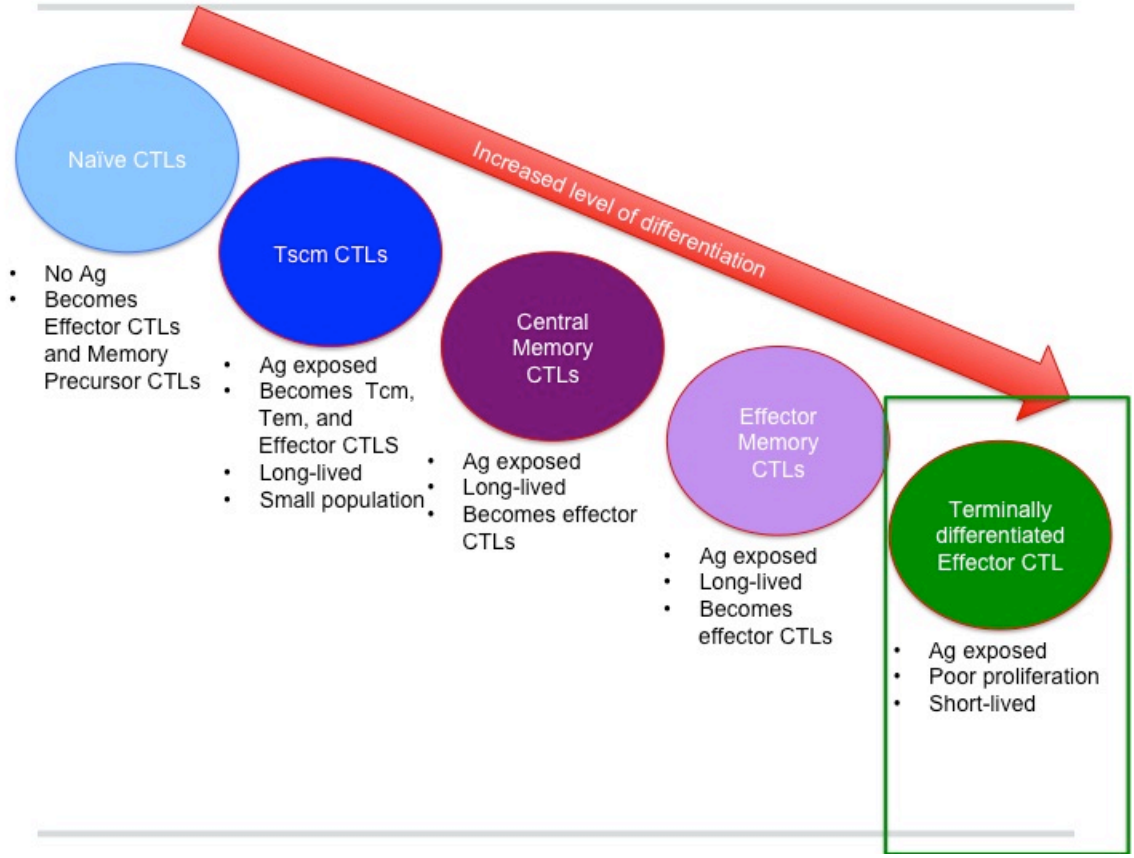


Figure 2: Progression of CTL Differentiation. Schematic of CTLs from least differentiated subsets to terminally differentiated CTLs.

Memory CTL subsets

Rather than continuing to push the T cells to a terminally differentiated state, ACT may benefit if the protocol promoted the formation of less differentiated CTL phenotypes, such as stem cell-like memory CTLs or central memory CTLs (Figure 2). A less differentiated cell is able to mount a more intense effector response when faced with an antigenic challenge. Antigen experienced memory CTLs are divided into subtypes capable to responding more vigorously when exposed to an antigen than a naïve CTL [113, 122, 123]. All memory subsets have the capability of maintaining a homeostatic population over a long period of time and differentiating into effector CTLs upon antigen reoccurrence [16, 124, 125]. Central memory T cells tend to be found in the peripheral lymphatic system, with a phenotype of IL-7R^{hi}, CD62L^{hi}, CCR7^{hi} and have a substantial proliferative response to the antigen [123, 126, 127]. Stem cell-like memory CTLs (T_{scm} cells) are the least differentiated memory subtype and have the capability of differentiating into central memory CTLs, and effector memory CTLs as well as effector CTLs [128]. This unique capacity of T_{scm} cells to differentiate into other memory subsets as well as effector CTLs gives these cells a more pronounced proliferative response to an antigen [128]. By focusing on this particular T_{scm} cells phenotype, high cell transfer number necessary for ACT may be reduced and the effector response increased [126].

T_{scm} cells were first discovered through WNT signal activation with the GSK3B inhibitor TWS119 [128, 129]. Since T_{scm} cells were discovered, the recognized phenotype CCR7+, CD62L+, CD27+, CD28+ and IL-7Ra+ has been used to identify and isolate these cells peripheral blood mononuclear cells (PBMC) in

humans and mice [128, 130, 131]. Unfortunately, this memory phenotype makes up a very small percentage of the memory cell population in the body [122, 129, 132]. *In vitro*, only a limited number of these cells can be generated through WNT signal activation possibly because WNT signaling may inhibit CTL activation [122, 129, 132]. A protocol to efficiently generate T_{scm} cells needs to be developed so these reactive cells can be used in a clinical setting.

Reprogramming to iPSCs

While the insertion of a genetically engineered chimeric antigen receptor is a popular approach to reduce the limitations of adoptive cell transfers, using regenerative techniques may prove to be more effective in providing younger effectors against the malignant cancer [133, 134]. By reprogramming these exhausted TILs back to induced pluripotent stem cell, the TILs can be guided into a younger, less differentiated state that can provide a stronger proliferative and effector response when encountering the antigen (Figure 3) [113, 128]. Reprogramming usually refers to the retroviral insertion of transcription factors that are used to push a fully differentiated somatic cell into a pluripotent state creating an induced pluripotent stem cell (iPSC) [135-137]. Traditionally, the Yamanaka factors OCT4, SOX2, Klf-4, and C-MYC (OSKM) are used, but by using SOX2, OCT4, and NANOG and removing the proto-oncogenes KLF4 and C-MYC, it may be possible to reduce oncogenic mutation rates [137-139].

SOX2, OCT4, and NANOG (SON) are known master controlling transcription factors of reprogramming [140]. In embryonic stem cells, the expression level of each of these transcription factors can either be used to sustain pluripotency

or guide differentiation [140]. These SON transcription factors act upon a complex network of signaling pathways during early embryonic development, including the WNT signaling pathway [140, 141]. When overexpressed in somatic cells, they guide the cells back to a pluripotent state and create induced pluripotent stem cells [142]. While the specific mechanism of action of SON in immune cells is unclear, these transcription factors have been used successfully to reprogram mature T cells into an induced pluripotent stem cell [143]. SOX2 and NANOG are normally expressed in only pluripotent embryonic and somatic stem cells and are not expressed in normal peripheral blood T cells [144-146]. The relationship between CTLs and OCT4 is a complicated one. Some CTLs even mount an immunological response against OCT4 [147]. OCT4 has two main isoforms OCT4A and OCT4B. OCT4A is one of the main transcription factors responsible for embryonic stem cell (ESC) pluripotency [148]. OCT4B, on the other hand, is unable to sustain this state of self-renewal in ESCs, but seems to be related to cell stress [148]. The isoform OCT4B is expressed in peripheral blood mononuclear cells, including CTLs, but the expression of the pluripotency marker OCT4A is still contested [149, 150].

An induced pluripotent stem cell has many of the same characteristics and capabilities of an embryonic stem cell including the ability to differentiate into endoderm, mesoderm, and ectoderm [135, 137]. These are the three tissues from which all of an organism's cells are derived [135, 137]. Induced pluripotent stem cells express genes closer to that of an embryonic stem cell then a differentiated cell such as the fibroblast. These iPSCs are defined by their ability to form teratomas, a type of tumor that contains endoderm, mesoderm, and ectoderm.

Retroviral reprogramming of a somatic cell into an induced pluripotent stem cell reduces number of ethical concerns that plague regenerative medicine when the generation of a specific cell type relies on harvesting human embryonic stem cells from embryos [137, 151]. The induction of pluripotency in somatic stem cells means that these cells can be guided toward many different cell fates. However, other studies have shown that these reprogrammed cells may still retain a sort of methylated memory of the original somatic cell's chromatin structure [152-155]. While this epigenetic memory may cause problems if the cell needs to be reprogrammed to a different cell type, this preference for the original somatic cell type may work to the advantage of ACT [113, 156].

T-iPSCs

Previous attempts to reprogram T cells back to pluripotency to create iPSCs (T-iPSCs) then guided forward have shown that the T cells products of T-iPSCs retain their original TCR [113, 156]. This retention may allow these rejuvenated cells to maintain the naturally diverse reactive T cell population against a variety of different antigens the cancer may possess [113, 156]. Since the newly reformed T cells are no longer antigenically exhausted, these younger cells are able to elicit an enhanced effector response in addition to expressing their original TCR [113, 156].

A common approach was to use the Sendai virus encoded with the Yamanaka factors SOX2, OCT4, C-MYC, and KLF4 [156, 157]. Generally, the CD3+ cells isolated from peripheral blood mononuclear cells of healthy humans were activated with anti-CD3/CD28 microbeads for 2 days before viral transduction could take place [156, 158, 159]. When using the Sendai virus method, the newly activated CD3+

lymphocytes are transduced with the Sendai virus while in T cell media including IL-7, IL-2, and IL-15 for 6 days before being reseeded onto mouse feeder fibroblasts. Once plated with these feeder cells, it takes as long as 40 days to form iPSCs from in vitro activated T cells (T-iPSCS) [156]. In order to reform functional, mature T cells, these T-iPSCs undergo a protocol that relies on a sequence of feeder cells with additional signals added to push the T cells through each stage of differentiation [156]. A final exposure to either anti-CD3 coated beads or PHA for activation preserved the original TCR and prevent TCRa rearrangement [156]. While this method did yield responsive, antigen specific CTLs, the generation of retroviral iPSCs has a few shortcomings.

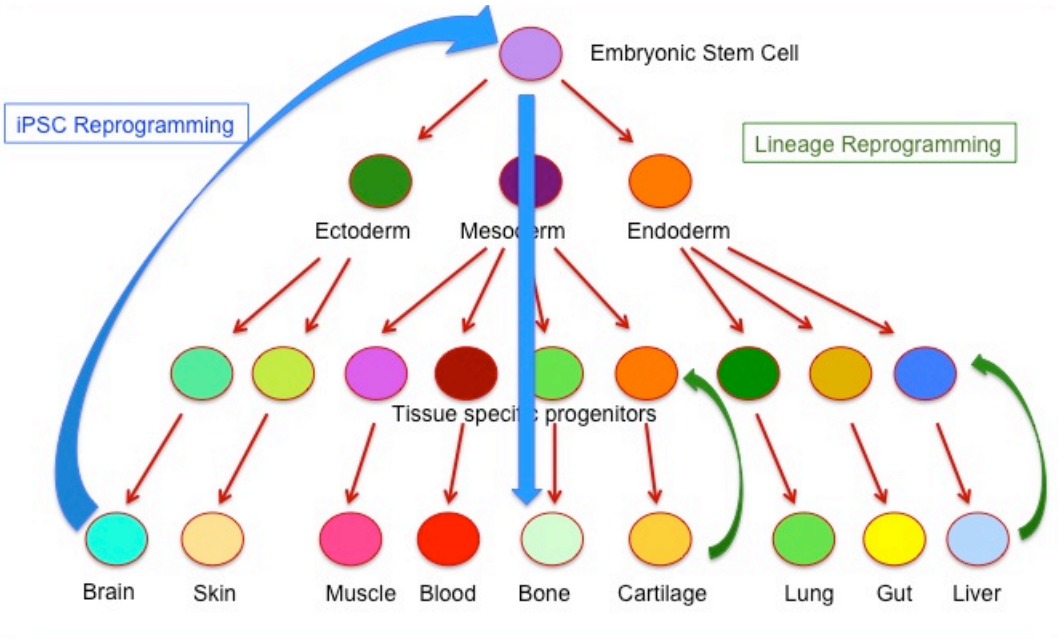


Figure 3: Regenerative Techniques. Diagram of general differences between induced pluripotent stem cell reprogramming and lineage reprogramming.

Lineage Reprogramming

Rather than pushing cells all the way back to a pluripotent state, lineage reprogramming, also known as transdifferentiation, directly reprograms somatic cells into either a precursor cell along the same developmental pathway or an entirely new somatic target cell (Figure 3) [160]. This shortens the generation time, because the somatic cell does not have to go through an induced pluripotent state. Two different approaches can be used to perform this lineage conversion.

The first, retrovirally inserts transcription factors associated with the target cell type, then plates the cells under stem cell culturing conditions to promote the formation of the precursor [160]. The second, uses conventional reprogramming transcription factors and cultures the cells under conditions favorable for that target lineage [160]. The transduction of the reprogramming transcription factors can be delivered retrovirally or by using cell-penetrating peptides to deliver the genes to the nucleus [160].

By directly converting the cell from one type to another, these reprogramed cells have the shorter reprogramming protocol when compared to a somatic cell pushed back to pluripotency then guided to the target lineage [160]. Though this method has not been demonstrated yet in T cells, human fibroblasts have been successfully reprogrammed into hematopoietic progenitor cells using the lentiviral transduction of OCT4 and culture conditions optimized for the generation of hematopoietic progenitor cells [161]. In this case, human fibroblasts transduced with OCT4 were cultured for 21 days on media with Flt-3 and stem cell factor (SCF), and

then CD45+ cells were transferred to media with hematopoietic cytokines (SCF, G-CSF, FLT3LG, IL-3, IL-6, and BMP-4) for 16 days [161]. The cells were tested for a variety of cell markers for pluripotency, hematopoietic cells, and mesodermal cells [161]. Further research needs to done to elucidate if T cells are amenable to lineage reprogramming and under what conditions would this conversion be optimal.

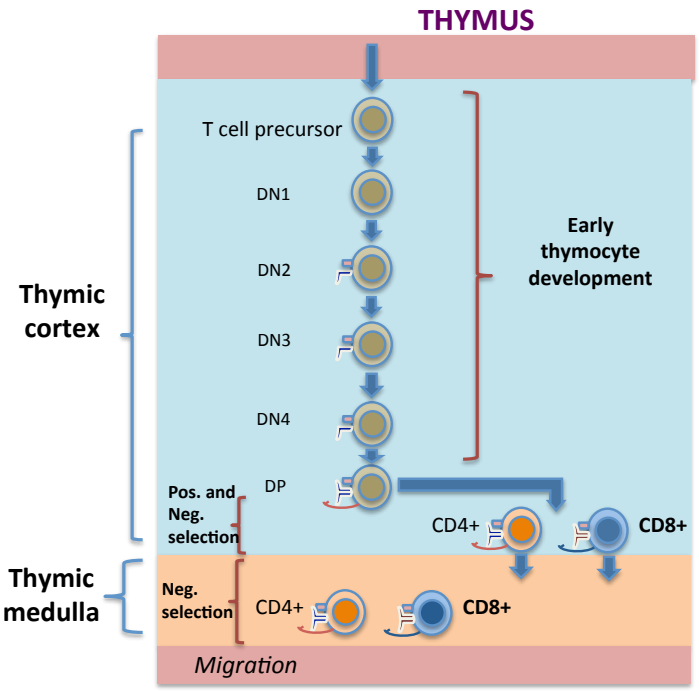


Figure 4: T cell development in the thymus. Depiction of T cell development as it moves through the double negative (DN) stages, the double positive (DP) stage, and single positive stage (SP). The positive and negative selection occurs in the DP and SP stages.

Difficulties with lineage reprogramming

One of the difficulties with lineage reprogramming using conventional transcription factors and specific culture conditions is pinpointing where along the lineage these cells have been reprogrammed [133, 160]. All blood cells, including CTLs, originally develop from hematopoietic stem cells in the bone marrow [16, 162]. In the case of T cells, these hematopoietic stem cells then differentiate into multipotent stem cells and then into lymphoid progenitor cells [16, 162]. Lymphoid progenitor cells then differentiate into early thymic progenitors cells [16, 162]. These early thymic progenitor cells migrate from the bone marrow to the thymus [16, 162]. If the CTLs were pushed too far back along the lineage into these progenitor cell types, then the resulting phenotype would be unable to respond to antigen stimulation because precursor cells would lack the appropriate machinery to recognize an antigenic challenge.

Once in the thymus, precursor T cells known as thymocytes undergo multiple rounds of TCR gene rearrangement (Figure 4) [16, 162, 163]. This random rearrangement with in the TCR genes is responsible for the T cell population's ability to protect against a wide variety of antigens [16, 162, 163]. As the thymocytes matures, the cell acquired components of the cytolytic signaling pathway, but they do not possess a mature TCR capable of correctly identifying the target antigen until the last double negative stage and the double positive stage [16, 162, 163]. At this point, the thymocytes undergo selection processes to ensure each possesses a functional TCR and prevent the development of autoimmune diseases [16, 162, 163]. If the CTLs are transdifferentiated into an early double negative (DN) thymocyte, they may

still be unable to respond to the antigen challenge because they do not possess the necessary costimulatory CD4 or CD8. Double positive cells may not possess a cytolytic ability. Neither of these thymocytes should exhibit the proliferative response and differentiation necessary to mount an attack against their target.

The younger, less differentiated phenotypes that arise from mature single positive T cells after exposure to their associated antigen would be the best target for lineage reprogramming the TICTLs. The most optimal subset of T cells would be the T_{scm} because of their ability to differentiate into many other CTL phenotypes. But if the cells are not pushed back far enough along the T cell lineage, they may remain in a tolerant, unproliferative state that is prone to apoptosis. Characterization of the expression for phenotypic markers at each stage in development and differentiation is essential to determining if the lineage-reprogrammed cell was successfully transdifferentiated into the target cell type.

Limitations of retroviral reprogramming

The use of retroviral transduction to generation of T-iPSCs or to induce lineage reprogramming has many weaknesses when applying this method to a clinical setting. The genes responsible for inducing the pluripotent state in these somatic cells are inserted into the genome, permanently altering it. This retroviral insertion may lead to gene mutation and tumorigensis [160, 164, 165]. The reprogramming efficiency of human somatic cells is low and the method of reprogramming the cells is usually more than 3 weeks in length [135, 160]. Since cells derived from a tumor environment may need to undergo an expansion protocol before reprogramming, at least an additional month of *in vitro* treatment is added before the cells are ready to be

transferred back into the patient. The lengthy protocol is not suited to the limited time most cancer patients have. The added risk of further mutation and generation of additional tumors reduces the appeal to patients already fighting against cancer. All of these concerns limit the application of retroviral reprogramming in a clinical setting [166].

Alternative reprogramming techniques are being explored to improve the safety and reduce the reprogramming time of somatic cells including plasmid transfection and a variety of protein delivery systems. So far the efficiency of reprogramming with these techniques has been minimal [160].

QQ-delivery

However, a fairly new nuclear delivery system known as the QQ-protein transduction technique is showing promise in the generation of iPSCs without the use of a retroviral system [167]. This new protocol for creating iPSCs has emerged recently from Wayne State University using a nuclear localizing reagent known as QQ [167]. The patented QQ reagent delivers the recombinant proteins directly to the nucleus of the cell and remains therefor two days as shown by codelivery with GFP [167]. The nature of this delivery system ensures a controlled exposure to the reprogramming transcription factors long enough to generate piPSCs. The risk for mutation and tumorigenesis is reduced, because the genes for these reprograming factors are not inserted into the genome. Instead, these pluripotent inducing proteins are delivered to the nucleus. This protocol claims to generate piPSCs within a week of exposure to either the Yamanaka factors (OSKM) or the master stem cell

regulators SOX2, OCT4, and NANOG (SON) [167].The patent also claims this technique using SON or OSKM has a near 100% efficiency of iPSC generation [167].

To generate iPSCs using this nuclear delivery system, the recombinant transcription factors were added to the QQ-reagent, then the somatic cells such as adult fibroblasts were treated using 24-hour cycles of QQ exposure for 3-12 hours followed by 21-12 hours of rest in QQ-free media [167]. In order to full reprogram somatic cells, they must undergo a minimum of 2-3 cycles of QQ-SON [167]. After 5-7 days, 500-1500 piPSC colonies have formed [167]. This unique feeder-free protocol that utilizes the protein transcription factors claims to reduce many of the concerns retroviral transduction by decreasing the reprogramming time, increasing reprogramming efficiency, and eliminating the chance of retroviral mutation. It is possible that the QQ delivery system can be used to reprogram these tolerant tumor infiltrating T cells to a younger phenotype without reverting back to pluripotency by adapting this delivery system to lineage reprogramming.

Combining lineage reprogramming and QQ-delivery

Lineage reprogramming using an alternative to retroviral transduction may prove to be safer and more efficient if the QQ nuclear protein delivery system is used. In addition to reducing the concerns associated with retroviral transduction and showing a higher efficiency of transcription factor nuclear translocation, this system only requires a few days to push to full pluripotency and allows only a transient exposure to the reprogramming transcription factors, which will allow greater control when fine tuning the optimum exposure time to SON. That being said, this approach to lineage reprogramming requires the development of culture conditions that

promote the target cell type. The culture conditions necessary to target specific phenotypes of CTLs using lineage reprogramming have yet to be elucidated. Utilizing conventional reprogramming transcription factors, SOX2, OCT4, and NANOG, and culturing the cells under T cell activation conditions is the best place to begin exploring T cell lineage reprograming. Under optimal *in vitro* activation conditions, naïve CTLs are primed to differentiate into effectors cells prepared to combat the antigen challenge and even capable of forming memory CTL [18, 19]. The SON transcription factors have successfully reprogramed exhausted T cells in retrovirus system, and have been used with the QQ nuclear protein delivery system [133, 167, 168]. By creating the conditions that promote young CTLs while being stimulated with SON transcription factors will most likely encourage the lineage reprogramming toward a stem cell like memory CTLs.

The need for a fast, efficient way to develop a large CTL population capable of reacting to a variety of cancer antigens is paramount in the war against cancer and may prove to be beneficial to adoptive cell transfer therapy. We want to investigate the first steps in optimizing the lineage reprogramming conditions necessary to produce a high number of younger CTLS, like stem cell-like memory CTLs, from exhausted tumor infiltrating CTLs. I hypothesis that treatment with SON under T cell activation conditions will yield highly proliferative, less differentiated CTL still capable of antigen recognition.

Chapter 2: Methods

Animal Model

OT-I mice (gifted by Dr. Mescher, University of Minnesota) possess a transgenic TCR are specific for the H-2Kb OVA257-264 of the Ovalbumin protein. These transgenic mice also exhibit the same background genetics as C57BL/6 mice. C57BL/6 mice were purchased from NCI or Charles River Laboratories. All mice were housed under pathogen-free conditions at University of Maryland, College Park. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at University of Maryland (R-15-22).

Cancer Model

EG7-OVA cells were obtained from Dr. Mescher (University of Minnesota) and stored at -80C. To recover the cryogenic vials were thawed in 37^{O}C water bath, then transferred to a 9ml of 25% FBS Allos medium and incubated at room temperature for 10minutes. The viability was checked with trypan blue before spinning down cells at room temperature and resuspended in 10ml of 25% FBS allos medium. G418 at 200ug/ml was used to select for OVA expressing cells and the cells cultured in 37^{O}C incubator with 5% CO2.

TICTL generation and harvest

OT-I mice were injected subcutaneously with $20x10^{6}$ EG7-$OVA_{257-264}$ cells per 200ul of DPBS. When the tumor reached 20mm in diameter, the mice were humanely euthanized with CO_2. The tumor was dissected out, minced, and digested for 2hours

with Collagenase D (400ug/ml), Hyaluronidase (2.5U/ml), and DNase (0.1mg/ml). Collagenase D was stopped with 0.1M EDTA after digestion. The cells were homogenized again into a single cell suspension before being washed with DPBS multiple times. Then the cells were resuspended in Allos media at 20million cells/ml and stained with 5ul CD8 PerCP/ml. The cells were then washed and resuspend at 20million cells/ml of Allos. Using the FACSAria II flow cytometry machine, the tumor cell suspension was sorted according to CD8+ expression. Depending on how many CD8+ T cells were isolated from the tumor, between $5x10^5$ or $1x10^6$ CD8+ T cells were then plated per well in Allos media on a flat-bottom microtiter with or without SON stimulation.

Naïve T cell purification

Naïve OT-I CTLs were isolated from inguinal, axillary, brachial, cervical and mesenteric lymph nodes (LN) as previously reported [20]. Briefly summarized, the LNs were pooled and homogenized into a single cell suspension. Then CD8+ CD44lo cells were isolated using negative selection. Cells were labeled and incubated with FITC antibodies for CD4, B220, I-A^b, and CD44. These FITC labeled cells were incubated with Anti-FITC magnetic Microbeads (Miltenyi Biotech). The labeled cell suspension was then passed through separation columns on a MACS magnet. Cells that passed through the column were collected and displayed >95% CD8+ and <0.5% CD44hi purity.

SON treatment

Cells received treatment using the QQ nuclear protein delivery technique (QQ-SON) from Dr. Jianjun Wang, Wayne State University (Patent #US20140242694 A1). QQ-SON consists of the nucleus targeting QQ-reagent and recombinant protein transcription factors SOX2, OCT4, and NANOG (SON) that delivers these reprogramming proteins to the nucleus of somatic cells. For each 24hour cycle treatment, the Naïve CTLs or TICTLs were exposed to 5ul QQ-SON in 1.5ml total Allos media for 5 hours before changing the media to 1.5ml Allos media without QQ-SON to allow the cells to rest for 19hours. When they are treated for 48hours they undergo two consecutive 24hour cycles as described above. After exposure to QQ-SON, the cells were transferred to a well with the three signals necessary to activation naïve CTLs and 1.5mls of Allos.

In vitro stimulation of CTLs

Flat-bottom microtiter wells in 24-well plates were coated with recombinant B7-1/Fc chimeric protein (R&D Systems) for costimulation and the antigen $OVA_{257-264}$ loaded onto DimerX H-2Kb:Ig fusion protein (BD Pharmingen) [20]. 2U/ml of murine rIL-12 (R&D Systems) were added to each coated well making these wells stimulated with all three activation signals (3SI) and 2.5U/ml of IL-2 was added as a survival signal. For the first antigen exposure after QQ-SON treatment, the cells were stimulated with 3SI for 4-5 days and incubated at 37 ^{O}C and 5% CO_2. For each additional exposure to antigen, $3x10^5$ cells were stimulated for 3 days under 3SI conditions in 1.5ml of Allos media. All controls not treated with QQ-SON were stimulated for 3 days with 3SI. Cells were plated in equal number ($3x10^5$ cells) in

1.5ml Allos media on uncoated wells without supplemental IL-2 or rIL-12 after the initial 4-5 day 3SI stimulation. Cells were identified with anti-CD45.2 mABs.

Adoptive cell transfer

In vitro cultured CTLs were adoptively transferred into wildtype C57BL/6NCr mice after stimulation with 3SI and QQ-SON treatment through intravenous injection through the tail vein with a volume of 300ul. Cell viability was determined using trypan blue and a hemocytometer. Blood was collected at various time points post transfer.

Intracellular staining

vitro cultured CTLs were stimulated in Allos with 0.2uM $OVA_{257-264}$ peptide and 1ul Brefeldin A (Biolegend) for 3.5hours at 37 ^{O}C and 5% CO2 in order to stain cells with IFNγ and TNFα. Directly conjugated fluorescent antibodies were purchased from BD Biosciences, eBioscience, or Biolegend. Granzyme B and BCL-2, and IFNγ and TNFα intracellular staining all follow the next protocol. 4% Paraformalin was used to fix the cells for 15minutes at 4^{O}C, then permeablized with saponin-containing Perm/Wash buffer (Biolegend) for 15minutes at 4^{O}C. After 5 minutes of incubation with Fc blocker (CD16/32), the cells are incubated with the conjugated antibodies for IFNγ -PE and TNFα-APC, and GZB-PE and BCL-2 PB separately for 30min at 4^{O}C. Then each sample of cells were washed with Perm/Wash buffer, followed by a wash with PBS with 2% FBS. These samples were then read with the FACSCanto II machine and analyzed with Flowjo.

Annexin V staining

Cells were washed twice with cold Biolegend's Cell Staining Buffer (Cat# 420201), then resuspended in Annexin V Binding Buffer. Pacific Blue Annexin V monoclonal antibody was added followed by 10ul of Propidium Iodide Solution. The cells were then gently vortexed and wrapped with foil to prevent light exposure while incubating for 15minutes at room temperature. An additional 400ul of Annexin V Binding Buffer was added and read at the FACS CANTO II flow cytometer. The results were analyzed using Flowjo.

CFSE staining

Before plating, cells were washed in a large volume of HBSS then resuspended in $5x10^6$ cells/ml. An equal volume of HBSS was put in a second tube and both were warmed in a 37 ^{O}C water bath for 15minutes. 22ul of 0.09m CFSE was added to the second tube, then the tubes were combined together, vortexing to mix. The CFSE and cell mixture was then incubated for 5minutes at 37 ^{O}C, mixing three times throughout. The cells were quenched with 40ml of cold Allos media, and then washed with an additional 40ml of cold Allos media. They were then resuspended at 200,000cells/ml to be plated. After 2 days of antigen stimulation, the cells were stained with CD25-APC following the Surface staining protocol.

Surface staining

For cell surface markers, the cells were washed and put in the 4^{O}C for 5minutes with Fc blocker. A conjugate antibody cocktail is added to incubate in the 4^{O}C for 30minutes. Directly conjugated fluorescent antibodies were purchased from BD

Biosciences, eBioscience, or Biolegend. Afterward, the cells were fixed with 4% Paraformalin for 15minutes in 4^{O}C before being washed with PBS containing 2% FBS. These samples were also read with the FACSCanto II machine and analyzed with Flowjo.

PCR

RNA was extracted using RNeasy Micro Kit (Qiagen) and concentration of RNA was determined using NanoDrop Spectrophotometer. cDNA was synthesized from the RNA using QuantiTech Reverse Transcription kit (Qiagen). For regular PCR, cDNA was diluted 1:1 with RNase- and DNase-free water. 1ul of cDNA template was added to 12.5ul GoTaq Green Master Mix, 10.5ul nuclease free water, 0.5ul Forward primer, and 0.5ul Reverse primer. Load the PCR tubes into the thermocycler for 2min at 95C for initial denaturation followed by 25, 30, or 35cycles of 30sec at 95C, 30sec 55C for annealing, and 1 minute at 72C for extension, and 5minutes at 72C for final extension. To determine, 10ul of this final mixture was loaded into each well in a 1% agarose gel with ethidium bromide along with a 1Kb DNA ladder. A Chemidoc was used to visualize the expression of each gene.

Statistical analysis

To determine significant differences between treatments, we used unpaired two-tailed Student's t-test in Prism (GraphPad Software).

Chapter 3: Results

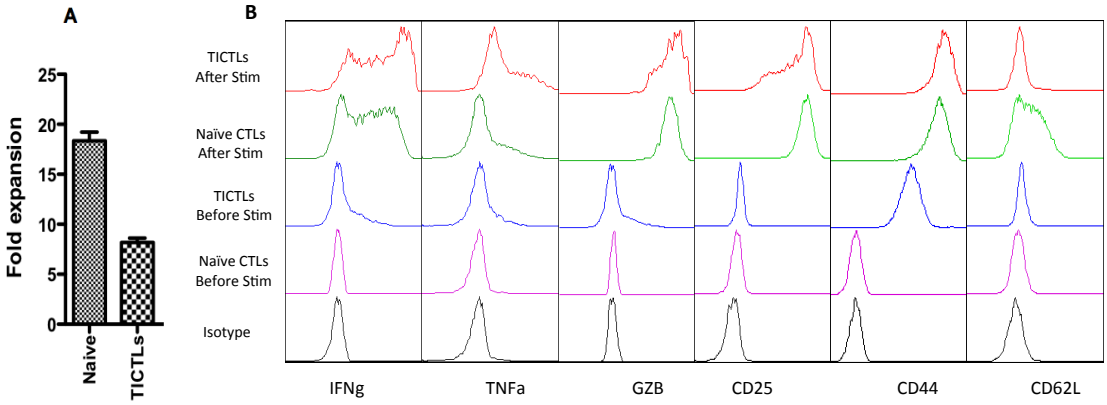
Experimental Model

The OT-I mouse model that we used has the same genetic background as C57/B6 mice. Their cells are marked with a congenital marker CD45.2 so the transferred cells can be tracked in recipient wildtype mice. This mouse model's T cells have T cell receptors that are transgenic for the OVA peptide SIINFEKL. Naïve CTLs were harvested and activated *in vitro* under the standard three signal activation conditions (3SI) [20]. To generate tumor-infiltrating OT-I CTLs, B6 mice, transferred with $2x10^6$ naïve OT-I cells, were injected subcutaneously with EG7-OVA, a lymphoma transgenically expressing the OVA antigen [24, 169]. The tumor was harvested when the tumor reached approximately $2000mm^3$ about 3-4 weeks after inoculation. The tumor-infiltrating CD8+ T cells (TICTLs) were isolated using flow cytometry as described in the Methods. Naïve CD8+ T cells, as a control, were isolated from the lymph nodes of OT-I mice and activated *in vitro* [20].

TICTLs as exhausted but responsive

TICTLs showed signs of exhaustion. In comparison to effector CTLs, TICTLs exhibited a $CD25^{lo}$, $CD44^{med}$, $CD62L^{lo}$ phenotype and minimal IFNγ, TNFα, and GZB, production before 3SI stimulation (Figure 5A). TICTLs also showed decreased expansion after *in vitro* antigen stimulation compared with stimulated effector CTLs (Figure 5B). Before antigen stimulation, the lowered proliferation combined with the terminally differentiated phenotype, and the impaired production of cytolytic molecules all implies that these antigen experienced TICTLs are exhausted and

unable to control the cancer in their current state [54, 170-172]. However, after 3SI stimulation TICTLs did show a increase in IFNγ, TNFα, and GZB, production equal to that of *in vitro* stimulated effector CTLs, despite their lowered expansion (Figure 5B). This indicates that TICTLs are still capable of responding to an antigen challenge, but only under the most optimal conditions [134, 170, 171]. The ability of these exhausted TICTLs to still be able to produce a cytolytic response is the reason that ACT is able cause tumor regression [13]. By starting off with older, exhausted TICTLs, ACT is limited to this terminally-differentiated phenotype that can only respond and kill in the short-term [13]. We aim to reinvigorate these exhausted TICTLs and make them younger, more proliferative cells using transient QQ-SON stimulation.



<u>Figure 5: TICTLs are exhausted but responsive terminally differentiated CTLs</u>: Naïve CTLs and isolated TICTLs were cultured under 3SI conditions for 3 days. (A) Fold expansion was calculated after 3 days of stimulation with 3SI (Antigen+B7+IL-12) (B) Cytolytic and activation related molecule expression was assessed using flow cytometry 3 days after 3SI stimulation.

Concurrent QQ-SON treatment of naïve T cells

In initial trials, QQ-SON stimulation was given concurrently with 3SI *in vitro* activation to isolated naïve CD8+ T cells for 3 days (Figure 6A). The SON stimulated cells exhibited similar cell proliferation, IFNγ, and GZB production and activation marker expression as *in vitro* activated CTLs without QQ-SON stimulation (Figure 6B). However, the behavior of the *in vitro* activated cells *in vivo* was drastically different between those exposed to QQ-SON. After transferring an equal number of cells, the SON treated CD8+ T cells were unable to survive *in vivo* (Figure 6C and Figure 6D). The naïve CTLs are sensitive to SON treatment, but they do not display any phenotypic changes compared to *in vitro* activated naïve CTLs without SON treatment. The reaction of naïve CTLs to QQ-SON stimulation implies that, while most of the cytolytic machinery seems to have remained functional, SON has altered a survival mechanism that is necessary under *in vivo* conditions. It appears that SON has affected the naïve CTL's ability survive, possibly by modifying their cell cycling related genes.

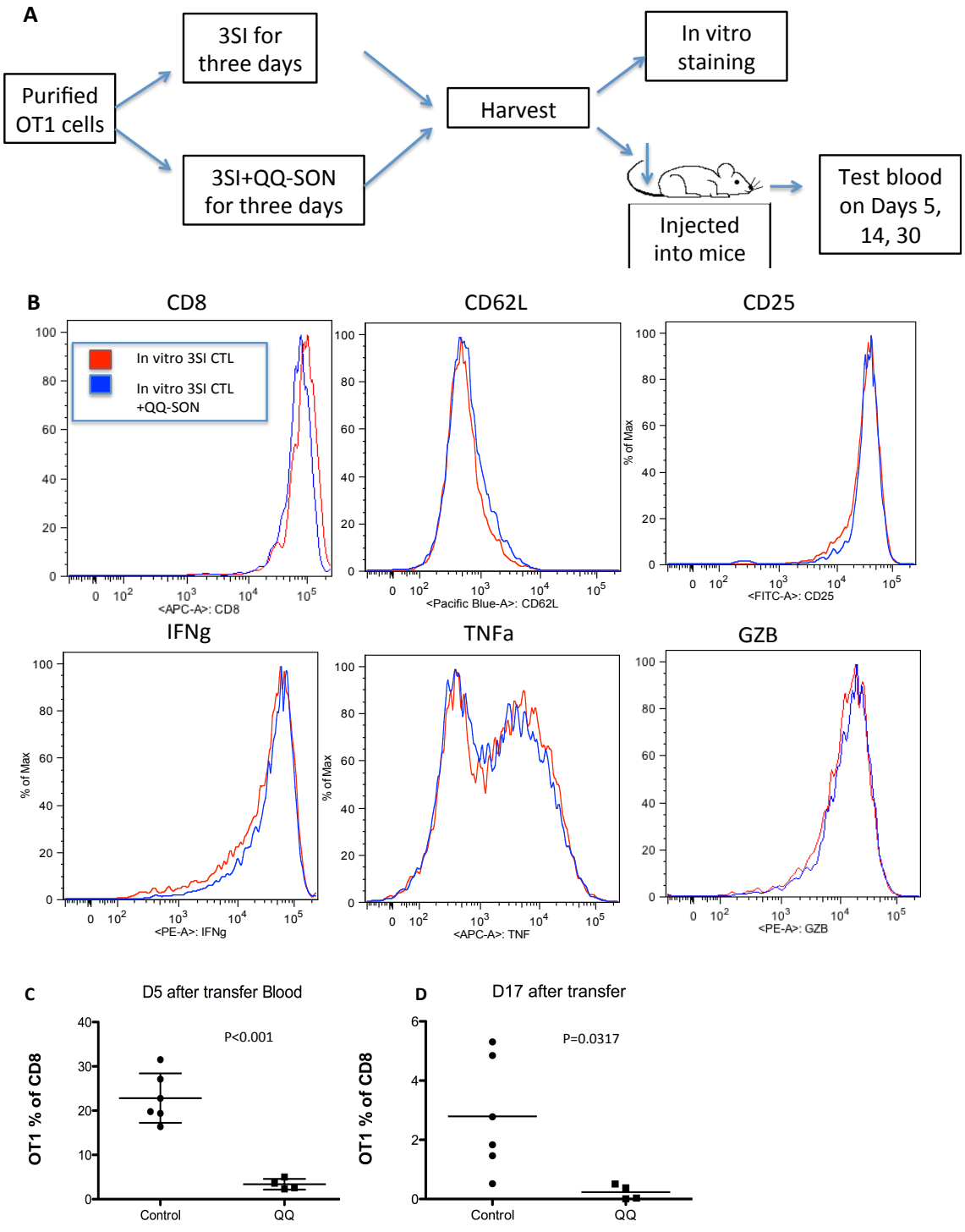

A
Purified OT1 cells
3SI for three days
3SI+QQ-SON for three days
Harvest
In vitro staining
Injected into mice
Test blood on Days 5, 14, 30
B
CD8
CD62L
CD25
In vitro 3SI CTL
In vitro 3SI CTL +QQ-SON
<APC-A>: CD8
<Pacific Blue-A>: CD62L
<FITC-A>: CD25
% of Max
IFNg
TNFa
GZB
<PE-A>: IFNg
<APC-A>: TNF
<PE-A>: GZB
C
D5 after transfer Blood
P<0.001
OT1 % of CD8
Control
QQ
D
D17 after transfer
P=0.0317

Figure 6: Concurrent treatment with QQ-SON of Naïve CTLs: OTI CD8+ T cells were purified and divided between two treatments. The first were exposed to only 3SI (antigen+ B7+ IL-12) stimulation for three days. The other combed 3SI stimulation with 0.5ug/ml at days 0,1, and 2. On the third day, the CD8+ T cells were harvested for staining (B) or injected intravenously into mice. The blood was collected from the mice on day 5 (C), 14 (D), and 30 after injection and stained. This was repeated three times

48hr QQ-SON treatment

We designed a lineage reprograming protocol based on previous CTL manipulations [19, 167]. In order to optimize the QQ-SON conditions, we tested both the naïve CTLs and the TICTLs derived from OT-I mice under two 24-hour cycles of QQ-SON treatment (Figure 7). Each 24-hour cycle consisted of 5 hours exposure to QQ-SON and 19hours of rest in QQ-free media. In order to fully reprogram somatic cells, cells must undergo a minimum of 2-3 cycles of QQ-SON [167]. After the SON cycles were completed, the cells were given an optional 48 hour rest period before being plated under three signal CTL activation conditions (3SI) with IL-2 for 4-5 days. These CTL activation conditions should indicate to the partially reprogrammed T cells that these SON-treated cells should remain T cells. 4-5 days of 3SI allowed the SON-treated cells to stabilize their proliferation. The SON-treated cells were then transferred to an uncoated plate or T25 flask. The cells were maintained by passaging every 2-3 days.

After treatment with two 24 hour cycles of QQ-SON (48 hour SON), the TICTLs (STICTLs) became a highly proliferative and remained so after 6 months of continuous passaging (Figure 8A and 8B). However, naïve CTLs treated under the same conditions with SONs died after 19 days *in vitro* regardless of antigen

stimulation (Data not shown). Conventionally, naïve CTLs stimulated with 3SI activation and without SON treatment only last for 12 days *in vitro* under optimal 3SI conditions and quickly died without antigen and cytokine stimulation (Figure 8A). The STICTLs also were larger in cell size than their effector control (Figure 8C). The STICTLs have clearly been altered by the SON treatment to increase their proliferation, unlike the naïve CTLs that could not survive *in vitro* for an extended period of time.

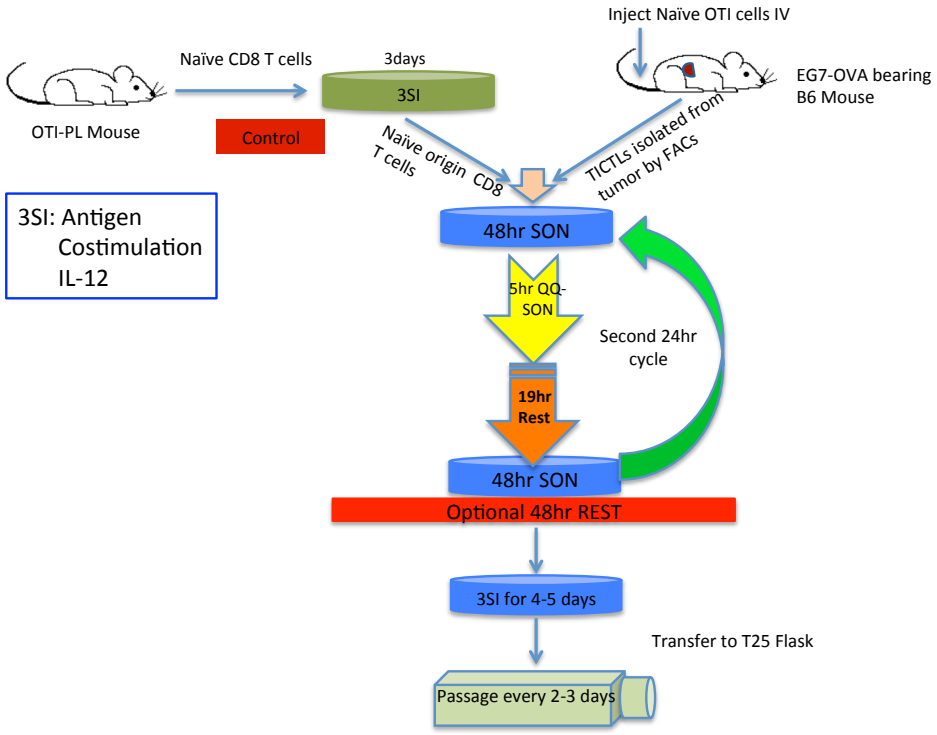


Figure 7: Experiment Design of Lineage Reprogramming with QQ-SON: Naïve CD8+T cells were purified from OTI mice then injected into tumor bearing (EG7-OVA) Wildtype mice (C57B6). The tumor was harvested when it reached about 1000mm3 and the CD8+ T cells were isolated from the tumor (TICTL). TICTLs and in vitro activation Naïve CTLs were plated under two different conditions. They were exposed either one or two 24 hour cycles. Each 24 hour cycle consisted of 5 hours of QQ-SON exposure and 19hours of rest, with an optional 48 hour rest period. The cells were then plated for 4-5 days of 3SI stimulation. After this they were transferred to unstimulated conditions.

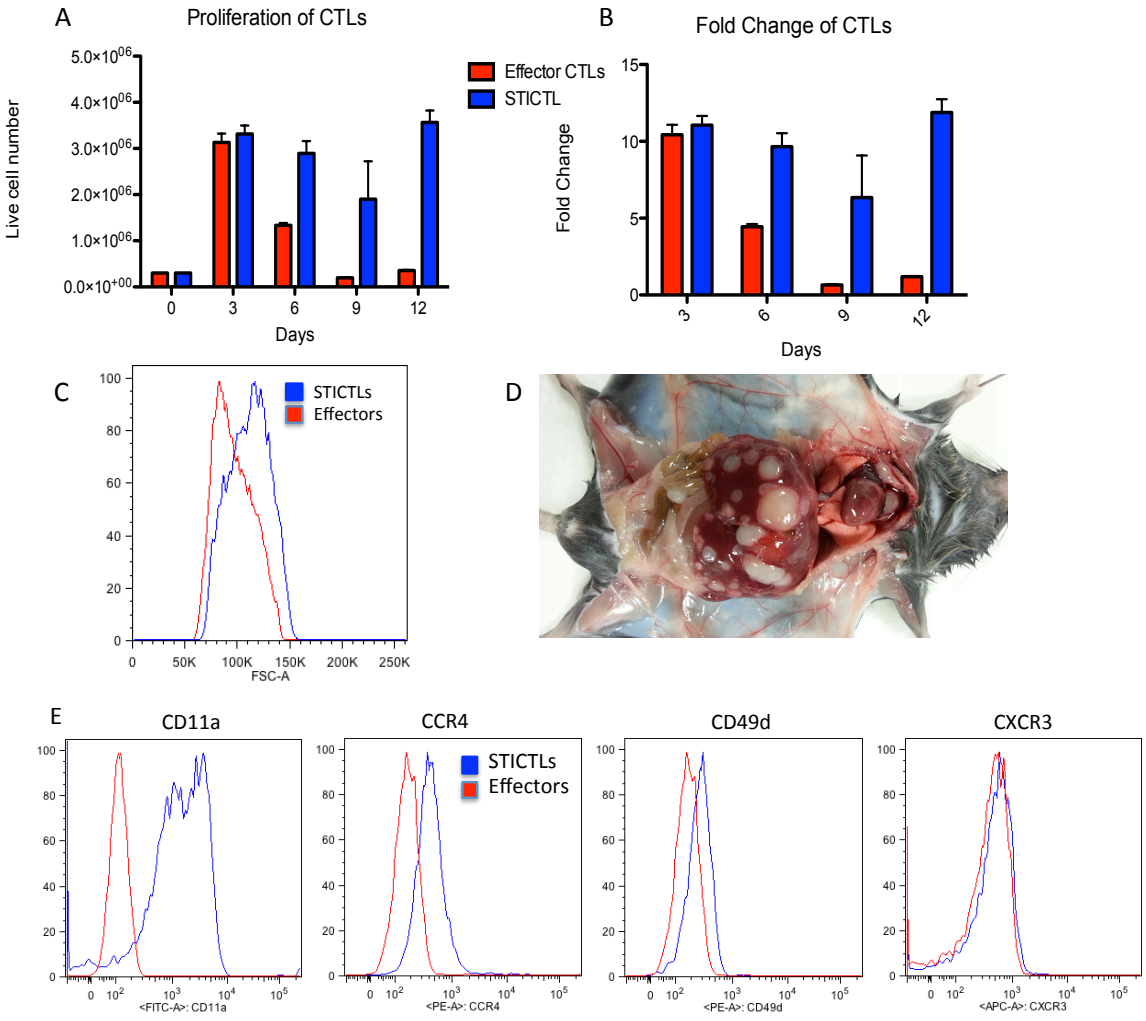


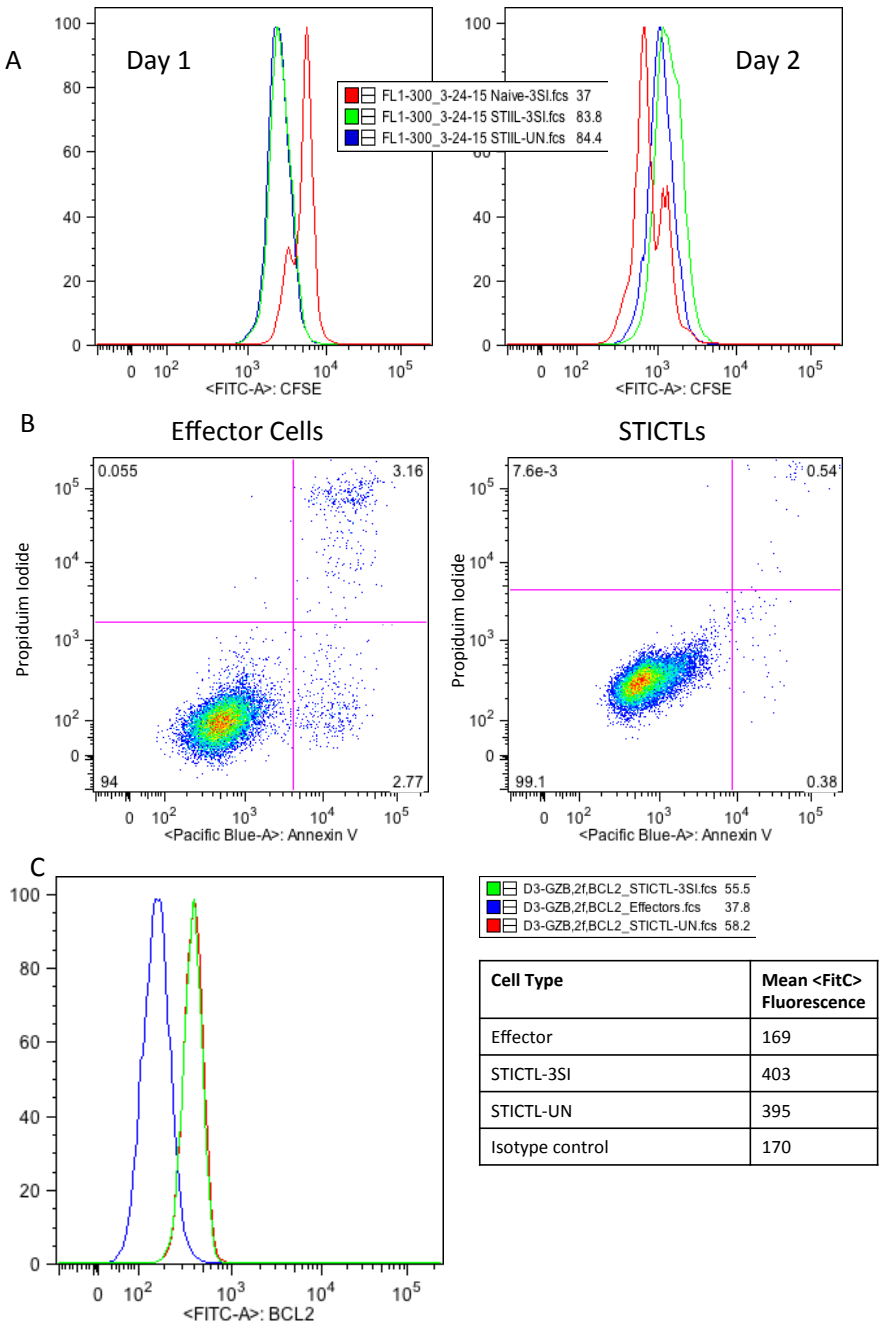
Figure 8: STICTLs endure *in vitro* and *in vivo* while Naïve CTLs falter: Naïve CTLs and STICTLs were plated on 3SI stimulation for 3 days. Every 3 days the cells were counted (A) and replated at $3x10^5$ cells per well with fresh media. (B) Fold change was calculated from the Day 3 cell counts compared to original $3x10^5$cells plated to each well and 5 replicates were averaged. (C) Naïve CTLs and STICTLs were read using a flow cytometer (D) Wildtype mice were intravenously injected with $2x10^6$ STICTLs cultured in antigen-free conditions; necropsies were performed 19 days after transfer. (E) STICTLs from unstimulated conditions were stained with liver associated adhesion molecules. This procedure and analysis was replicated four times with similar results.

STICTLs proliferate in liver after transfer

Next, we wanted to see if these SON treated TICTLs (STICTLs) behaved *in vivo* as they did *in vitro*. We wondered if this sustained proliferative response would result in the formation of a tumor. Wildtype mice were injected intravenously with different concentrations of STICTLs; the STICTLs were able to persist. When STICTLs were injected intravenously at $2x10^6$ or more, the persistence of the cells manifested as cystic masses in the livers in two out of three mice and sometimes exhibited partial paralysis of the hind legs (Figure 8D). A necropsy showed that all other tissues including the brain, spinal cord, lung, kidney, and intestines were mass free (Supplemental). The definitive borders of the cysts and the clear localization to the liver led us to believe that these masses were most likely not malignant. The cells isolated from the cysts displayed the similar passage survivability and proliferative capacity as the STICTLs (Data not shown). To determine why these STICTLs migrated to the liver, we examined the expression of common liver adhesion markers (Figure 8E). STICTLs expressed CD11a, CCR4, CD49d, and CXCR3 all of which support the localization to the liver.

Unlike SON treated effector cells, these STICTLs were able to persist *in vivo* and continue to proliferate. Their behavior *in vivo* was very different from transferred effector cells. STICTLs were not detectable in the blood one day after injection (Data not Shown). Instead, the STICTLs localized to the liver rather than circulating around to secondary lymphoid tissue. The dichotomy between the persistent *in vivo* survival of STICTLs and failure for SON- treated *in vitro*-activated naïve CTLs indicates that

the SON treatment interacts very differently with the exhausted TICTLs compared to the effector CTLs (Figure 6C).



Cell Type	Mean <FitC> Fluorescence
Effector	169
STICTL-3SI	403
STICTL-UN	395
Isotype control	170

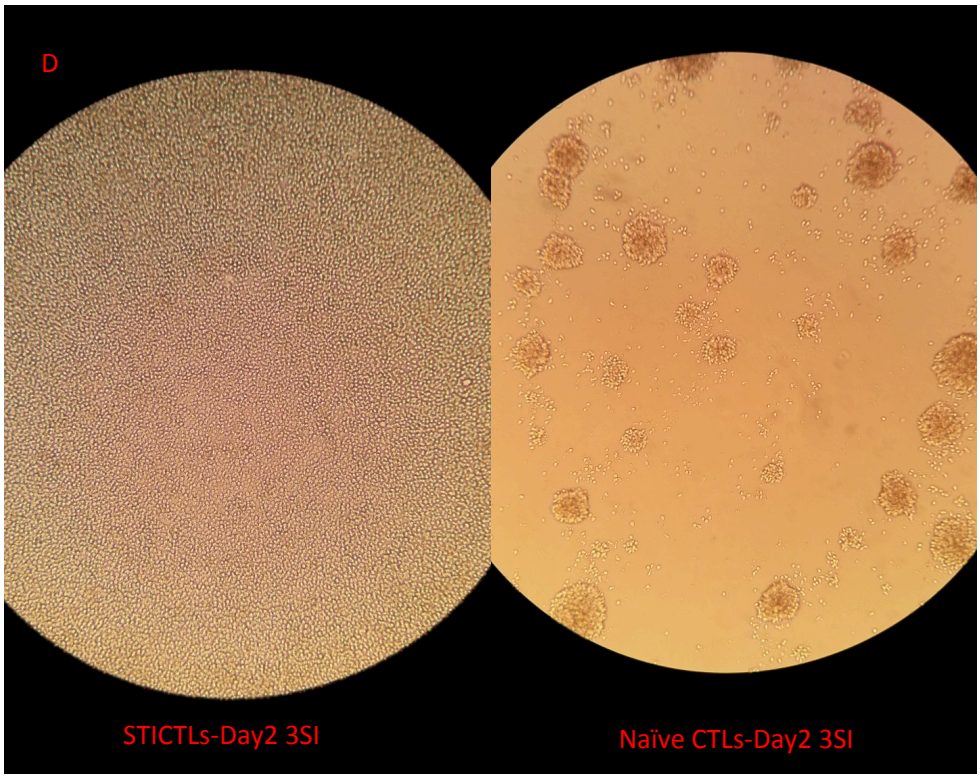


Figure 9: STICTLs proliferate at a steady rate and resist apoptosis: (A) CFSE stained cells STICTLs and in vitro activated naïve CTLs were read on day 2 after plating. (B)Also after 3 days with or without *in vitro* 3SI stimulation, STICTLs and *in vitro* activated naïve CTLs were stained with Annexin V and Propidium Iodide. (C) STICTLs and *in vitro* activated naïve CTLs were intracellularly stained with BCL-2 after 3 days of culture with or without 3SI stimulation. (D) 2 days after 3SI stimulation STICTLs display confluent coverage instead of cell aggregation. This experiment was replicated four times with similar findings.

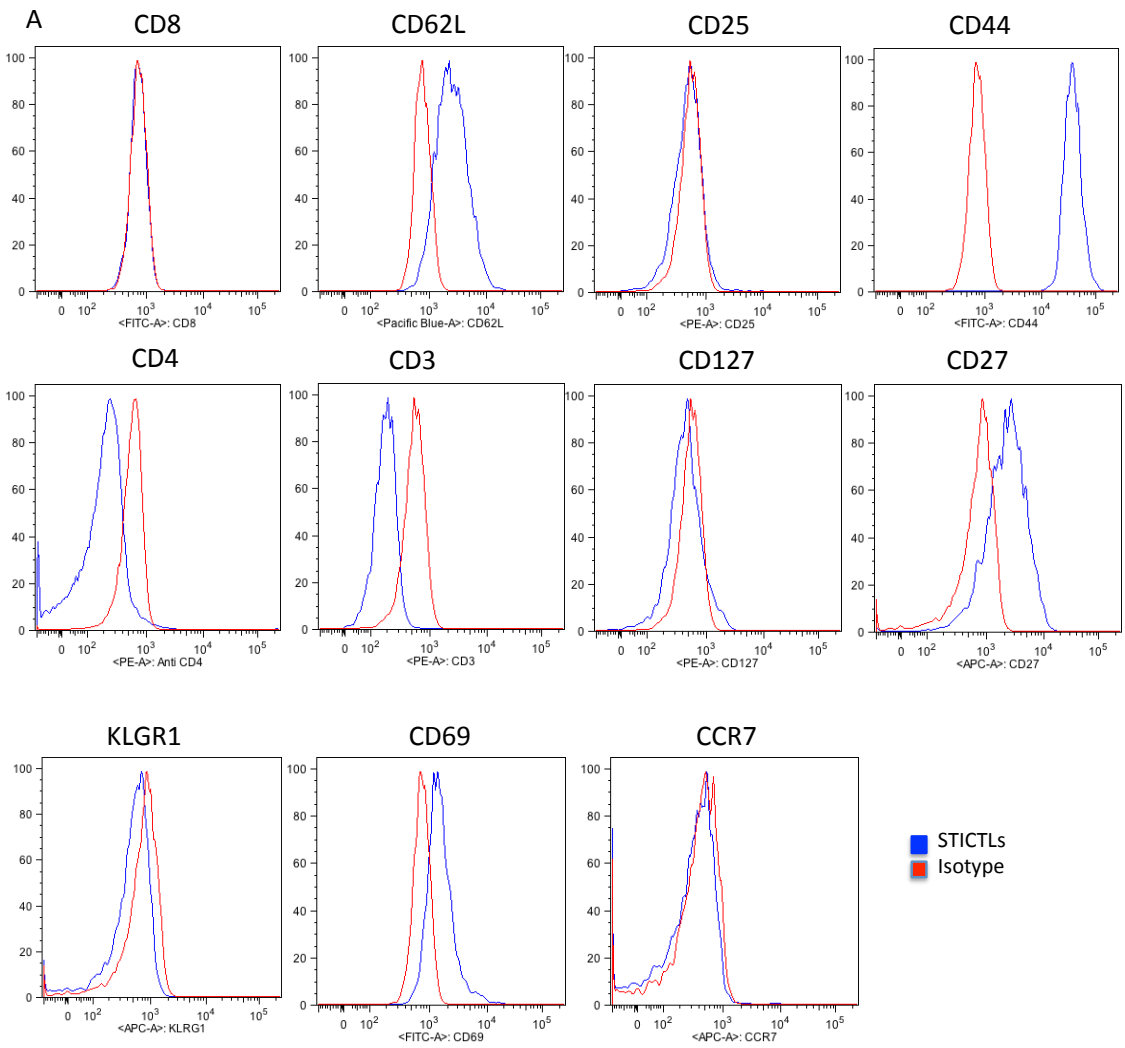
STICTLs show reduced apoptosis in vitro

We wanted to discern whether the STICTLs enhanced survival was from a decreased apoptotic rate or if the proliferative rate increased. CFSE labeling was used to determine the rate of cell division of the STICTLs and *in vitro* activated naïve CTLs. The STICTL demonstrated a similar proliferation speed as the *in vitro* activated naïve CTLs on day 2 of expansion regardless of the presence of antigen (Figure 9A). STICTLs were able to maintain a consistent proliferation rate compared to that of the naïve stimulated cells.

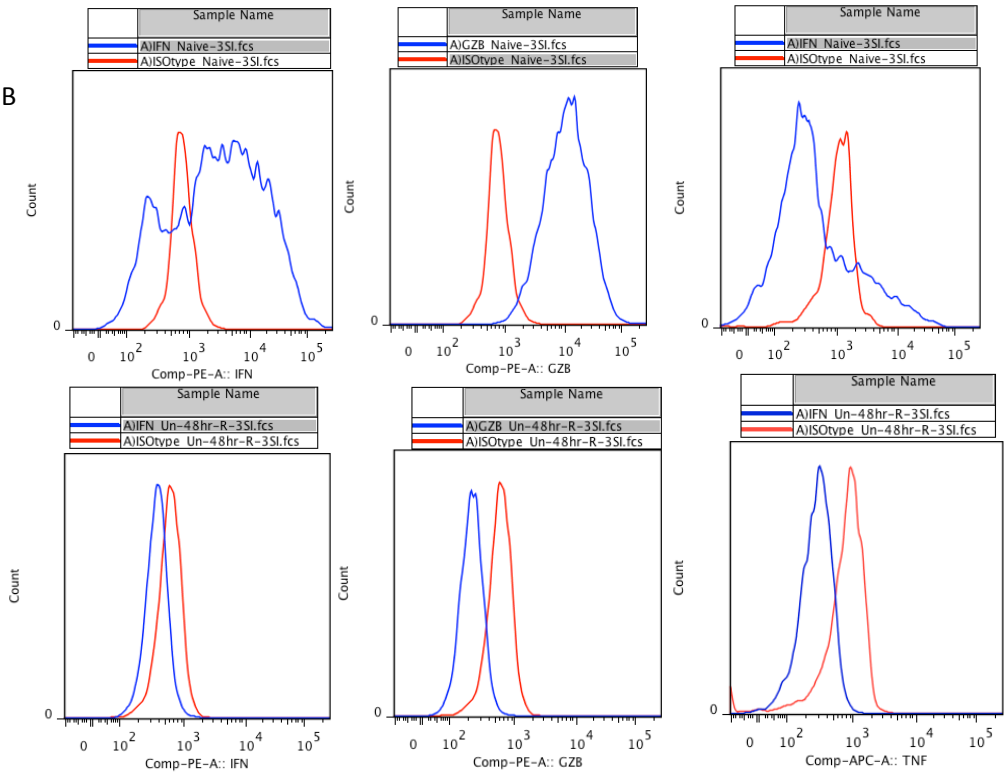
To determine the percentage of the live cells that are entering the early apoptotic stage and if there are any late stage apoptotic cells present, we used an Annexin V and Propidium Iodide staining (Figure 9B). The STICTLs showed less than 1% of the live cell population entering into either the early or late stage of apoptosis, while naïve CTLs displayed almost 5% of their live cell population at peak expansion on day 2 of optimal 3SI stimulation (Figure 9B). STICTLs also exhibited an increased expression BCL-2, an anti-apoptotic oncogene related to enhanced survival, compared to that of effector cells (Figure 9C) [173-175]. The STICTLs appears to have a lower rate of apoptosis and a steady rate of proliferation compared to *in vitro* activated naïve CTLs.

Despite exhibiting signs of increased survival and a stable proliferation rate, the STICTLs clearly did not react to antigen and cytokine stimulation. Instead of displaying the aggregate behavior characteristic of CTL activation, usually on day 2 of antigen exposure, the STICTLs showed a confluent coverage of the well with

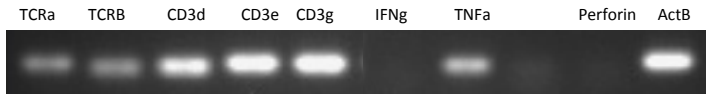
antigen stimulation (Figure 9D). The change in STICTL behavior from that of naïve CTLs lead us to question if the STICTLs were responding to antigen stimulation.


A
CD8
CD62L
CD25
CD44
<FITC-A>: CD8
<Pacific Blue-A>: CD62L
<PE-A>: CD25
<FITC-A>: CD44
CD4
CD3
CD127
CD27
<PE-A>: Anti CD4
<PE-A>: CD3
<PE-A>: CD127
<APC-A>: CD27
KLGR1
CD69
CCR7
<APC-A>: KLRG1
<FITC-A>: CD69
<APC-A>: CCR7
STICTLs
Isotype

B


Sample Name
A)IFN_Naive-3SI.fcs
A)ISOtype_Naive-3SI.fcs
Count
Comp-PE-A:: IFN
Sample Name
A)GZB_Naive-3SI.fcs
A)ISOtype_Naive-3SI.fcs
Comp-PE-A:: GZB
Sample Name
A)IFN_Naive-3SI.fcs
A)ISOtype_Naive-3SI.fcs
Sample Name
A)IFN_Un-48hr-R-3SI.fcs
A)ISOtype_Un-48hr-R-3SI.fcs
Comp-PE-A:: IFN
Sample Name
A)GZB_Un-48hr-R-3SI.fcs
A)ISOtype_Un-48hr-R-3SI.fcs
Comp-PE-A:: GZB
Sample Name
A)IFN_Un-48hr-R-3SI.fcs
A)ISOtype_Un-48hr-R-3SI.fcs
Comp-APC-A:: TNF

C


TCRa
TCRB
CD3d
CD3e
CD3g
IFNg
TNFa
Perforin
ActB

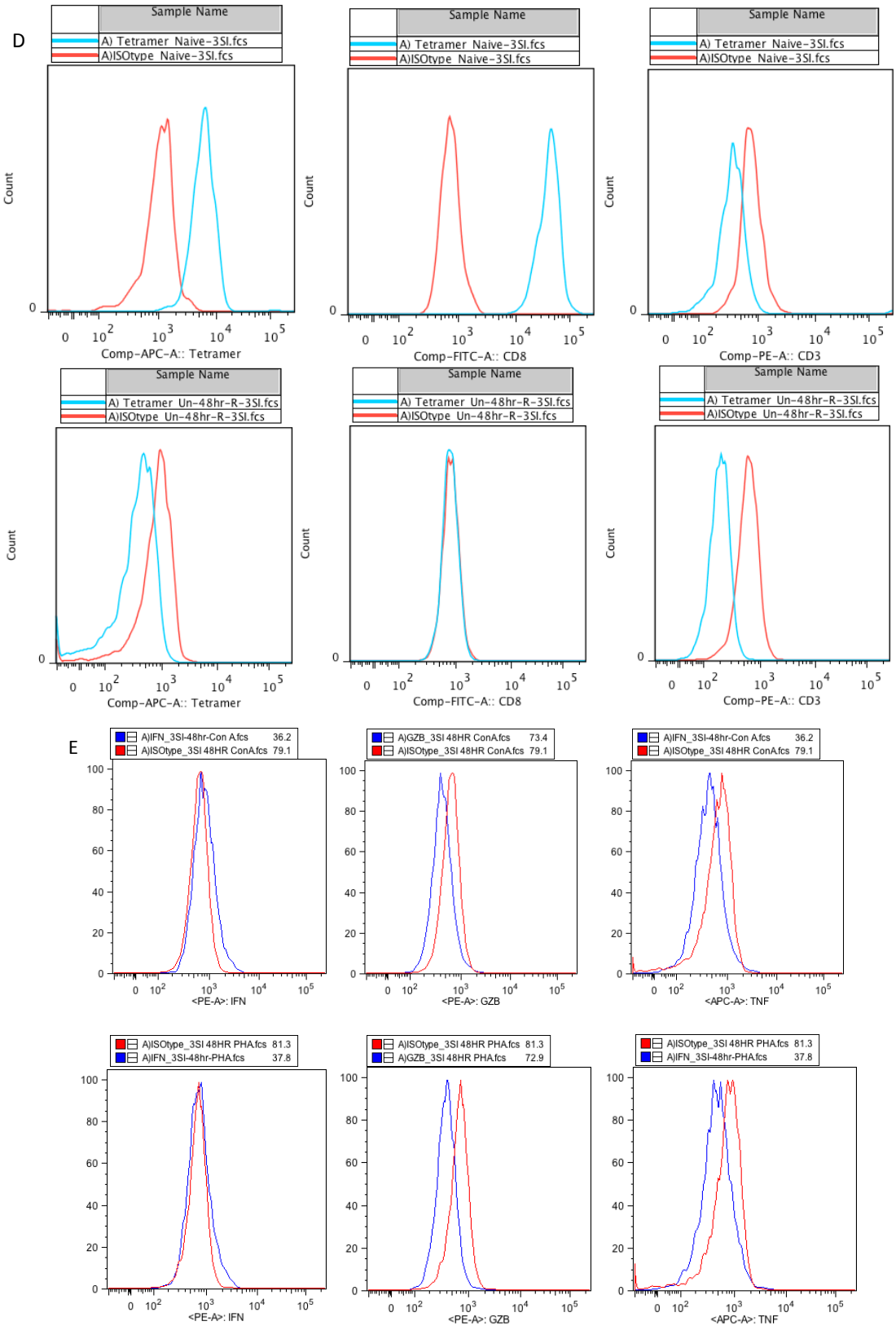

D
Sample Name
A) Tetramer Naive-3SI.fcs
A)ISOtype Naive-3SI.fcs
Count
Comp-APC-A:: Tetramer
Comp-FITC-A:: CD8
Comp-PE-A:: CD3
A) Tetramer Un-48hr-R-3SI.fcs
A)ISOtype Un-48hr-R-3SI.fcs
E
A)IFN_3SI-48hr-Con A.fcs 36.2
A)ISOtype_3SI 48HR ConA.fcs 79.1
A)GZB_3SI 48HR ConA.fcs 73.4
<PE-A>: IFN
<PE-A>: GZB
<APC-A>: TNF
A)ISOtype_3SI 48HR PHA.fcs 81.3
A)IFN_3SI-48hr-PHA.fcs 37.8
A)GZB_3SI 48HR PHA.fcs 72.9

Figure 10: Phenotypic expression of activation and cytolytic markers in STICTLs: (A) Expression of phenotypic and activation markers 3 days after 3SI antigen stimulation. (B) Cytolytic molecule expression after 3 days of 3SI stimulation. (C) mRNA transcription of STICTLs under unstimulated conditions. (D) Tetramer binding expression after 3 days of 3SI stimulation. (E) Cytolytic molecule expression after 3 days of PHA or ConA cross-linking stimulation. Each experiment was replicated four times with similar results

STICTLs are unable to respond to antigen stimulation

In order to determine if the SON treatment changed the ability of STICTLs to respond to an antigen, we challenge the STICTLs with 3SI antigen stimulation *in vitro* for three days. The STICTLs did not exhibit any of the changes characteristic of CTL activation. The $CD25^{lo}$, $KLRG1^{lo}$, $CD27^{med}$, $CD44^{hi}$, $CD62L^{hi}$ phenotype displayed by the STICTLs before antigen stimulation initially lead us to believe that the STICTLs were close to a central memory phenotype (Figure 10A). After antigen stimulation, the STICTLs remained the unchanged in their phenotypic expression and proliferation. The STICTLs did not up-regulate activation marker expression, such as CD25 and CD69, usually displayed by effector CTLs (Figure 10A). In fact, the STICTLs completely abolished CD8, the hallmark cell surface marker of cytotoxic T lymphocytes (Figure 6A) [35, 124, 176]. Furthermore, the STICTLs did not produce IFNγ, TNFα, or GZB, even after optimal 3SI stimulation (Figure 10B and Figure 10C). This is contrary to the exhausted TICTLs that were exposed to antigen *in vitro* (Figure 8B). Antigen stimulation was unable to even induce the transcription of these cytolytic molecules in STICTLs (Figure 10D). This inability to produce effector molecules and no indication of activation in the face of continuous proliferation indicates that the STICTLs are neither a memory nor effector CTL. Somehow, the SON treatment has caused a disregulation in the effector pathways of the TICTLs.

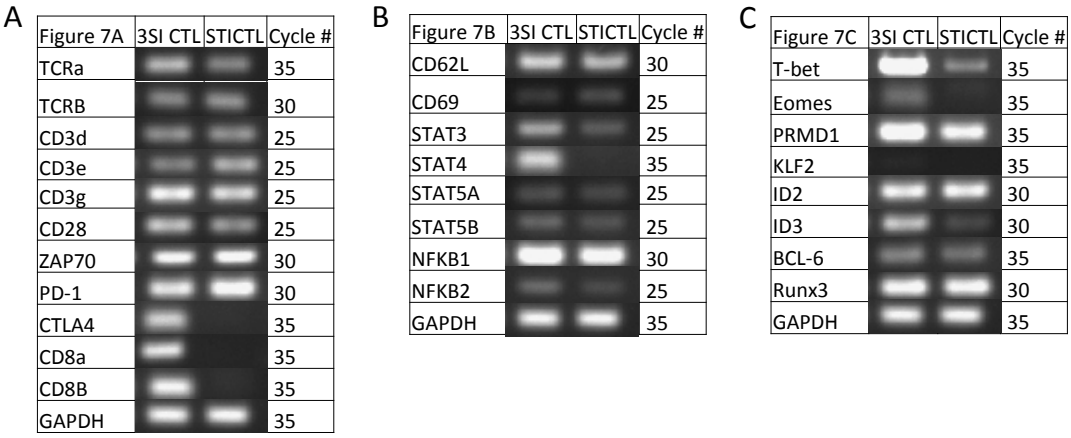


Figure 11: Downstream signaling components of TCR and cytokine pathways: Regular PCR was run at 25, 30, or 35 cycles to visualize the differential gene expression in naïve CTLs, *in vitro*-activated naïve CTLS (Effector CTLs), and STICTLs from unstimulated conditions. (A) Parts at CTL activation interface (B) effector function intermediaries (C) Key effector function transcription factors. Each gene was sequenced and PCR was repeated twice.

Effector response is altered by SON treatment

The arrest of activation markers in the STICTLs led us to question whether the cytolytic machinery was intact and capable of functioning or if SON treatment had completely incapacitated the effector response. First, we tested if the TCR is actively able to bind an MHC class I tetramer (Figure 10D) [177]. Tetramer binding is reliant on CD8 to adhere to the side of MHC class I of the tetramer, so the abolishment of CD8 expression in the STICTLs may explain the STICTLs' inability to bind the tetramer (Figure 10D). We then treated the STICTLs with PHA or ConA, to bypass the CD8 and other costimulatory mechanisms to see if the TCR signaling pathway could be jump-started (Figure 10E). PHA (phytohaemagglutinin) and ConA (Concanavalin A) are both plant derived lectins that are known to aggluntinate lymphocytes, especially T cells, thus activating them through the crosslinking of their TCRs without relying on the presence of CD8 [178]. PHA and ConA treated STICTLs continued to expand, but still failed to produce an effector response (Figure 10E). The lack of activation markers and effector protein production from ConA or PHA stimulation demonstrates that the TCR has been disconnected from the downstream signaling components of the TCR signaling pathway.

We looked at important components of the TCR signaling pathway on the RNA level using reverse transcriptase PCR (RT-PCR) (Figure 11). STICTLs did express a complete TCR/CD3 complex, CD28 for costimulation, and ZAP70 for initial signal transduction, all of which assist in initiating the TCR signaling pathway at the activation synapse (Figure 11A). NFKB, an essential transcription factor for the TCR signaling pathway, was also positively expressed (Figure 11B). The main piece

missing from the synapse is CD8 (Figure 11A). The lack of both CD8α and CD8β may inhibit the activation of LCK and further TCR signal transduction [179]. It appears that SON treatment kept most of the TCR signaling machinery intact, except for the down-regulation CD8.

When we looked at other genes related to the effector function and T cell activation, we saw that STICTLs clearly do not function like 3SI effector CTLs. On the transcriptional level, STICTLs still expressed PD-1, an activation marker, and CD62L, an adhesion molecule to localize to the secondary lymphoid tissue. The STICTLs exhibited a decreased expression of the activation markers CD69, BCL-6, ID3, and BLIMP-1 (PRMD1), all of which contribute to the level of CTL activation and differentiation. The effector related markers, T-bet, Eomes, and STAT4, were undetectable in STICTLs.

The inhibition of the effector molecule production leads us to believe that the SON transcription factors have essentially uncoupled these T cells from the TCR signaling pathway, but is appears that most of the effector machinery is intact.

SON changed cytokine receptor expression and signaling

Antigen stimulation, costimulation, and IL-12 act as the main survival signals for CTLs. The changes in the TCR signaling pathway, raises the suspicion SON treatment may have also changed the STICTLs cytokine signaling pathways. STAT4 is a major player of signal transduction in the IL-12 signaling pathway. STAT4's absence in STICTLs may indicate that transduction of the third signal of activation could be impaired (Figure 11B).

In addition to the presence of these three activation signals, CTLs also utilize the survival cytokines IL-2, for effector CTLs, and IL-7, often for memory CTLs [121, 180]. STICTLs exhibit a down regulation of both IL-2Rα (CD25) and IL-7Rα (CD127) (Figure 12A, Figure 12B and Figure 12C). STAT5 plays a role in both IL-2 and IL-7 signaling pathways and is expressed within STICTLs, along with STAT3 (Figure 11B)[121, 180]. The impaired expression of these cytokine receptors suggests that the STICTLs are also no longer reliant on IL-2 and IL-7 for continued cell survival. The SON treatment appears to have altered the cytokine signaling pathways in addition to the TCR signaling pathway.

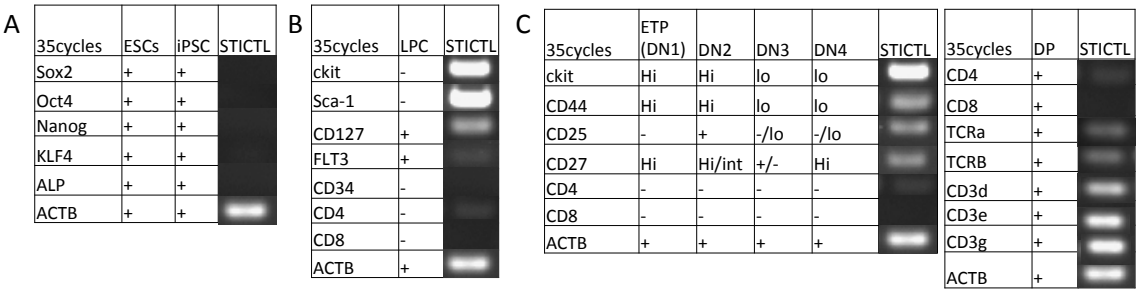


Figure 12: Precursor markers of the T cell lineage: (A) induced pluripotent stem cell (iPSC) and embryonic stem cell (ESC) markers run under 35cycles of PCR in naïve CTLs, *in vitro*-activated naïve CTLS (Effector CTLs), and STICTLs from unstimulated conditions. (B) Common Lymphoid Progenitor (LPC) cell markers run at 35 cycles of PCR in naïve CTLs, *in vitro*-activated naïve CTLS (Effector CTLs), and STICTLs from unstimulated conditions. (C) Thymocyte cell markers from early thymocytes progenitor (ETP) though the double negative (DN) stages to Double positive [206] run for 35cycles of PCR in naïve CTLs, *in vitro*-activated naïve CTLS (Effector CTLs), and STICTLs from unstimulated conditions. Each gene was sequenced and PCR was repeated twice with similar results

STICTLs have not been reverted to progenitors

The seemingly unlimited clonal expansion of STICTLs combined with the down regulation of CD8α and CD8β lead us to question if the SON treatment may have pushed the exhausted TICTLs into a phenotype more akin to a precursor cell instead of a stem cell-like T cell phenotype.

First, we looked at common stem cell markers for ESCs and iPSCs [137, 167] (Figure 12A). STICTLs did not express SOX2, OCT4, NANOG, or ALP, which demonstrates STICTLs are not iPSCs. Next, we compared STICTLs to the lymphocyte precursor markers from T cell progenitors Lymphoid Progenitors (LPC) (Figure 12B) [10, 16, 162, 181]. The STICTL's strong expression of ckit and Sca-1 indicated that they were not LPCs. This Sca-1 expression is seen on DN thymocytes as well as memory T cells and could be related to the STICTLs ability to maintain a proliferative population [182, 183].

Once, we established that the STICTLs are not progenitors; we began looking along the T cell lineage to see if the STICTLs were pushed into a thymocyte state. The STICTLs did still express a complete T cell receptor, so the STICTLs can still be identified as T cells. STICTLs were compared against each stage of T cell development in the thymus (Figure 12C) [16, 162, 163]. The obvious lack of CD8 and CD4 surface expression definitively indicates that the STICTLs are not double positive thymocytes [184]. The high ckit expression, intermediate level of CD44, in addition to this abolishment of CD8 and CD4 leads us to believe that the STICTLs are more similar to an early double negative stage.

A

Figure 9A	Naïve CTL	3SI CTL	STICTL	Cycle #
PI3K				30
PTEN				30
PDK1				35
AKT1				25
AKT2				30
AMPK				30
E2F1				30
GAPDH				35

B

Figure 9B	Naïve CTL	3SI CTL	STICTL	Cycle #
Cyclin D1				35
CDK2				35
CDK4				30
CDK6				35
HIF1a				25
p53				30
Tert				35
GAPDH				35

C

Figure 9C	Naïve CTL	3SI CTL	STICTL	Cycle #
C-myc				35
Runx1				30
Runx2				25
Runx3				30
GAPDH				35

Figure 13: Cell cycling and proliferation genes expressed by STICTLs: Cell cycling and proliferation genes run for 35, 30, or 25 cycles using reverse transcriptase PCR to get a differential expression. (A) AKT related genes (B) Cell cycling related genes (C) C-myc related genes. Each gene was sequenced and PCR repeated twice with similar results.

Proliferation genes

Since it is clear that the steadily proliferating STICTLs are not dependent on traditional antigen and cytokine signals to survive, we wondered if they were using an alternative proliferation pathway. Conventionally, activated CTLs use the AKT Map Kinase pathway to signal for proliferation after antigen stimulation [12, 185]. Using reverse transcriptase PCR, we looked at AKT as well as E2F1, PTEN and PI3K, and the inhibitory regulator of PDK1, PTEN [185]. Each of these components was expressed by the STICTLs (Figure 13A). The STICTLs also expressed the AKT alternative, AMPK (Figure 13A) [185].

The expression of most of the cell cycling genes is not very different between the STICTLs and the naïve and effector CTLs (Figure 13B). However, Cyclin D1 expression was completely abated in both naïve and effector CTLs, in sharp contrast against the clear positive expression in STICTLs (Figure 13B). The increased expression of Cyclin D1 is often associated with cancer growth [186].

The STICTLs also expressed higher level of the proto-oncogene C-MYC (Figure 13C). C-MYC has been linked to cell proliferation and is related to uncontrolled growth [138]. Increased C-MYC expression leads to the activation of cyclins and may control E2F1 expression [187, 188]. E2F1 is a transcription factor that promotes the cell cycling progression from G1 to S phase [187, 188]. E2F1 and Runx1, Run2, and Runx3 are all expressed in STICTLs (Figure 13B and Figure 13C). The Runx family has been identified as a group of myc-collaborating genes [189].

To see if the STICTLs possessed the capability of controlling their population growth, we looked at one of the main cell cycling checkpoint genes, p53 [190]. This

tumor-suppressor gene inhibits the activity of CDK2, CDK4, and CDK6 which in turn reduces cell division [190]. When a cell enters a hypoxic environment, such as a tumor, HIF is stimulated through the PI3K/Akt/mTOR pathway [191]. HIF in turn aids in the stimulation of p53. Though p53 and HIF1α are expressed by STICTLs, the proliferation appears to be unhindered (Figure 13B). The expression of p53 and HIF1α indicates that the STICTLs may use another the mechanism for their unlimited proliferation.

Regardless, it is clear that the STICTLs are capable of proliferating seemingly indefinitely. While the SON treatment has uncoupled the TICTLs dependence on CTL activation signals to survive, the STICTLs still express the cell proliferation and cell cycling machinery similarly to naïve and effector CTLs, with additional proliferation-related genes like Cyclin D1.

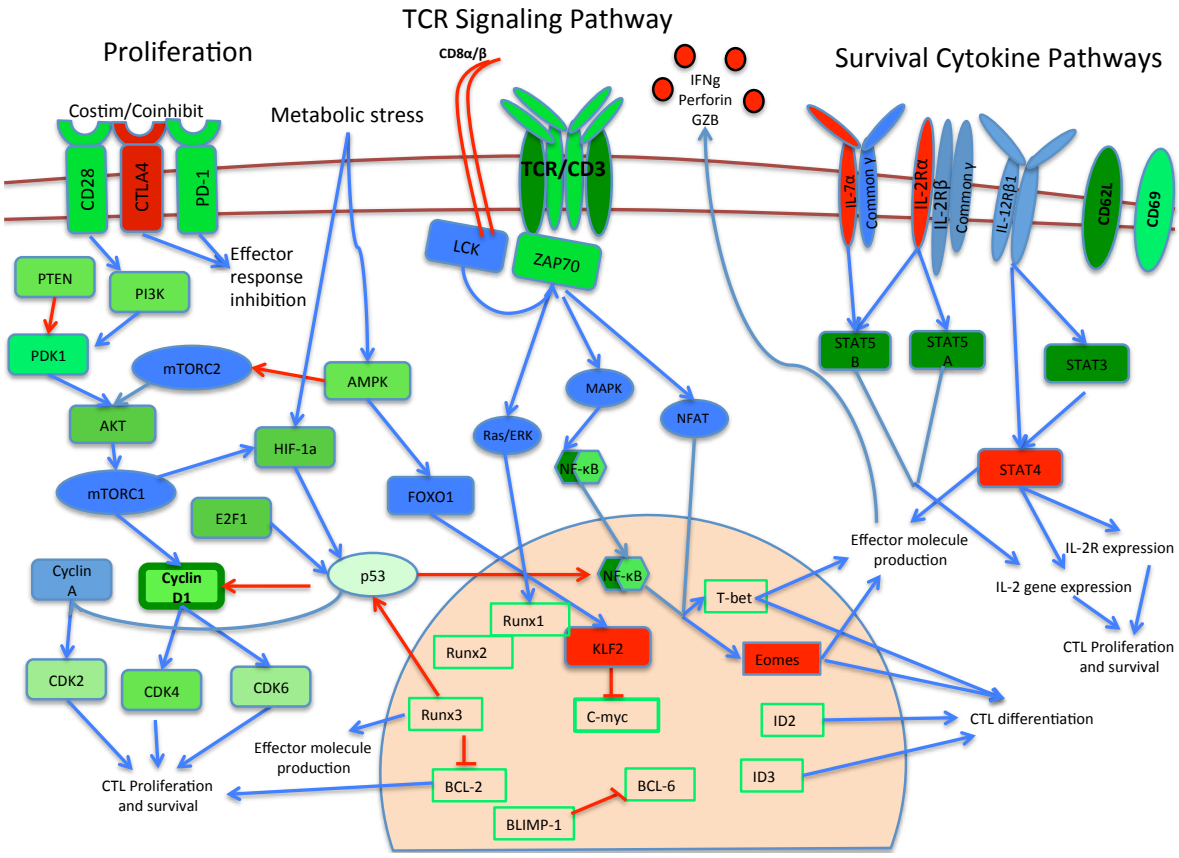


Figure 14-Summary of STICTL pathways: Green shapes indicate positive expression in STICTLs. Red shapes show negative expression in STICTLs, blue shapes are untested. Red arrow for inhibitory interaction, Blue arrow indicates next interaction.

Chapter 4: Discussion

Only the STICTLs exhibited sustained proliferation in the absence of antigen or cytokine stimulation. Whether under concurrent QQ-SON or the same treatment conditions as the STICTLs, the naïve and effector cells could not maintain a steadily propagating population, the way the STICTLs did *in vitro* and *in vivo*. STICTLs demonstrate steady proliferation as shown by CFSE labeling. The STICTLs also appear to be slightly resistant to apoptosis and higher expression of BCL-2 compared with effector CTLs. A closer look at the proliferation pathway, we see that STICTLs express many of the main cell cycling components used by CTLs. Though these cell cycling pieces are present within the STICTLs, we cannot yet say if each one is functioning. In fact the STICTLs may express more cell cycling related genes, including Cyclin D1, than effector or naïve CTLs. Still, we do not yet know which alteration to the cell cycling pathway is contributing the most to the STICTL's proliferation.

In vitro, STICTLs did not need the same survival signals normally utilized by CTLs in order to survive. The STICTLs did not exhibit any change in response to antigen stimulation. When exposed to optimal *in vitro* antigen stimulation the STICTLs did not accelerate the proliferation rate, aggregate in clumps, or produce any effector molecules such as GZB, or IFNγ. We used cross-linking of the TCRs in an attempt to jumpstart the effector response, but STICTLs still remained unresponsive.

We took a closer look at the components of the TCR signaling pathway and a few of the crucial cytokine signaling pathways. At the activation interface, the

STICTLs appeared to possess a complete TCR/CD3 complex as well as CD28 costimulatory molecule. The essential downstream components of the TCR signaling pathway ZAP70 and NFKB were also positively expressed in the STICTLs. STICTLs do express PD-1, an activation marker, but it may be a remnant of the terminally differentiated state the TICTLs were previously in. STICTLs did not exhibit an increased expression of other activation markers like CD69 or CD25. Though STICTLs possess the necessary parts to initiate activation, STICTLs do not display any outward indication of being activated after optimal 3SI stimulation. The lack of response by the STICTLs suggests that QQ-SON treatment disengages the TCR signaling pathway. Whole transcriptome analysis may enable the identification of the mechanism of this disengagement caused by SON protein reprogramming.

However, STICTLs exhibit changes in genes related to the effector function. They do not express Eomes and exhibit decreased expression of T-bet. Eomes and T-bet are major regulators of T cell differentiation, with T-bet influencing the effector function and Eomes memory formation capabilities [12, 192, 193]. Eomes and T-bet are the transcription factors that interact with Runx3 and BLIMP-1 to induce the effector response [193-196]. Without adequate expression of these effector function regulators, the STICTLs would not mount an effector response.

The survival signals, which are critical for effector CTLs, are dispensable for STICTLs. The STICTLs did not need an antigen signal or costimulation *in vitro* to initiate population expansion. STAT4, an important part of the IL-12 signaling pathway, is also missing from the STICTLs [22]. Because the TICTLs were reactive to the antigen initially, we expected STAT4 to be expressed. Though IL-12 may use

an alternative signaling pathway such as using other STAT, SON treatment has potentially altered the pathways of IL-12, one of the critical third signal cytokines for effector CTLs. STICTLs also show a down-regulation of both IL-2 and IL-7 survival cytokine receptors. IL-2 and IL-7 are known to enhance CTL survival [121, 180]. These three signals and survival cytokines are normally essential for effector CTL survival. The combination of these changes to the initiation of the effector function clearly demonstrates that the SON treatment uncoupled the survival mechanism from the antigen and cytokine signaling CTLs normally rely on.

The most surprising key component missing from the TCR signaling pathway was CD8. On both the protein and mRNA level the expression of CD8 was abolished. There have been cases of a down-regulation of CD8 during the early stages of *Listeria monocytogenes* or vaccinia virus infections, but only on the protein level, mRNA transcription of CD8 continued [197]. The TICTLs that were treated with SON were isolated based on their expression of CD8, indicating that this complete down-regulation is the result of the SON treatment. The low transcription of CD4 mRNA was unexpected, but the expression level is less than the residual transcription seen in CD8+ naïve and CD8+ effector CTLs.

The down-regulation of these hallmark surface proteins of T cells lead us to question whether the SON treatment may have pushed the TICTLs into a precursor stage. The STICTLs do not express SOX2, OCT4, NANOG or ALP (Figure 12A), which are characteristics of an iPSC, so STICTLs are not iPSC. STICTLs displayed a low or negative expression of activation markers CD69, KLRG1, CD25, and CD127 suggesting these are not effector cells [12].The STICTLs display some cell markers

similar to those used to identify central memory T cells, such as a high level of CD44, CD62L, and low expression of CD25. However, the low expression of CCR7 and their inability to respond to an antigen demonstrates that the STICTLs are not central memory CTLs [25, 114, 198]. Compounded with an absence of the protein expression of both CD8 and CD4, STICTLs cannot be classified as a mature T cell or a double positive thymocyte. Because of this, we looked further back along the T cell lineage to see what the STICTLs have become.

The markers of thymocytes stages signify that STICTLs may have settled within a double negative stage of development. Though the STICTLs do not express CD8 and CD4, they do possess a complete TCR/CD3 complex that lets us continue to designate these cells as T cells (Figure 11). The clear expression of ckit, CD44, and CD27 (Figure 12) further supports that the TICTLs were pushed too far back along the T cell lineage by SON to become a cell-type closer to an early double negative thymocytes [16, 162, 163].

The behavior of STICTLs *in vivo* differed to that of than that transferred effector CTLs. Rather than circulating in the blood or locating to secondary lymph tissue, these STICTLs localize to the liver of the recipient mice, where they settle and continue to proliferate. We looked at common liver adhesion molecules. The STICTLs positively expressed CD11a along with slight expression of CCR4, CXCR3, and CD49d [199-204].

When other CTLs migrate to the liver they usually follow a chemokine gradient and roll along the vasculature expressing adhesion receptors that allow them to bind to the endothelium [199, 200]. The adhesion molecules expressed direct the

lymphocytes to their destination [199, 200]. CXCR3 encourages localization to the liver sinusoids and liver associated lymphocytes display an increased expression of CD11a, and low expression of CD49d [200, 204]. CCR4 has been noted on liver localizing Tregs as well [200, 201]. Terminally differentiated CTLs not specific to liver-related infections are believed to migrate to the liver to apoptose and be broken down [199, 205]. But instead of apoptosis, the STICTLs survive, possibly due to the alterations SON treatment has made on the STICTL survival mechanism [199, 205]. The enhanced survival STICTLs display both *in vivo* and *in vitro*, may be due to the up-regulation of certain genes related to cell cycling, such as Cyclin D1.

Though a few of the genes expressed by STICTLs are associated with the unlimited growth of cancer cells, the targeted migration of the STICTLs to a particular organ suggests that the STICTLs have not become malignant. The clear borders seen on the cystic formations in the liver also suggest that the STICTLs simply continue proliferating in a confined space and are not actively invading the surrounding tissue. However, the up-regulation of genes, such as Cyclin D1 and C-MYC, may still be related to the unlimited growth displayed by the STICTLs. Regardless of the functionality of these STICTLs, the transient exposure to SON has clearly enhanced the proliferation of the TICTLs.

We found the effector and naïve cells react very differently than exhausted TICTLs to SON treatment using a nuclear protein deliver system. This difference in response to the master regulator SON transcription factors could be related to the exhausted state the antigenic experienced TICTLs faced while in the tumor. It is possible that by being exposed to the harsh confusing immunosuppressive

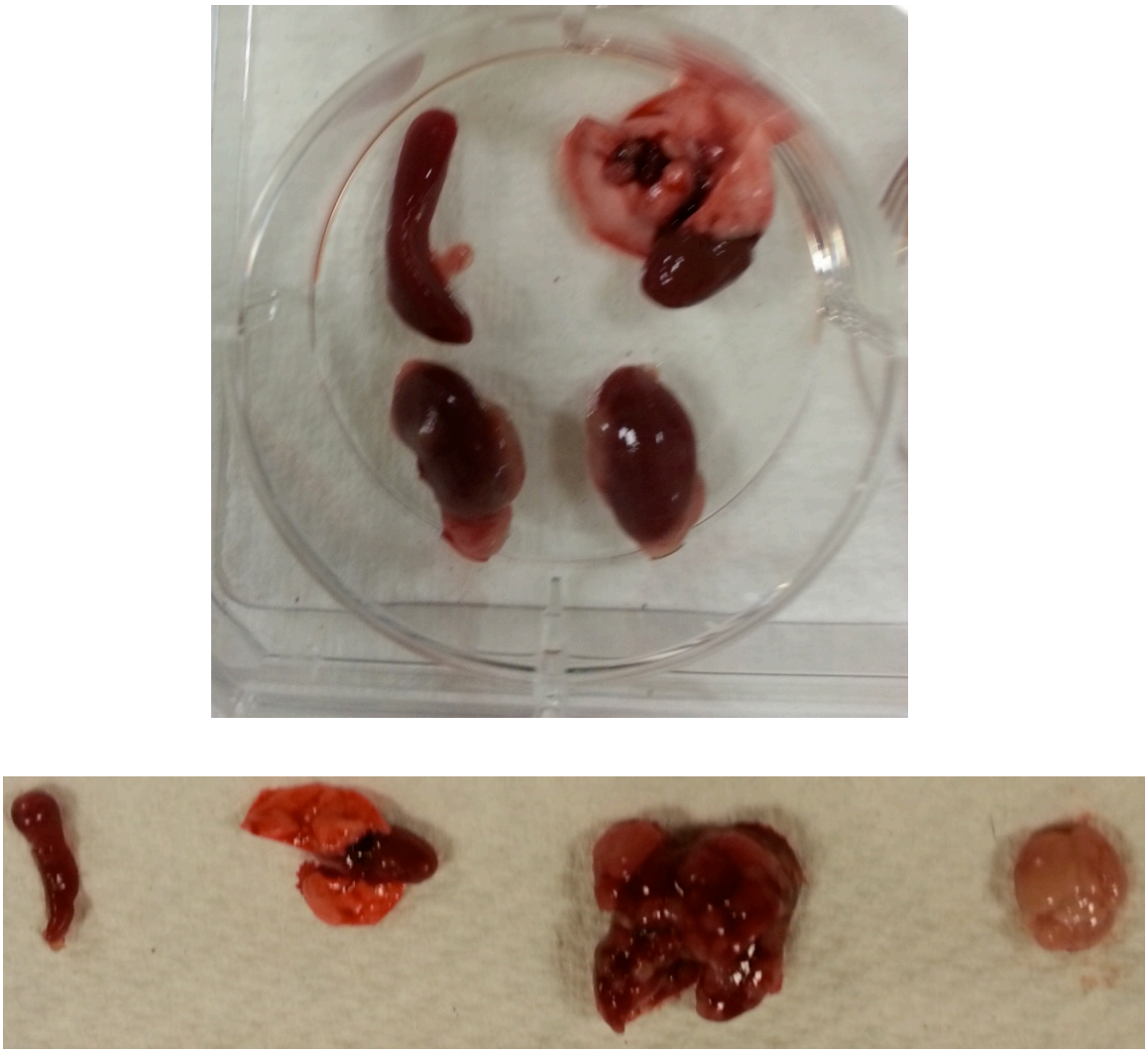
microenvironment of the tumor increased the susceptibility of the TICTLs to alteration by SON, while the naïve and effector CTLs remained more fixed in terms of their survivability.

More investigation is needed to gain a more complete picture of how SON acts on the T cell genome. An in depth look at the differences between the exhausted TICTLs and naïve and effector CTLs may shed light on why the TICTLs were more receptive to SON manipulation. Other exhausted T cells created by chronic infections could also become more reactive to reprogramming. An aging immune system show signs of senescence and exhaustion, so perhaps the elderly exhausted T cells are susceptible to this method of reprogramming. By understanding these differences between the exhausted CTLs and reactive CTLs, we may be able to increase the efficiency of T cell reprogramming.

It may be beneficial for future research to explore different avenues to reinstate the effector function of these STICTLs, possibly by treating them with another round of effector related transcription factors such as T-bet and Eomes. T-bet and Eomes are two major regulators of the effector response and their expression plays a big role in the CTL's response to antigen stimulation [193, 195]. Direct treatment of the TICTLs with other lineage related transcription factors might also help to directly push them to that young intermediate stem cell-like phenotype that maintains the effector response to an antigen as well as an expandable population. Most importantly, further investigation needs to be performed to find how these three transcription factors acts on the T cell genome and why the exhausted TICTLs are more susceptible to the alterations made by the SON transcription factors.

In summary, treatment with the pluripotency master regulators SOX2, OCT4, and NANOG proteins seems to disengage the TICTLs from the normal effector response (Figure 14). SON treatment also instills seemingly unlimited proliferation in these cells by removing the TICTLs' reliance on traditional CTL survival signals, such as antigen or cytokine stimulation. Though the TICTLs are clearly altered in their survival mechanism, they have not been reprogrammed into a typical stem cell stage. Nevertheless, we show here that tumor-infiltrating CTLs are uniquely sensitive to lineage programming using transcription factors delivered directly to nuclei as proteins. This sensitivity to protein lineage reprogramming may prove to be useful when rejuvenating CTLs for immunotherapy in cancers or chronic infections in aging populations.

Supplemental Figure



Supplemental Figure: A) The cyst free spleen, heart, lung, and kidneys of a mouse injected intravenously with $2x10^6$ STICTLs. B) Cyst free spleen, heart, lung, and brain with a cystic liver of a mouse injected intravenously with $2x10^6$ STICTLs.

Bibliography

References

1. Siegel, R., et al., *Cancer statistics, 2014*. CA Cancer J Clin, 2014. **64**(1): p. 9-29.
2. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2015*. CA Cancer J Clin, 2015. **65**(1): p. 5-29.
3. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. Cell, 2011. **144**(5): p. 646-74.
4. Apetoh, L., S. Ladoire, G. Coukos, F. Ghiringhelli *Combining immunotherapy and anticancer agents: the right path to achieve cancer cure?* Annals of Oncology, 2015.
5. Ascierto, P., *Immunotherapies and novel combinations: the focus of advances in the treatment of melanoma* Cancer Immunology, Immunotherapy, 2015. **64**(3): p. 271-274.
6. Crompton, J.G., et al., *Reprogramming antitumor immunity*. Trends Immunol, 2014. **35**(4): p. 178-85.
7. Hinrichs, C.S., S.A. Rosenberg, *Exploiting the curative potential of adoptive T-cell therapy for cancer*. Immunological Reviews, 2014. **257**(1): p. 56-71.
8. Rosenberg, S.A., et al., *Adoptive cell transfer: a clinical path to effective cancer immunotherapy*. Nat Rev Cancer, 2008. **8**(4): p. 299-308.
9. Sharma, P. and J.P. Allison, *The future of immune checkpoint therapy*. Science, 2015. **348**(6230): p. 56-61.
10. McGray, A.J., et al., *Immunotherapy-induced CD8+ T cells instigate immune suppression in the tumor*. Mol Ther, 2014. **22**(1): p. 206-18.
11. Schreiber, R.D., L. J. Old, M. J. Smyth, *Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion*. Science, 2011. **331**(6024): p. 1565-1570.
12. Kaech, S.M. and W. Cui, *Transcriptional control of effector and memory CD8+ T cell differentiation*. Nat Rev Immunol, 2012. **12**(11): p. 749-61.
13. Crompton, J.G., Sukumar, M., and Restifo, N.P., *Uncoupling T-cell expansion from effector differentiation in cell-based immunotherapy*. . Immunological Reviews, 2014. **257**(1): p. 264-276.
14. Klebanoff, C.A., L. Gattinoni, and N.P. Restifo, *Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy?* J Immunother, 2012. **35**(9): p. 651-60.
15. Hultcrantz, M., et al., *The target cell response to cytokines governs the autoreactive T cell repertoire in the pancreas of NOD mice*. Diabetologia, 2009. **52**(2): p. 299-305.
16. Kindt, T.J., B.A.Osbourne, R.A. Goldsby, *Kuby Immunology*. Sixth ed.
17. Parkin, J. and B. Cohen, *An overview of the immune system*. The Lancet, 2001. **357**(9270): p. 1777-1789.

18. Curtsinger, J.M., C. S. Schmidt, A. Mondino, D.C. Lins, R.M. Kedl, M.K. Jenkins, M.F. Mescher, *Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells*. The Journal of Immunology, 1999. **162**(6): p. 3256-3262.
19. Mescher, M.F., J.M. Curtsinger, P. Agarwal, K.A. Casey, M. Gerner, C.D. Hammerbeck, F. Popescu, Z. Xiao *Signals required for programming effector and memory development by CD8+ T cells*. Immunological Reviews, 2006. **211**(1): p. 81-92.
20. Xiao, Z., et al., *Programming for CD8 T cell memory development requires IL-12 or type I IFN*. J Immunol, 2009. **182**(5): p. 2786-94.
21. Curtsinger, J.M., J.O. Valenzuela, P. Agarwal, D. Lins, M.F. Mescher, *Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation*. Journal of Immunology (Baltimore, Md.: 1950), 2005. **174**(8): p. 4465-4469.
22. Vignali, D.A. and V.K. Kuchroo, *IL-12 family cytokines: immunological playmakers*. Nat Immunol, 2012. **13**(8): p. 722-8.
23. Curtsinger, J.M. and M.F. Mescher, *Inflammatory cytokines as a third signal for T cell activation*. Curr Opin Immunol, 2010. **22**(3): p. 333-40.
24. Boissonnas, A., et al., *In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor*. J Exp Med, 2007. **204**(2): p. 345-56.
25. Perret, R. and F. Ronchese, *Memory T cells in cancer immunotherapy: which CD8 T-cell population provides the best protection against tumours?* Tissue Antigens, 2008. **72**(3): p. 187-94.
26. Zou, W., *Immunosuppressive networks in the tumour environment and their therapeutic relevance*. Nat Rev Cancer, 2005. **5**(4): p. 263-74.
27. Harimoto, H., et al., *Inactivation of tumor-specific CD8(+) CTLs by tumor-infiltrating tolerogenic dendritic cells*. Immunol Cell Biol, 2013. **91**(9): p. 545-55.
28. Kim, R., M. Emi, and K. Tanabe, *Functional roles of immature dendritic cells in impaired immunity of solid tumour and their targeted strategies for provoking tumour immunity*. Clin Exp Immunol, 2006. **146**(2): p. 189-96.
29. Malyguine, A.M., Strobl, S.L., Shurin, M.R., *Immunological monitoring of the tumor immunoenvironment for clinical trials*. Cancer Immunology, Immunotherapy, 2011.
30. Sideras, K., et al., *Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies*. Cancer Treat Rev, 2014. **40**(4): p. 513-22.
31. Vitale, M., et al., *Effect of tumor cells and tumor microenvironment on NK-cell function*. Eur J Immunol, 2014. **44**(6): p. 1582-92.
32. Gajewski, T.F., H. Schreiber, and Y.X. Fu, *Innate and adaptive immune cells in the tumor microenvironment*. Nat Immunol, 2013. **14**(10): p. 1014-22.
33. Oleinika, K., et al., *Suppression, subversion and escape: the role of regulatory T cells in cancer progression*. Clin Exp Immunol, 2013. **171**(1): p. 36-45.
34. Wynn, T.A., *Myeloid-cell differentiation redefined in cancer*. Nat Immunol, 2013. **14**(3): p. 197-9.

35. Mahnke, Y.D., et al., *The who's who of T-cell differentiation: human memory T-cell subsets*. Eur J Immunol, 2013. **43**(11): p. 2797-809.
36. Vitali, C., et al., *Migratory, and not lymphoid-resident, dendritic cells maintain peripheral self-tolerance and prevent autoimmunity via induction of iTreg cells*. Blood, 2012. **120**(6): p. 1237-45.
37. Ma, Y., G.V. Shurin, Zhu Peiyuan, M.R. Shurin, *Dendritic Cells in the Cancer Microenvironment*. Journal of Cancer, 2013. **4**(1): p. 36-44.
38. Topalian, S.L., C.G. Drake, and D.M. Pardoll, *Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity*. Curr Opin Immunol, 2012. **24**(2): p. 207-12.
39. Hao, N.B., et al., *Macrophages in tumor microenvironments and the progression of tumors*. Clin Dev Immunol, 2012. **2012**: p. 948098.
40. Noy, R. and J.W. Pollard, *Tumor-associated macrophages: from mechanisms to therapy*. Immunity, 2014. **41**(1): p. 49-61.
41. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system*. Nat Rev Immunol, 2009. **9**(3): p. 162-74.
42. Gabrilovich, D.I., *Myeloid-Derived Suppressor Cells and Tumor Escape*. AACR Education Book, 2011. **2011**(1): p. 99.
43. Bingisser, R.M., P.A. Tilbrook, P.G. Holt, U.R. Kees, *Macrophage-Derived Nitric Oxide Regulates T Cell Activation via Reversible Disruption of the Jak3/STAT5 Signaling Pathway*. The Journal of Immunology, 1998. **160**(12): p. 5729-5734.
44. Ostrand-Rosenberg, S., *Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity - Springer*. Cancer Immunology, Immunotherapy, 2010. **59**: p. 1593-1600.
45. K. Wing, Y.O., P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, S. Sakaguchi, *CTLA-4 Control over Foxp3+ Regulatory T Cell Function*. Science, 2008. **322**(5899): p. 271-275.
46. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism*. Nat Rev Immunol, 2005. **5**(8): p. 641-54.
47. Rodriguez, P.C., et al., *Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes*. Cancer Res, 2009. **69**(4): p. 1553-60.
48. Yu, J., et al., *Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer*. J Immunol, 2013. **190**(7): p. 3783-97.
49. P.C. Rodriguez, D.G.Q., A.C. Ochoa, *l-arginine availability regulates T-lymphocyte cell-cycle progression*. Blood, 2007. **109**(4): p. 1568-1573.
50. Schwartz, R.H., *T cell anergy*. Annu Rev Immunol, 2003. **21**: p. 305-34.
51. Mellor, A.L. and D.H. Munn, *IDO expression by dendritic cells: tolerance and tryptophan catabolism*. Nat Rev Immunol, 2004. **4**(10): p. 762-74.
52. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in tumor immunity*. Int J Cancer, 2010. **127**(4): p. 759-67.
53. Zou, W., *Regulatory T cells, tumour immunity and immunotherapy*. Nat Rev Immunol, 2006. **6**(4): p. 295-307.

54. Spranger, S., R.M. Spaapen, Y. Zha, J. Williams, Y. Meng, T.T. Ha, T.F. Gajewski, *Up-Regulation of PD-L1, IDO, and Tregs in the Melanoma Tumor Microenvironment Is Driven by CD8+ T Cells*. Science Translational Medicine, 2013. **5**(200): p. 200ra116.
55. Karimi, S., S. Chattopadhyay, and N.G. Chakraborty, *Manipulation of regulatory T cells and antigen-specific cytotoxic T lymphocyte-based tumour immunotherapy*. Immunology, 2015. **144**(2): p. 186-96.
56. Chen, M.L., et al., *Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo*. Proc Natl Acad Sci U S A, 2005. **102**(2): p. 419-24.
57. Baitsch, L., P. Baumgaertner, E. Devevre, S.K. Raghav, A. Legat, L. Barba, S. Wieckowski, H. Bouzourene, B. Deplancke, P. Romero, N. Rufer, D.E. Speiser, *Exhaustion of tumor-specific CD8+ T cells in metastases from melanoma patients*. Journal of Clinical Investigation, 2011. **121**(6): p. 2350-2360.
58. Dowlatshahi, M., V. Huang, A.E. Gehad, Y. Jiang, A. Calarese, J.E. Teague, A.A. Dorosario, J. Cheng, P. Nohiem, C.F. Schanbacher, M. Thakuria, C.D. Schmults, L.C. Wang, R.A. Clark, *Tumor-Specific T Cells in Human Merkel Cell Carcinomas: A Possible Role for Tregs and T-Cell Exhaustion in Reducing T-Cell Responses*. Journal of Investigative Dermatology, 2013. **133**(7): p. 1879-1889.
59. Yu, P. and Y.X. Fu, *Tumor-infiltrating T lymphocytes: friends or foes?* Lab Invest, 2006. **86**(3): p. 231-45.
60. Hadrup, S., M. Donia, and P. Thor Straten, *Effector CD4 and CD8 T cells and their role in the tumor microenvironment*. Cancer Microenviron, 2013. **6**(2): p. 123-33.
61. Kim, R., M. Emi, and K. Tanabe, *Cancer immunosuppression and autoimmune disease: beyond immunosuppressive networks for tumour immunity*. Immunology, 2006. **119**(2): p. 254-64.
62. Shiao, S.L., et al., *Immune microenvironments in solid tumors: new targets for therapy*. Genes Dev, 2011. **25**(24): p. 2559-72.
63. Koreth J., H.T.K., S.M. McDonough, B. Bindra, E.P. Alyea, P. Armand, C. Cutler, V.T. Ho, N.S Treister, D.C. Bienfang, P. Sashank, D. Tzachanis, R.M. Joyce, D.E. Avigan, J. Ritz, R.J. Soiffer, *Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease*. New England Journal of Medicine, 2011. **365**(22): p. 2055-2066.
64. Colombo, M.P.a.S.P., *Regulatory T-cell inhibition versus depletion: the right choice in cancer immunotherapy*. Nature Reviews Cancer, 2007. **7**(11): p. 880-887.
65. Shiao, S.L., A.P. Ganesan, H.S. Rugo, L.M. Coussens, *Immune microenvironments in solid tumors: new targets for therapy*. Genes Dev, 2011. **25**(24): p. 2559-2572.
66. Finn, L., S.N. Markovic, and R.W. Joseph, *Therapy for metastatic melanoma: the past, present, and future*. BMC Med, 2012. **10**: p. 23.
67. Walker, L.S. and D.M. Sansom, *Confusing signals: recent progress in CTLA-4 biology*. Trends Immunol, 2015. **36**(2): p. 63-70.

68. Lipson, E.J. and C.G. Drake, *Ipilimumab: an anti-CTLA-4 antibody for metastatic melanoma*. Clin Cancer Res, 2011. **17**(22): p. 6958-62.
69. Mahoney, K.M., G.J. Freeman, and D.F. McDermott, *The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma*. Clin Ther, 2015. **37**(4): p. 764-782.
70. Fellner, C., *Ipilimumab (Yervoy) Prolongs Survival In Advanced Melanoma*. Pharmacy and Therapeutics, 2012. **37**(9): p. 503-530.
71. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma*. N Engl J Med, 2010. **363**(8): p. 711-23.
72. Charlton, J.J., et al., *Programmed death-1 shapes memory phenotype CD8 T cell subsets in a cell-intrinsic manner*. J Immunol, 2013. **190**(12): p. 6104-14.
73. Marzuka, A., et al., *Melanoma Treatments: Advances and Mechanisms*. J Cell Physiol, 2015.
74. *Nivolumab approved for lung cancer*. Cancer Discovery, 2015. **5**(5): p. OF1.
75. Sim, G.C. and L. Radvanyi, *The IL-2 cytokine family in cancer immunotherapy*. Cytokine Growth Factor Rev, 2014. **25**(4): p. 377-90.
76. Dutcher, J.P., D. Schwartzentruber, H.L. Kaufman, S.S. Agarwala, A.A. Tarhini, J.N. Lowder, M.B. Atkins, *High dose interleukin-2 (Aldesleukin) - expert consensus on best management practices*. Journal of Immunotherapy of Cancer, 2014. **2**(1): p. 26.
77. Rosenberg, S.A., *IL-2: the first effective immunotherapy for human cancer*. J Immunol, 2014. **192**(12): p. 5451-8.
78. Nelson, B.H., *IL-2, Regulatory T Cells, and Tolerance*. The Journal of Immunology, 2004. **172**(7): p. 3983-3988.
79. Bunimovich-Mendrazitsky, S., S. Halachmi, N. Kronik, *Improving Bacillus Calmette-Guérin (BCG) immunotherapy for bladder cancer by adding interleukin 2 (IL-2): a mathematical model*. Mathematical medicine and biology: a journal of the IMA, 4-16-2015.
80. Coulie, P.G., et al., *Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy*. Nat Rev Cancer, 2014. **14**(2): p. 135-46.
81. Turcotte, S., et al., *Phenotype and function of T cells infiltrating visceral metastases from gastrointestinal cancers and melanoma: implications for adoptive cell transfer therapy*. J Immunol, 2013. **191**(5): p. 2217-25.
82. Blanchard, T., P.K. Srivastava, and F. Duan, *Vaccines against advanced melanoma*. Clin Dermatol, 2013. **31**(2): p. 179-90.
83. McDermott, D., et al., *Efficacy and safety of ipilimumab in metastatic melanoma patients surviving more than 2 years following treatment in a phase III trial (MDX010-20)*. Ann Oncol, 2013. **24**(10): p. 2694-8.
84. Koya, R.C., et al., *BRAF inhibitor vemurafenib improves the antitumor activity of adoptive cell immunotherapy*. Cancer Res, 2012. **72**(16): p. 3928-37.
85. Dudley, M.E., J. Wunderlich, M.I. Nishimura, D. Yu, J.C Yang, S.L. Topalian, D.J. Schwartzentruber, P. Hwu, F.M. Marincola, R. Sherry, S.F. Leitman, S.A. Rosenberg, *Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma*. Journal of Immunology (Hagerstown, Md.: 1997), 2001. **24**(4): p. 363-373.

86. Banchereau, J., V. Pascual, and A. O'Garra, *From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines*. Nat Immunol, 2012. **13**(10): p. 925-31.
87. Murphy, G.P., B.A. Tjoa, S.J. Simmons, J. Jarisch, V.A. Bowes, H. Ragde, M. Rogers, A. Elgamal, G.M. Kenny, O.E. Cobb, R.C. Ireton, M.J. Troychak, M.L. Salgaller, A.L. Boynton, *Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: A phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease*. The Prostate, 1999. **38**(1): p. 73-78.
88. Neller, M.A., J.A. Lopez, and C.W. Schmidt, *Antigens for cancer immunotherapy*. Semin Immunol, 2008. **20**(5): p. 286-95.
89. Queirolo, P., V. Picasso, and F. Spagnolo, *Combined BRAF and MEK inhibition for the treatment of BRAF-mutated metastatic melanoma*. Cancer Treat Rev, 2015.
90. Welsh, S.J., P.G. Corrie, *Management of BRAF and MEK inhibitor toxicities in patients with metastatic melanoma*. Therapeutic Advances in Medical Oncology, 2015. **7**(2): p. 122-136.
91. Chapman, P.B., et al., *Improved survival with vemurafenib in melanoma with BRAF V600E mutation*. N Engl J Med, 2011. **364**(26): p. 2507-16.
92. Lidsky, M., G. Antoun, P. Speicher, B. Adams, R. Turley, C. Augustine, D. Tyler, F. Ali-Osman, *MAP kinase hyper-activation and enhanced NRAS expression drive acquired vemurafenib resistance in V600E BRAF melanoma cells*. Journal of Biological Chemistry, 2014.
93. Fisher, R.a.J.L., *Vemurafenib: a new treatment for BRAF-V600 mutated advanced melanoma*. Cancer Management and Research, 2012. **4**: p. 243-252.
94. Su, F., et al., *Resistance to selective BRAF inhibition can be mediated by modest upstream pathway activation*. Cancer Res, 2012. **72**(4): p. 969-78.
95. Mackiewicz-Wysocka, M., L. Krokowicz, J. Kocur, J. Mackiewicz, *Resistance to vemurafenib can be reversible after treatment interruption: a case report of a metastatic melanoma patient*. Medicine, 2014. **93**(27): p. e157.
96. Miao, B., Z. Ji, L. Tan, M. Taylor, J. Zhang, H.G Coi, D.T. Frederick, R. Kumar, J.A. Wargo, K.T. Flaherty, N.S. Gray, H. Tsao, *EPHA2 Is a Mediator of Vemurafenib Resistance and a Novel Therapeutic Target in Melanoma*. Cancer Discovery, 2015. **5**(3): p. 274-287.
97. Arina, A., et al., *Adoptively transferred immune T cells eradicate established tumors despite cancer-induced immune suppression*. J Immunol, 2014. **192**(3): p. 1286-93.
98. Donia, M., et al., *Methods to improve adoptive T-cell therapy for melanoma: IFN-gamma enhances anticancer responses of cell products for infusion*. J Invest Dermatol, 2013. **133**(2): p. 545-52.
99. Dudley, M.E., et al., *Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma*. J Clin Oncol, 2005. **23**(10): p. 2346-57.
100. Mazzarella, T., et al., *Ex vivo enrichment of circulating anti-tumor T cells from both cutaneous and ocular melanoma patients: clinical implications for*

adoptive cell transfer therapy. Cancer Immunol Immunother, 2012. **61**(8): p. 1169-82.
101. Wu, R., et al., *Adoptive T-cell therapy using autologous tumor-infiltrating lymphocytes for metastatic melanoma: current status and future outlook*. Cancer J, 2012. **18**(2): p. 160-75.
102. Itzhaki, O., D. Levy, D. Zikich, A.J. Treves, G. Markel, J. Schachter, M.J. Besser, *Adoptive T-cell transfer in melanoma*. Immunotherapy, 2012. **5**(1): p. 79-90.
103. Dudley, M.E. and S.A. Rosenberg, *Adoptive-cell-transfer therapy for the treatment of patients with cancer*. Nat Rev Cancer, 2003. **3**(9): p. 666-75.
104. Phan, G.Q., S.A. Rosenberg, *Adoptive Cell Transfer for Patients With Metastatic Melanoma: The Potential and Promise of Cancer Immunotherapy*. Cancer Control, 2013. **20**(4): p. 289-297.
105. Sadeghi, A., et al., *Rapid expansion of T cells: Effects of culture and cryopreservation and importance of short-term cell recovery*. Acta Oncol, 2013. **52**(5): p. 978-86.
106. Nguyen, L.T., et al., *Expansion and characterization of human melanoma tumor-infiltrating lymphocytes (TILs)*. PLoS One, 2010. **5**(11): p. e13940.
107. Chong, A.S., D.E. Bier, W.J. Grimes, E.M. Hersh, *Gamma-irradiated peripheral blood mononuclear cells can express LAK activity*. International Journal of Cell Cloning, 1991. **9**(1): p. 65-77.
108. Riddell, S.R., P.D. Greenberg, *Adding large number of non-dividing peripheral blood mononuclear cells as feeder cells to t-cells in culture medium, activating t-cell receptor complex, adding vector and incubating producing antigen-specific and restricted cells*, 1996, Fred Hutchinson Cancer Research Center: United States.
109. Rosenberg, S.A.a.N.P.R., *Adoptive cell transfer as personalized immunotherapy for human cancer*. Science, 2015. **348**(6230): p. 62-68.
110. Takayama, T., et al., *Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial*. The Lancet, 2000. **356**(9232): p. 802-807.
111. Yee, C., *The use of endogenous T cells for adoptive transfer*. Immunological Reviews, 2014. **257**(1): p. 250-263.
112. Funke, L., O. Prummer, H. Schresenmeier, D. Hardt, M. Weiss, F. Porzsolt, R. Arnold, H. Heimpel, *Capillary leak syndrome associated with elevated IL-2 serum levels after allogeneic bone marrow transplantation*. Annals of Hematology, 1994. **68**(1): p. 49-52.
113. Gattinoni, L., C.A. Klebanoff, and N.P. Restifo, *Paths to stemness: building the ultimate antitumour T cell*. Nat Rev Cancer, 2012. **12**(10): p. 671-84.
114. Klebanoff, C.A., L. Gattinoni, N.P. Restifo, *CD8+ T-cell memory in tumor immunology and immunotherapy*. Immunological Reviews, 2006. **211**: p. 214-224.
115. Jensen, M.C. and S.R. Riddell, *Designing chimeric antigen receptors to effectively and safely target tumors*. Curr Opin Immunol, 2015. **33**: p. 9-15.
116. Curran, K.J.a.R.J.B., *Chimeric Antigen Receptor T Cells for Cancer Immunotherapy*. Journal of Clinical Oncology, 2015.

117. Woo, S.R., L. Corrales, and T.F. Gajewski, *Innate immune recognition of cancer*. Annu Rev Immunol, 2015. **33**: p. 445-74.
118. Barrett, D.M., et al., *Chimeric antigen receptor therapy for cancer*. Annu Rev Med, 2014. **65**: p. 333-47.
119. Hillerdal, V. and M. Essand, *Chimeric antigen receptor-engineered T cells for the treatment of metastatic prostate cancer*. BioDrugs, 2015. **29**(2): p. 75-89.
120. Grupp, S.A., et al., *Chimeric antigen receptor-modified T cells for acute lymphoid leukemia*. N Engl J Med, 2013. **368**(16): p. 1509-18.
121. Mackall, C.L., T.J. Fry, and R.E. Gress, *Harnessing the biology of IL-7 for therapeutic application*. Nat Rev Immunol, 2011. **11**(5): p. 330-42.
122. Kaech, S.M. and E.J. Wherry, *Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection*. Immunity, 2007. **27**(3): p. 393-405.
123. Kallies, A., *Distinct regulation of effector and memory T-cell differentiation*. Immunol Cell Biol, 2008. **86**(4): p. 325-32.
124. Flynn, J.K. and P.R. Gorry, *Stem memory T cells (TSCM)-their role in cancer and HIV immunotherapies*. Clin Transl Immunology, 2014. **3**(7): p. e20.
125. Luckey, C.J., et al., *Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells*. Proc Natl Acad Sci U S A, 2006. **103**(9): p. 3304-9.
126. Harty, J.T. and V.P. Badovinac, *Shaping and reshaping CD8+ T-cell memory*. Nat Rev Immunol, 2008. **8**(2): p. 107-19.
127. Kaech, S.M., E.J. Wherry, and R. Ahmed, *Effector and memory T-cell differentiation: implications for vaccine development*. Nat Rev Immunol, 2002. **2**(4): p. 251-62.
128. Gattinoni, L., et al., *A human memory T cell subset with stem cell-like properties*. Nat Med, 2011. **17**(10): p. 1290-7.
129. Gattinoni, L., et al., *Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells*. Nat Med, 2009. **15**(7): p. 808-13.
130. Fergusson, J.R., V.M. Fleming, and P. Klenerman, *CD161-expressing human T cells*. Front Immunol, 2011. **2**: p. 36.
131. Lugli, E., et al., *Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells*. Nat Protoc, 2013. **8**(1): p. 33-42.
132. Xiao, Z., et al., *Wnt signaling inhibits CTL memory programming*. Mol Immunol, 2013. **56**(4): p. 423-33.
133. Crompton, J.G., M. Rao, and N.P. Restifo, *Memoirs of a reincarnated T cell*. Cell Stem Cell, 2013. **12**(1): p. 6-8.
134. Restifo, N.P., M.E. Dudley, and S.A. Rosenberg, *Adoptive immunotherapy for cancer: harnessing the T cell response*. Nat Rev Immunol, 2012. **12**(4): p. 269-81.
135. Liu, S.P., et al., *Induced pluripotent stem (iPS) cell research overview*. Cell Transplant, 2011. **20**(1): p. 15-9.
136. Tite, J.P., X.M. Gao, C.M. Hughes-Jenkins, M. Lipscombe, D. O'Callaghan, G. Dougan, F.Y. Liew, *Anti-viral immunity induced by recombinant nucleoprotein of influenza A virus. III. Delivery of recombinant nucleoprotein*

to the immune system using attenuated Salmonella typhimurium as a live carrier. Immunology, 1990. **70**(4): p. 540-546.
137. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors*. Cell, 2006. **126**(4): p. 663-76.
138. Miller, D.M., et al., *c-Myc and cancer metabolism*. Clin Cancer Res, 2012. **18**(20): p. 5546-53.
139. Pasi, C.E., et al., *Genomic instability in induced stem cells*. Cell Death Differ, 2011. **18**(5): p. 745-53.
140. Rizzino, A., *Concise review: The Sox2-Oct4 connection: critical players in a much larger interdependent network integrated at multiple levels*. Stem Cells, 2013. **31**(6): p. 1033-9.
141. Wang, Z., et al., *Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells*. Cell Stem Cell, 2012. **10**(4): p. 440-54.
142. Bar-Nur, O., et al., *Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells*. Cell Stem Cell, 2011. **9**(1): p. 17-23.
143. Giorgetti, A., et al., *Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2*. Cell Stem Cell, 2009. **5**(4): p. 353-7.
144. Thompson, L.H., et al., *A LIF/Nanog axis is revealed in T lymphocytes that lack MARCH-7, a RINGv E3 ligase that regulates the LIF-receptor*. Cell Cycle, 2014. **9**(20): p. 4213-4221.
145. Wen, X., et al., *Downregulation of the transcription factor KLF4 is required for the lineage commitment of T cells*. Cell Res, 2011. **21**(12): p. 1701-10.
146. Gelebart, P., et al., *Aberrant expression and biological significance of Sox2, an embryonic stem cell transcriptional factor, in ALK-positive anaplastic large cell lymphoma*. Blood Cancer J, 2012. **2**: p. e82.
147. Dhodapkar, K.M., et al., *Natural immunity to pluripotency antigen OCT4 in humans*. Proc Natl Acad Sci U S A, 2010. **107**(19): p. 8718-23.
148. Wang, X. and J. Dai, *Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology*. Stem Cells, 2010. **28**(5): p. 885-93.
149. Kotoula, V., S.I. Papamichos, and A.F. Lambropoulos, *Revisiting OCT4 expression in peripheral blood mononuclear cells*. Stem Cells, 2008. **26**(1): p. 290-1.
150. Zangrossi, S., et al., *Oct-4 expression in adult human differentiated cells challenges its role as a pure stem cell marker*. Stem Cells, 2007. **25**(7): p. 1675-80.
151. Nishikawa, S., R.A. Goldstein, C.R. Nierras, *The promise of human induced pluripotent stem cells for research and therapy*. Nature Reviews Molecular Cell Biology, 2008. **9**(9): p. 725-729.
152. Cahan, P. and G.Q. Daley, *Origins and implications of pluripotent stem cell variability and heterogeneity*. Nat Rev Mol Cell Biol, 2013. **14**(6): p. 357-68.
153. Kim, K., et al., *Epigenetic memory in induced pluripotent stem cells*. Nature, 2010. **467**(7313): p. 285-90.

154. Nissenbaum, J., et al., *Global indiscriminate methylation in cell-specific gene promoters following reprogramming into human induced pluripotent stem cells*. Stem Cell Reports, 2013. **1**(6): p. 509-17.
155. Plath, K. and W.E. Lowry, *Progress in understanding reprogramming to the induced pluripotent state*. Nat Rev Genet, 2011. **12**(4): p. 253-65.
156. Nishimura, T., et al., *Generation of rejuvenated antigen-specific T cells by reprogramming to pluripotency and redifferentiation*. Cell Stem Cell, 2013. **12**(1): p. 114-26.
157. Minagawa, A. and S. Kaneko, *Rise of iPSCs as a cell source for adoptive immunotherapy*. Hum Cell, 2014. **27**(2): p. 47-50.
158. Kishino, Y.T.S., J. Fujita, S. Yuasa, S. Tohyama, A. Kumitomi, R. Tabei, K. Nakajima, M. Okada, A. Hirano, H. Kanazawa, K. Fukuda, *Derivation of Transgene-Free Human Induced Pluripotent Stem Cells from Human Peripheral T Cells in Defined Culture Conditions*. PLoS One, 2014. **9**(5).
159. Seki, T., et al., *Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells*. Cell Stem Cell, 2010. **7**(1): p. 11-4.
160. Li, S. and Q. Li, *A promising approach to iPSC-based cell therapy for diabetic wound treatment: direct lineage reprogramming*. Mol Cell Endocrinol, 2014. **393**(1-2): p. 8-15.
161. Szabo, E., et al., *Direct conversion of human fibroblasts to multilineage blood progenitors*. Nature, 2010. **468**(7323): p. 521-6.
162. Koch, U. and F. Radtke, *Mechanisms of T cell development and transformation*. Annu Rev Cell Dev Biol, 2011. **27**: p. 539-62.
163. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision*. Nat Rev Immunol, 2002. **2**(5): p. 309-22.
164. Pan, C., et al., *Reprogramming human fibroblasts using HIV-1 TAT recombinant proteins OCT4, SOX2, KLF4 and c-MYC*. Mol Biol Rep, 2010. **37**(4): p. 2117-24.
165. Zhang, H., et al., *Reprogramming of somatic cells via TAT-mediated protein transduction of recombinant factors*. Biomaterials, 2012. **33**(20): p. 5047-55.
166. Zhao, T., et al., *Immunogenicity of induced pluripotent stem cells*. Nature, 2011. **474**(7350): p. 212-5.
167. Wang, J., Q. Li, *A protein-induced pluripotent cell technology uses thereof*, 2014, Wayne State University: USA.
168. Vizcardo, R., et al., *Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells*. Cell Stem Cell, 2013. **12**(1): p. 31-6.
169. Moore, M.W., F.R. Carbone, M.J. Bevan, *Introduction of soluble protein into the class I pathway of antigen processing and presentation*. Cell, 1988. **54**(6): p. 777-785.
170. Gothert, J.R., *Bypassing T cell 'exhaustion'*. Nat Immunol, 2013. **14**(11): p. 1114-6.
171. Schurich, A., et al., *The third signal cytokine IL-12 rescues the anti-viral function of exhausted HBV-specific CD8 T cells*. PLoS Pathog, 2013. **9**(3): p. e1003208.

172. Wherry, E.J., *Molecular Basis of T-Cell Exhaustion*. Blood, 2013. **122**(21).
173. Chen, L., et al., *Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function*. Mol Cell, 2005. **17**(3): p. 393-403.
174. Cleary, M.L., S.D. Smith, J. Sklar, *Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation*. Cell. **47**(1): p. 19-28.
175. Vaux, D.L., S. Cory, J.M. Adams, *Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells*. Nature. **335**(6189): p. 440-442.
176. Seder, R.A., R. Ahmed, *Similarities and differences in CD4+ and CD8+ effector and memory T cell generation*. Nature Immunology, 2003. **4**(9): p. 835-842.
177. Altman, J.D., P.A.H. Moss, P.J.R. Goulder, D.H. Barouch, M.G. Mcheyzer-Williams, J.I. Bell, A.J. McMichael, M.M. Davis, *Phenotypic Analysis of Antigen-Specific T Lymphocytes*. Science, 1996. **274**(5284): p. 94-96.
178. Ceuppens, J.L., M.L. Baroja, K. Lorre, J.V. Damme, A. Billiau, *Human T cell activation with phytohemagglutinin. The function of IL-6 as an accessory signal*. The Journal of Immunology, 1988. **141**(11): p. 3868-3874.
179. Cronin, S.J.F., J.M. Penninger, *From T-cell activation signals to signaling control of anti-cancer immunity*. Immunological Reviews, 2007. **220**(1): p. 151-168.
180. Levy, D.E., C.K. Lee, *What does Stat3 do?* Journal of Clinical Investigation, 2002. **109**(9): p. 1143-1148.
181. Challen, G.A., et al., *Mouse hematopoietic stem cell identification and analysis*. Cytometry A, 2009. **75**(1): p. 14-24.
182. Holmes, C. and W.L. Stanford, *Concise review: stem cell antigen-1: expression, function, and enigma*. Stem Cells, 2007. **25**(6): p. 1339-47.
183. Whitmire, J.K., B. Eam, and J.L. Whitton, *Mice deficient in stem cell antigen-1 (Sca1, Ly-6A/E) develop normal primary and memory CD4+ and CD8+ T-cell responses to virus infection*. Eur J Immunol, 2009. **39**(6): p. 1494-504.
184. Deepak, D., et al., *The influence of growth hormone replacement on peripheral inflammatory and cardiovascular risk markers in adults with severe growth hormone deficiency*. Growth Horm IGF Res, 2010. **20**(3): p. 220-5.
185. Finlay, D. and D.A. Cantrell, *Metabolism, migration and memory in cytotoxic T cells*. Nat Rev Immunol, 2011. **11**(2): p. 109-17.
186. Musgrove, E.A., C.E. Caldon, J. Barraclough, A. Stone, R.L. Sutherland, *Cyclin D as a therapeutic target in cancer*. Nature Reviews Cancer, 2011. **11**(8): p. 558-572.
187. Johnson, D.G., J.K. Schwarz, W.D. Cress, J.R. Nevins, *Expression of transcription factor E2F1 induces quiescent cells to enter S phase*. Nature, 1993. **365**(6444): p. 349-352.
188. O'Donnell, K.A., et al., *c-Myc-regulated microRNAs modulate E2F1 expression*. Nature, 2005. **435**(7043): p. 839-43.

189. Ito, Y., S.C. Bae, and L.S. Chuang, *The RUNX family: developmental regulators in cancer*. Nat Rev Cancer, 2015. **15**(2): p. 81-95.
190. Golias, C.H., A. Charalabopoulos, K. Charalabopoulos, *Cell proliferation and cell cycle control: a mini review*. International Journal of Clinical Practice, 2004. **58**(12): p. 1134-1141.
191. Deberardinis, R.J., J.J. Lum, G. Hatzivassiliou, C.B. Thompson, *The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation*. Cell Metabolism, 2008. **7**(1): p. Jan 2008.
192. Banerjee, A., et al., *Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche*. J Immunol, 2010. **185**(9): p. 4988-92.
193. Intlekofer, A.M., et al., *Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin*. Nat Immunol, 2005. **6**(12): p. 1236-44.
194. Cruz-Guilloty, F., et al., *Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs*. J Exp Med, 2009. **206**(1): p. 51-9.
195. Li, G., et al., *T-Bet and Eomes Regulate the Balance between the Effector/Central Memory T Cells versus Memory Stem Like T Cells*. PLoS One, 2013. **8**(6): p. e67401.
196. Lotem, J., D. Levanon, V. Negreanu, D. Leshkowitz, G. Friedlander, Y. Groner, *Runx3-mediated Transcriptional Program in Cytotoxic Lymphocytes*. PLoS One, 2013. **8**(11): p. e80467.
197. Xiao, Z., M.F. Mescher, and S.C. Jameson, *Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation*. J Exp Med, 2007. **204**(11): p. 2667-77.
198. Sprent, J. and C.D. Surh, *T cell memory*. Annu Rev Immunol, 2002. **20**: p. 551-79.
199. Lalor, P.F., P. Shields, A.J. Grant, D.H. Adams, *Recruitment of lymphocytes to the human liver*. Immunol Cell Biol, 2002. **80**(1): p. 52-64.
200. Oo, Y.H. and D.H. Adams, *The role of chemokines in the recruitment of lymphocytes to the liver*. J Autoimmun, 2010. **34**(1): p. 45-54.
201. Oo, Y.H., et al., *Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver*. J Immunol, 2010. **184**(6): p. 2886-98.
202. Tuncer, C., et al., *The regulation of T-cell recruitment to the human liver during acute liver failure*. Liver Int, 2013. **33**(6): p. 852-63.
203. Ohira, H.T.U., T. Torimura, R. Kasukawa, , *Leukocyte adhesion molecules in the liver and plasma cytokine levels in endotoxin-induced rat liver injury*. Scandinavian Journal of Gastroenterology, 1995. **30**(10): p. 1027-1035.
204. Garía-Barcina, M., I. Bidaurrazaga, V. Naeud, P. Bioulac-Sage, C. Balabaud, F. Vidal-Vanaclocha, M. Winnock, *Variations in the expression of cell-adhesion molecules on liver-associated lymphocytes and peripheral-blood lymphocytes in patients with and without liver metastasis*. International Journal of Cancer. Journal International Du Cancer, 1995. **61**(4): p. 475-470.
205. Park, S., D. Murray, B. John, N.I. Crispe, *Biology and significance of T-cell apoptosis in the liver*. Immunology and Cell Biology, 2002. **80**(1): p. 74-83.