AUGMENTATION OF ANTI-MYELOMA T CELLS

AUGMENTATION OF ANTI-MYELOMA ENGINEERED T CELLS BY PHARMACOLOGICAL OR GENETIC INTERVENTIONS

By

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Lay Abstract

Despite advances in early detection and treatment, cancer remains the leading cause of death in Canada and takes a large toll on the healthcare system and the lives of many. Multiple myeloma is an aggressive and difficult to treat blood cancer, particularly for people with advanced and relapsed disease. Therapies that employ white blood cells have been an effective method for treating myeloma. Our lab has devised a method to generate white blood cells for the treatment of myeloma that is expected to be well tolerated and efficacious. The work described in this thesis is focused on developing strategies that enhance the effectiveness of these white blood cells to improve their anti-tumor activity against multiple myeloma.

Abstract

Multiple myeloma is an aggressive plasma cell cancer that consistently acquires multi-drug resistance and relapses despite initial treatment successes. Patients may go through greater than 10-lines of therapy, highlighting the need for more effective treatment options. Immunotherapies are the latest evolution in targeted cancer treatments, and thus far have displayed impressive results in several hematological cancers, including multiple myeloma. T cells possess robust anti-tumor functions which can be harnessed and refined for the treatment of cancers. Genetic engineering of T cells to express a chimeric antigen receptor (CAR) confers antigen-specific tumor-targeting, and adoptive transfer of patient-derived CAR-engineered T (CAR T) cells has been efficacious in relapsed/refractory multiple myeloma. Despite the high efficacy, CAR T cell therapy for myeloma is associated with serious adverse events, which limits dose levels and patient eligibility.

We have developed a novel synthetic antigen receptor platform, called the T cell antigen coupler (TAC) receptor, which has shown comparatively higher efficacy with a reduced pro-inflammatory profile compared with CAR T cells in pre-clinical models. The TAC receptor was purpose-built to co-opt the natural T cell activation machinery and lacks the costimulatory signaling typically incorporated in CAR designs. This thesis investigates strategies to augment TAC T cell function against for multiple myeloma through the evaluation of ancillary pharmacological and protein stimuli that would complement the anti-tumor functions of TAC T cells without modifying the TAC receptor design.

In **chapter 2**, I investigated a strategy combining TAC T cells with the SMAC mimetic LCL161 to provide transient costimulatory effects. While LCL161 boosted TAC T cells survival and proliferation, the drug also enhanced susceptibility of TAC T cells to apoptosis and offered no advantage to the TAC T cells when challenged with myeloma.

In **chapter 3**, I engineered TAC T cells to secrete IL-27 in an attempt to modulate the myeloma microenvironment and support T cell cytolytic function. IL-27 did not enhance the anti-tumor activity of TAC T cells but forced expression of IL-27 led to a reduction in the production of pro-inflammatory cytokines without altering cytotoxicity.

In **appendix I**, I describe the process of optimizing CRISPR/Cas9 editing of primary TAC T cells. This methodology was required for much of the work in chapter 2.

In **appendix II**, I describe an assessment of mRNA-engineering as a method to produce TAC T cells. This approach proved to be therapeutically futile and was not pursued beyond the work described herein.

The work presented here highlights methods of combining TAC T cells with a clinically relevant SMAC mimetic, or the cytokine IL-27, and provides insights into the biological mechanisms that are affected by these approaches.

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*S: supplemental

List of Abbreviations and Symbols

Abbreviations

ABD	Antigen-binding domain
ACT	Adoptive cell transfer
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AICD	Activation-induced cell death
ANOVA	Analysis of variance
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ATCC	American Type Culture Collection
ATP	Adenosine 5′-triphosphate
AUC	area-under-the-curve
autoSCT	autologous stem cell transplant
BAD	BCL-2-associated agonist of cell death
BAFF	B cell activating factor
BCL-2	B cell lymphoma 2
BCMA	B cell maturation antigen
BCR	B cell receptor
BIM	BCL-2-like protein 11
BiTE	Bi-specific T cell engager
BP	Biological processes
CAR	Chimeric antigen receptor
CAR T cell	Chimeric antigen receptor-engineered T cell
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
cIAP	Cellular inhibitor of apoptosis
CR	Complete response
CRISPR	Clustered regularly interspaced short palindromic repeats
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
CTV	CellTrace Violet
DARPin	Designed ankyrin repeat protein
Dex	Dexamethasone
DGA	Differential gene analysis
DIABLO	Direct IAP binding protein with low pI
DMEM	Dulbecco’s Modified Eagle’s Medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E:T	Effector-to-target ratio
EBI3	Epstein-Barr virus induced gene 3
EDTA	Ethylenediaminetetraacetic acid
EF-1α	Elongation factor-1 alpha
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase 1 or 2
FADD	Fas-associated death domain protein
FAIM3	Fas apoptotic inhibitory molecule 3
FBS	Fetal bovine serum
FcγR	Fc gamma receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GO	Gene ontology
GPRC5G	G protein–coupled receptor, class C, group 5, member D
gRV	Gammaretrovirus
GVHD	Graft-versus-host disease
HEK293	Human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HR	Hazard ratio
i.p.	Intraperitoneal
i.v.	Intravenous
IAP	Inhibitor of apoptosis
ICANS	Immune effector cell-associated neurotoxicity syndrome
ICE	Inference of CRISPR Edits
ICOS	Inducible T cell costimulator
ICU	Intensive care unit
IFN	Interferon
IgG	Immunoglobulin G
IKK	IκB kinase
IL-	Interleukin
IMiD	Immunomodulatory drugs
IR	Infrared

ITAM	Immunoreceptor tyrosine-based activation motif
IVT	*In vitro* transcribed
JAK	Janus kinase
KO	Knock-out
LAGE-1	L antigen family member 1
LFA-1	Lymphocyte function-associated antigen 1
LLPC	Long-lived plasma cell
LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia-1
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MRD	Minimal residual disease
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NE	Not electroporated
NF-κB	Nuclear factor kappa B
NFAT	Nuclear factor of activated T cells
NGFR	Nerve growth factor receptor
NIK	NF-κB-inducing kinase
NK cell	Natural killer cell
NKG2D	Natural killer group 2, member D
NKT cell	Natural killer T cell
NRG	NOD.Cg-Rag1^{tm1Mom}Il2rg^{tm1Wjl}/SzJ
NS or ns	Not significant
NY-ESO-1	New York esophageal squamous cell carcinoma 1
OE	Over-expressed
ORR	Overall response rate
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PE	Phycoerythrin
PerCP-Cy5.5	peridinin chlorophyll protein-cyanine 5.5
PFS	Progression-free survival
PI	Proteosome inhibitor
PI3K	Phosphoinositide 3-kinases

PLAT-E	Platinum-E
pMHC	Peptide-MHC complex
PROTAC	Proteolysis targeting chimera
PS	Phosphatidylserine
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
RAG1/2	Recombination-activating gene 1 or 2
RCF	Relative centrifugal force
rhIL-	recombinant human interleukin
RIPA	Radioimmunoprecipitation assay buffer
RIPK1	Receptor-interacting protein kinase 1
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPMI	Roswell Park Memorial Institute
RRMM	Relapsed/refractory multiple myeloma
RT	Room temperature
RV	Retrovirus
scFv	Single-chain variable fragment
sCR	Stringent complete response
SD	Standard deviation
SEM	Standard error of mean
sgRNA	Single-guide RNA
SLAMF7	SLAM family member 7
SMAC	Second mitochondria-derived activator of caspase
SM	SMAC mimetic
STAT	Signal transducer and activator of transcription
TAC	T cell antigen coupler
TBS	Tris-buffered saline
TCR	T cell receptor
TGF	Transforming growth factor
T_H	T helper
TIL	Tumor-infiltrating lymphocyte
TIL/Tc	Tumor-infiltrating T cell
TM	Transmembrane
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
TRAC	TCR α-chain constant
TRAIL	TNF-related apoptosis-inducing ligand
TRUCK	T cells redirected for antigen-unrestricted cytokine-initiated killing

TU	Transducing units
UTR	Untranslated region
XIAP	X-linked IAP

Symbols

α	alpha
β	beta
°C	degrees Celsius
d	day(s)
δ	delta
Δ	delta
ε	epsilon
γ	gamma
κ	kappa
μ	micro
%	percent
ζ	zeta

Declaration of Academic Achievement

This document was independently authored by Arya Afsahi, with editorial support from Dr. Jonathan Bramson. The studies and experiments described were conceptualized, conducted, and analyzed by Arya Afsahi in collaboration with Dr. Jonathan Bramson, with the noted assistance below:

- Figures 2-1 and 2-2 were generated using assets from BioRender.
- The anti-BCMA TAC and CAR utilized in all studies were designed and optimized by Dr. Ksenia Bezverbnaya[1, 2].
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Chapter 1 – Introduction

1.1 Carcinogenesis

Cancer initiation and development is a complex process that typically unfolds over the timespan of years to decades. With the exception of a small subset of malignancies formed by specific mutational events[3] or viral infection[4-6], the majority of cancers are stochastic and begin through neoplastic transformation of somatic cells. Transformed cells arise gradually as a byproduct of random DNA replication errors, genetic predisposition, and/or environmental factors that damage the genome[7-9]. Tissues that undergo constant self-renewal or change such as the blood, breast, lung, and colon, or those that are exposed to environmental damage such as skin (through ultraviolet rays) or colon and lung (through carcinogen exposure) are most likely to acquire mutations and pass them along to daughter cells.

The accumulation of mutations and resultant genetic instability eventually confer capacity for uncontrolled proliferation, leading to the development of cancer[10]. As cancerous cells begin to amass and establish, released growth factors and cytokines initiate the formation of a microenvironmental niche to support the growing tumor. The exact composition and make-up of a tumor microenvironment will vary based on anatomical location and tumor-type, but generally it will be comprised of vasculature and accessory cells, such as fibroblasts and stromal cells[11, 12]. The ultimate progression of cancer is the dissemination of neoplastic cells to distant sites in a process known as the invasion-metastasis cascade[13]. This involves acquisition of an aggressive phenotype that allows tumor cells to attain motility, invade locally, and intravasate into the vasculature and arrive at a distal location and once again establish growth programs.

Ultimately, the unrestrained growth of the tumor and its metastases will deprive healthy tissues of nutrients due to the large energy demand, physically disrupt the function of organ systems invaded, and possibly secrete factors that upset natural physiology. Each of these mechanisms negatively affect the health of afflicted patients and contribute to their eventual death if left untreated.

1.2 Conventional cancer therapies

The challenges in cancer treatment

Although all cancers share a common defining feature of unrestricted growth of self-tissue, in reality cancer represents a large category of illnesses which can diverge considerably in their features and attributes. Consequently, the notion that there exists a possible “cure to cancer” is disingenuous given the highly complex and variable nature of the disease. The individual differences are

in part dictated by the particular somatic mutations and genetic instabilities that led to formation of a malignant neoplasm, and the differing physiology of the tissues of origin. This diversity translates from inter-tumor heterogeneity, where there is variation between patients of the same cancer, to intra-tumor heterogeneity, where tumors within a patient are often comprised of subpopulations which deviate in both phenotype and gene expression[14-16]. As one can imagine, the clinical implication is that an individual's cancer may not respond to the same treatment course that another with the same cancer would. Disease management becomes a situation where no single pharmacological or medical intervention may catch the entire tumor population.

Underpinning these issues is the fundamental challenge in cancer treatment – design of anti-cancer agents that effectively and safely discriminate cancerous tissue from healthy tissue. An effective cancer therapy that completely spares surrounding healthy tissue is an aspirational treatment; however, with the exception of certain virally-induced malignancies that express foreign antigens, cancer is a disease entirely of the self and thus collateral damage to similar but non-cancerous tissue is often a reality.

Surgery, radiation, and chemotherapy

Conventional cancer treatment modalities can be broadly classified as loco-regional or systemic, and these treatments typically target and exploit commonalities and weaknesses among tumors such as genetic instability and rapid division. In the case of solid tumors, surgery can be an effective remedy, especially for non-metastasized and well contained tumors. For example, minimally invasive surgery followed by adjuvant radiation or hormonal therapy is the standard of care for stage I/II breast cancers and relapses remain below 20%[17]. Surgery for cancer continues to be utilized and improved to this day[18], however many tumors are not surgically accessible or have metastasized, can be too close to vital organs, or the pre-operative health of the patient will not tolerate surgical procedures. Furthermore, there is inherent risk that surgery will enhance early recurrence or trigger metastases due to shedding of cancer cells during removal and/or induction of inflammation that can sustain tumor growth[19]. Targeted ionizing radiation (x-ray or gamma) or systemically delivered drugs (chemotherapy) can be used in conjunction with surgery as *adjuvant* (post-treatment) or *neo-adjuvant* (pre-treatment) therapy to further increase the likelihood of success. Many chemotherapeutic drugs function by targeting cells undergoing division, which includes tumor cells and cells undergoing regular self-renewal such as bone marrow or the lining of the GI tract. It is for this reason that chemotherapy can be extremely arduous and damaging to the body[20, 21], having known long term risks that can even increase the risk of secondary

neoplasms[22, 23]. Radiotherapy uses focused ionizing radiation directed at the tumor to induce DNA damage and in turn initiate cell death due to their already unstable genetic state. However, it too can cause long-term damage to the body through radiation burns and minimally increase the chance of secondary malignancy[24].

Unfortunately not all cancers are amenable to these strategies[20, 21], and cancer remains the leading cause of death in Canada, accounting for roughly 26% of all deaths in 2020[25]. In the case of metastatic disease, previously disseminated tumor cells can remain dormant and hidden for a considerable length of time – even as long as decades – before emergence and progression into active disease once again[26-28]. Other cancers, such as multiple myeloma, are aggressive and remain largely incurable despite impressive strides in treatment over the last two decades[29].

1.3 Multiple myeloma

The plasma cell is the workhorse of the humoral arm of the adaptive immune system. Responsible for the constitutive and extended release of pathogen-specific antibodies used in host defense, long-lived plasmas cells (LLPC) primarily reside in the bone marrow amongst a niche that supports their survival and longevity[30]. Here the LLPC interacts with supportive accessory cells and receives a myriad of signals and factors such as APRIL[31-33], BAFF[34], IL-6[33], and integrin-mediated cell signaling[32] that are necessary for these cells to thrive. B cell maturation antigen (BCMA) is a growth receptor crucial for this on LLPCs and functions through its interactions with its ligands APRIL and BAFF[35].

Neoplastic transformation of LLPC is thought to first occur when a founder clone in the germinal center receives a genetic hit that initiates an immortalization process, often acquired through chromosomal abnormalities such as hyperdiploidy or translocations involving the immunoglobulin heavy chain during somatic hypermutation[36-38]. This process results in a subclinical neoplastic state known as monoclonal gammopathy of undetermined significance (MGUS). As further mutational events accumulate, the plasma cell clone attains greater proliferative potential and infiltration of the bone marrow[39], progressing into smouldering multiple myeloma before and finally reaching the terminus of active multiple myeloma[40]. Many clinical manifestations of multiple myeloma are a result of its invasion of the bone marrow and the ensuing consequences. Expansion of the myeloma represses hematopoiesis by limiting the space for production of blood cells, leading to anemia in most patients. Additionally, multiple myeloma is coupled with very high incidence of bone fracture and associated mortality[41, 42] and hypercalcemia[43, 44], which is due to the multiple myeloma-mediated remodeling of the bone marrow niche towards dominance of

bone-resorbing osteoclasts[45]. Finally, production of monoclonal antibody by myeloma cells (known as M protein) leads to accumulation and deposition of immunoglobulin light-chains in the kidneys resulting in renal failure[46]. Combined, these pathophysiological effects result in multi-organ damage that is aggressive and fatal.

Conventional treatment strategies

Modern myeloma treatment is a task complicated by disease state, patient health, stem-cell transplant eligibility, and availability/approval of drugs in the patient's jurisdiction. New drugs and novel therapies for myeloma are being developed at a high rate, and although the treatment landscape may be confusing due to extensive clinical trials combing drugs[47], it is an undoubted accomplishment that improves patient outcomes and quality of life. Treatment regimens for newly diagnosed multiple myeloma typically consist of a combination of proteosome inhibitor (PI), immunomodulatory drug (IMiD), and steroid – the cited guideline generally consisting of bortezomib (PI), lenalidomide (IMiD), and dexamethasone (dex; steroid)[48, 49] – as induction therapy prior to autologous stem-cell transplantation (autoSCT), if the patient is eligible. The inclusion of autoSCT improves progression-free survival (PFS), overall survival (OS) rate, and minimal residual disease (MRD) rate[50]. However, the unfortunate reality is that the median age of myeloma diagnosis is 65-70[44] and some of these patients may be too frail to survive autoSCT.

Despite impressive strides made by these therapies to extend survival and quality of life, multiple myeloma is often a chronically recurring cancer. Following successful treatment with frontline agents, patients typically relapse with resistant disease that renders drugs of the same class ineffective[51]. Therefore, relapsed/refractory multiple myeloma (RRMM) requires the use of agents naïve to the treatment plan[52], and in the face of this challenge researchers continue to make strides in drug discovery.

Next-generation chemotherapeutics

Newer treatment options are emerging that are highly targeted towards tumor-associated targets to increase efficacy and reduce adverse events. Two novel anti-cancer small molecule drugs are in the pipeline for heavily pre-treated RRMM patients. Selinexor is a first-in-class selective inhibitor of nuclear export drug that that inhibits the activity of exportin 1, which is a nuclear export protein that inactivates tumor suppressor proteins and promotes oncogenesis at high levels[53, 54]. Phase 3 trial data investigating the use of selinexor with bortezomib and dexamethasone found the drug to be generally well-tolerated with reduced peripheral neuropathy, and better median PFS and ORR in patients who had

received 1-3 prior lines of treatment[55]. Similarly, melflufen is a novel first-in-class alkylating peptide-drug conjugate that targets aminopeptidases highly expressed in myeloma cells which cleave the drug to release its alkylating payload. The phase 3 OCEAN trial showed an improvement to PFS with melflufen and dexamethasone vs pomalidomide and dexamethasone[56], however post-hoc analysis and post-trial data highlighted a concern that the drug had a reduced overall survival which lead to a rapid removal of the drug from the marketplace[57]. Despite these disappointing results currently, refinement of the drug and trial design may lead to improved results in the future.

Additional targeted therapies exist that represent a useful avenue in the treatment of specific subgroups in myeloma. For example, a recent phase 3 trial investigated the use of the BCL-2 inhibitor, venetoclax, in RRMM patients that harbor a t(11;14) translocation which results in higher expression of the antiapoptotic protein BCL-2. This trial had impressive results with increased PFS, ORR, and MRD[58] and highlights the potential of targeted therapies.

Over the last two decades, a novel and extremely promising class of anti-cancer treatments has become clinically viable in myeloma and many other malignancies. Immunotherapeutic approaches utilize aspects of the immune system, such as monoclonal antibodies targeting tumor-associated antigens, antibodies to reverse inhibited anti-tumor immune cells, or cellular treatments that expand and modify immune cells outside the body to arm them against cancer as live-cell treatments. These therapies have revolutionized treatment in several previously untreatable cancers. To understand the potential of these modalities, we must first examine the extensive interplay and balance between cancer and the immune system.

1.4 Cancer and the immune system

The human immune system, which can be broadly classified into the innate and adaptive branches, consists of a complex network of cells, tissues, and soluble mediators that have evolved to respond not only to external pathogens, but also mediate tissue repair and sequester inorganic material. Many immune cells circulate systemically and access tissues via the arteriovenous and lymphatic vessels, while others reside in tissues to act as sentinels for pathogen entry.

Cancer immunosurveillance

Vertebrate immune systems have historically been thought only to protect against and reduce damage from foreign entity invasion. However, in the early 20th century, the idea that the immune system also plays part in controlling neoplasms began to develop. By the 1960-70s the hypothesis of cancer

immunosurveillance was formally proposed by Burnet[59, 60], who postulated that in any long-lived organism heritable somatic genetic changes would push cells towards malignancy and the resultant antigenic potential would trigger immune cell involvement and tumor control. He expanded that this would be a relatively frequent sub-clinical occurrence throughout one's life. The implication of this hypothesis would mean a failure in immune control would impose an increase in cancer occurrence. The next 20 years saw attempts at validating this hypothesis experimentally in mice, but despite these efforts truly convincing studies did not manifest and the immunosurveillance hypothesis fell out of favour until the late 20th/early 21st century when clearer evidence emerged[61]. Nowadays, one need only look at the incidence of cancer in organ transplant patients undergoing immunosuppression to observe the link between cancer control and immune function. Indeed, the frequency of cancer in organ transplant patients is significantly greater than matched individuals[62-64], and this observation is seen in both virally-induced and non-viral malignancies[65, 66]. The latter distinction is of particular importance; were the observation only present in virally-induced cancers then the scope of cancer immunity would be limited to the prevention of those infections rather than control of neoplasm itself. Finally, the presence of immune cell infiltrates in many tumors have been prognostically linked with overall survival. CD8+ and/or CD4+ T lymphocyte infiltration of colorectal[67, 68], ovarian[69, 70], gallbladder[71], lung[72, 73], and some breast cancers[74] has been correlated with greater overall survival and/or progression-free survival; however, in some cases infiltration by these T cell populations is negatively correlated with survival, indicating that there is no one-size-fits-all approach to tumor infiltrating lymphocyte (TIL) prognostic value[75-77]. In such cases, greater stratification of the TIL population may provide better insight[78, 79].

Immunosurveillance is unfortunately not a perfect process; immune-evasion by cancer is a defining hallmark and necessity of tumor formation[80]. The dynamic interplay between the immune system and burgeoning cancer cells is a process known as cancer immunoediting[81, 82]. In the earliest stage of cancer development, an *elimination* phase consisting of attack and destruction of cancer cells by the immune cells is initiated. Should the immune pressure fail to completely eradicate the nascent neoplastic cells, a dynamic equilibrium establishes. The *equilibrium* phase can last for years and is a sub-clinical interaction whereby cancerous cells may subvert immune processes and withstand selective pressures. Paradoxically, it is this extrinsic tumor control by the immune system that sculpts and selects for tumor cells able to evade and escape immune clearance. In this *escape* phase the tumor mass has undergone genetic and epigenetic alterations allowing it to completely breach immune control and signals the start of clinical manifestations.

Despite the failure in tumor control by natural immune processes, the anti-cancer potential of the immune system can be refined and harnessed therapeutically. An understanding of the molecular basis for cancer recognition by the immune system is necessary to understand the extent to which such approaches may developed and applied.

Target recognition by the immune system

Innate immunity refers to the more primitive and evolutionarily ancient branch of the immune system[83]. Innate immune cells utilize germline encoded receptors, such as pattern recognition receptors, to detect evolutionarily important pathogen- or danger-associated molecular patterns and ligands[84]. At the core of many tumor masses is a necrotic and hypoxic environment which is host to cells undergoing various forms of cell death. Metabolites and intracellular components released by these cells are part of a series of danger signals that draw in macrophages and immature neutrophils. These cells are often subverted by the tumor to facilitate its growth and suppress anti-tumor immune responses[85-88], generating an environment, similar to that of a healing wound, which supports vascularization and tissue growth[89, 90]. Natural killer (NK) cells of the innate system also utilize an additional set of germline receptors. These cells integrate signaling from inhibitory and activating receptors that respond to ligands present or absent, respectively, on healthy tissues. This functionality is not binary and requires a net activating or inhibitory signal to stimulate or repress effector functions such as cytotoxicity or cytokine release[91]. These receptors form the basis of the *"missing self"* and *"altered self"* mechanisms. The absence of self-identifying receptors (ie. *missing self*) and the induction of stress ligands (ie. *altered self*) on a cell can result in NK cell attack[92-94]. For example, downregulation of MHC class I receptors, critical receptors obligate to the function of T lymphocytes of the adaptive branch of the immune system, is a well-known method of immune evasion by cancers[95]. However, the presence of MHC class I on cells is a universal repressor signal to prevent NK attack on healthy tissues. In theory this should incite NK cell targeting; however, cancer cells can exploit non-classical MHC I receptors such as HLA-G[96, 97] to maintain a "do not attack" signal to NK cells. Germline encoded receptors of the innate immune system, although effective in rapid response to pathogens, do not have the breadth of coverage necessary to combat the pace of microbial evolution or account for the subversion of normal tissue homeostasis by cancers[98]. Furthermore, in the context of cancer recognition germline encoded receptors present an inflexible design that cannot account for *de novo* mutation of antigens (referred to as *neo-antigens*) that can distinguish tumor cells from healthy tissues.

Adaptive immunity refers to the B and T lymphocyte lineages within jawed vertebrates that have evolved elegantly to respond to virtually any antigen[99],

including mutated tumor antigens. These lymphocytes are unique amongst immune cells in that almost every B or T cell bears an antigen receptor with unique target specificity. Known simply as the B cell receptor or T cell receptor (BCR, TCR), each are encoded within the genome in the format of numerous segments within their respective loci. During development of T or B cells, these segments are somatically rearranged in a process known as V(D)J recombination to generate a TCR or BCR with unique binding capacity[100, 101]. Critically important to this function are the RAG-1/2 recombinase and terminal deoxynucleotidyl transferase enzymes, which facilitate the transposition of segments[102, 103] in an imprecise and stochastic manner to generate diversity[102, 103]. The theoretical maximum diversity by this process is in the range of 10^{15} to 10^{20} unique sequences[104, 105]. Compounding the repertoire diversity are allelic polymorphisms granting additional variation to the V(D)J segments in both B and T cells[106-108].

Of these two lymphocyte populations, conventional αβT cells have the most potent direct anti-tumor function. However, naturally occurring anti-tumor T cells are rare and many tumors remain largely non-immunogenic despite the presence of mutated proteins [109, 110].

Central tolerance – a restriction on natural anti-cancer T cells

At the crux of cancer immunity is effective discrimination of self from non-self[111], a vital requirement to prevent autoimmunity and damage to the host by the immune system. As cancerous cells arise from self-tissues, there are inherent limitations in their recognition by T lymphocytes due to selection processes during T cell development.

T cell development is an intricate and orchestrated activity that begins with migration of thymic-seeding progenitors from the bone marrow to the thymus. Although the identity of these progenitors remains controversial[112, 113], the differentiation of the progenitors to thymocytes, and finally mature naïve T cells, is better understood[114-116]. Conventional αβT cells bear a heterodimeric TCR comprised of a TCRα and TCRβ chain, which associates with the CD3 receptor complex for function[117]. The TCR-CD3 complex, in association with a CD4 or CD8 co-receptor, binds to peptide antigens processed from proteins and presented in the context of a major histocompatibility complex (MHC) molecule. Briefly, conventional T cell development is stepwise and begins foremost with rearrangement of the TCRβ gene; thymocytes which have generated a functional TCRβ receptor receive survival signals and proceed to TCRα locus rearrangement. Next, immature T cells undergo positive selection in the thymus cortex where only cells that have generated a functioning TCR capable of binding peptide-MHC (pMHC) weakly or moderately are given survival signals. The ability to bind either

MHC class I or II determines their differentiation to the CD4+ or CD8+ subset, respectively[118]. T cells with TCRs that bind too strongly to self-peptide in MHC are deleted by negative selection[119]. Finally, the immature T cells traverse to the thymus medulla where they are exposed to medullary thymic epithelial cells and antigen presenting cells (APC) that are rich in expression of many tissue-type antigens. This exposure selects self-reactive T cells to be deleted or differentiated to a regulatory phenotype[120] to prevent autoimmunity. The ultimate culmination of T cell education is the generation of T cells capable of responding to peptide presented in the context of self-MHC with minimal self-reactivity.

As one can imagine, this process removes most T cells capable of responding against cancers, which arise from self. While mutations and aberrant protein expression, such as overexpression or addiction of oncogenic antigens[121-123], can result in abnormalities that trigger detection by immune cells, the magnitude of these natural responses may be insufficient when we consider tolerance mechanisms, the heterogenous nature of cancers, and immunoediting.

Under normal circumstances these factors limit the clinical utility of naturally occurring anti-tumor immune cells which are not specific to mutated neo-epitopes without additional intervention.

1.5 Cancer immunotherapy and the advent of immuno-oncology

Immunotherapy – the utilization of immune cells, soluble factors, and antibodies – has become one of the leading breakthroughs in cancer treatment over the last 2 decades, ushering in new treatment options and enhancing prognosis of many previously dismal diseases[124].

Modes of immunotherapy in multiple myeloma

The modern era of molecular biology and genetic engineering now allows scientist to bypass natural limitations of anti-cancer immunity. As immune function and action are precise and generally target-oriented, immunotherapies can be developed which are *antigen-specific*, those that exert their effect directly on cells bearing a known target antigen, or *antigen-agnostic*, strategies that utilize pathways or mechanisms without knowing antigenic determinants on the tumor cells.

In *antigen-specific* strategies the choice of target antigen is one of *the* prime considerations to avoid unwanted pathology. Tumor antigens lay across a spectrum ranging from tumor-specific to tumor associated (refer to Figure 1-1)[125]. Neo-antigens and oncofetal/cancer testis antigens (antigens normally only expressed in germline tissues or during development) represent the most specific tumor antigens. However, the identification of neo-antigens is challenging,

mutations are very individual[110] and many cancers do not express oncofetal/cancer testis antigens. Instead, the majority of targeted tumor antigens include overexpressed proteins or differentiation antigens also present on non-essential tissues[125]. Antigenic choice is further nuanced by the method and scope of the immunotherapy. For example, live cell treatments may have higher inherent cytotoxicity than antibody therapies, and therefore targeting antigens with greater overlap with healthy cells poses greater risk of adverse events. Therefore, the choice of antigen may require consideration of the typical toxicities associated with a particular treatment modality[126].

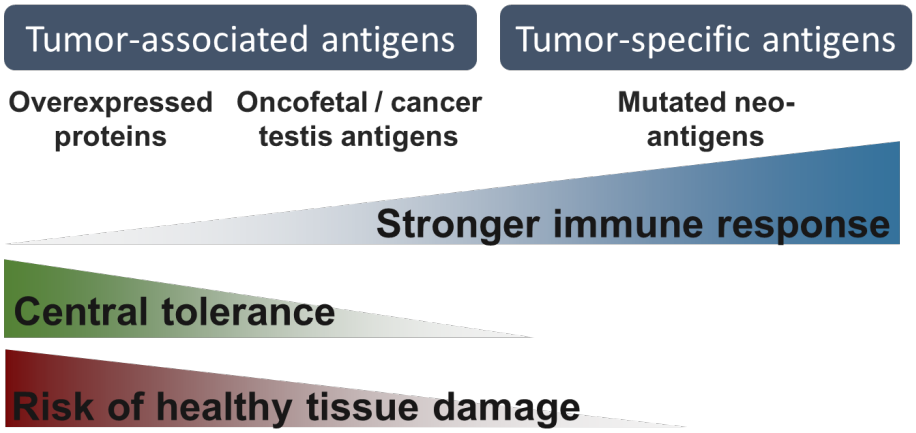

Figure 1-1. Tumor antigen-expression on healthy tissues correlates with risk of autoimmunity.

i. Monoclonal antibodies (mAb)

Generation and modification of therapeutic monoclonal antibodies is an established technology; investigational antibodies can be generated in chimeric, humanized, or fully human backbones and/or conjugated with an active functional payload to be delivered to target cells[127].

Antibodies targeting specific myeloma antigens can be categories as either naked or drug conjugated. Naked antibodies bind to and function directly against the target cell by agonism/antagonism of receptors or direct cytotoxicity. Alternatively, opsonized cancer cells can be deleted through antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), or complement-dependent cytotoxicity (CDC). Antibody-drug conjugates (ADC) link a functional group, such as a cytokine, drug, or radionuclide, to the specificity of an antibody to deliver precise treatment to cancerous cells. In the case of

conjugated cytotoxic compounds, this approach aims to reduce systemic spread of the chemotherapeutic.

Bi-specific antibodies are recombinant proteins genetically engineered to have dual-specificity, granting the ability to bind and link two different targets. Within the myeloma space this method has been utilized with bi-specific T cell engagers (BiTE). Acting as a hybrid solution between monoclonal antibody therapies and T cell-based cell therapies, BiTEs redirect circulating T cells *in vivo* towards a chosen antigenic target by grafting together the binding domain of a tumor antigen-specific antibody and the binding domain of an antibody specific to the CD3 complex, which is invariant amongst αβT cells[128]. Systemic delivery of BiTEs will label circulating T cells within the body and re-direct them to the target antigen.

ii. Live cell products

Treatment of cancer patients with a tumor-reactive immune cell population falls under the category of adoptive cell transfer (ACT) therapies and represents the first "living drug" therapy to enter the market for cancer treatment. In its current form, ACT is primarily a personalized medicine that uses *ex vivo* manipulation and growth of patient-derived anti-tumor immune cells which are then infused as therapy following a lymphodepleting regimen.

ACT of tumor-reactive T cells can be broadly categorized as the enhancement of pre-existing anti-tumor cells, or the generation of novel anti-tumor cells *ex vivo*. The former is best exemplified by tumor-infiltrating T cell (TIL/Tc) therapy, an *antigen-agnostic* treatment. In this approach tumor samples are excised, and cells are extracted from the mass to harvest TIL/Tc. These TIL/Tc are revitalized, cultured to high numbers, and infused into the patient as a large bolus for treatment. TIL therapy is largely applicable to solid tumors and is beyond the scope of this document, for further reading refer to refs[129, 130]. Finding and expanding naturally occurring anti-tumor cells is a difficult endeavor and may not be possible in hematological malignancies or cancers that lack natural immune infiltration[79]. Genetic engineering is an increasingly attractive tool to circumvent these limitations by generating *de novo* tumor-reactivity. To do so, cells are harvested, engineered, and expanded *ex vivo*, then infused into the patient in high numbers. With synthetic biology, researchers have developed novel artificial receptors that redirect specificity of immune cells towards a chosen tumor antigen.

Of the engineered cell products in pre-clinical and clinical development, T cell-based approaches are the most advanced and well-studied.

1.6 Engineered T cells for cancer

T cell therapies present both advantages and challenges over other immunotherapy formats, and in their current incarnation come with trade-offs for impressive clinical efficacy. Inherent biological characteristics of T cells, patient health, and the availability of knowledge, reagents, and systemic processes for clinical manufacture of GMP T cell products contribute to these pros and cons.

A brief overview of T cell biology and function

Following education and maturation, naïve T cells egress from the thymus and enter circulation, beginning their tenure rotating through secondary lymphoid tissues until they encounter their cognate antigen presented by APCs. Ligation of the TCR-CD3 complex with pMHC (known as *signal one*) results in spatial rearrangement of membrane proteins and receptors into the immunological synapse, a focal contact zone between the T cell and the target cell that segregates signaling molecules necessary for activation[131]. During initial activation, naïve T cells require costimulatory ligands (known as *signal two*) provided by APCs concurrent to binding peptide antigen, otherwise activation fails and the T cell enters a state of hyporesponsiveness[132]. Upon activation, T cells will proliferate to exceptional numbers, and distribute systemically to exert cytotoxicity and effector function.

Conventional αβT cells are broadly categorized within CD4+ or CD8+ subsets, and both contribute to anti-tumor responses. CD8+ cytotoxic T lymphocytes (CTL) mediate direct cellular cytotoxicity and tumor de-bulking through soluble- and receptor-mediated mechanisms. Granule exocytosis is the predominant method of inducing target cell death. Cytotoxic perforin and granzyme molecules are released by the T cell into the synaptic cleft to initiate apoptosis in the target cell[133]. Death receptors, such as FasL/Fas, are also utilized by CTLs to trigger an apoptotic program in target cells[134, 135]. The major role of CD4+ helper T (T_H) cells in tumor immunity is in regulation of other immune cells, although they are infrequently capable of direct cytotoxicity[136, 137]. T_H1 cells facilitate CD8+ cytotoxic T cell anti-tumor responses[138-140] and produce a number of cytokines critical to promote anti-tumor control, such as IFNγ and TNFα[141-144].

Furthermore, both CD4+ and CD8+ T cells mediate serial and multiplex killing by binding and inducing cell death in target cells sequentially and/or simultaneously, respectively[145-147]. The lytic machinery is spatiotemporally uncoupled during this process, allowing the targeting of surrounding cancer cells irrespective of antigen[145, 148], an important quality in controlling an antigen-heterogenous tumor populations.

When used as living drugs, T cells have the potential to disseminate and traffic to tumors and distal metastatic sites[149] where they may persist for extended time (upwards of years) to exert tumor control, allowing greater penetrance than the passive diffusion of conventional drugs.

T cell engineering and synthetic antigen receptors

Research and development of engineered T cell products has advanced comparatively further than other cellular candidates for ACT. In addition to the anti-tumor qualities of T cells, early understanding of T cell culture conditions[150, 151], availability of reagents, and permissibility of genomic engineering in T cells may be factors that have facilitated this progress. Recombinant transgene expression is mainly achieved using retroviral vectors such as lentiviruses for genomic integration, and these systems were developed using the HIV-1 genomic backbone, which evolved to primarily infect human T cells[152-154].

Genetic engineering of bulk T cells to redirect antigen specificity can be achieved using two approaches: **i) transgenic TCRs**, or **ii) synthetic antigen receptors**.

i. Transgenic TCRs

Some antigens such as NY-ESO-1 (an intracellular cancer testis antigen with high tumor-restricted expression[155]) are present in many cancers but are only visible to the immune system through expression of their processed peptides on MHC. Antigen-processing and presentation utilizes all proteins within a cell, theoretically granting T cell access to the entire proteome. However, the difficulty in TCR-engineering lies in acquiring TCR sequences against these peptide antigens. TCRs may be harvested through sampling of TILs or screened against a library of neoepitopes[156], however this process is laborious and can be highly personalized due to the nature of neo-antigen generation[157]. In multiple myeloma, T cells bearing an affinity-enhanced TCR targeting a shared NY-ESO-1 and LAGE-1 epitope are under clinical investigation and have shown moderate responses[158].

However, transgenic TCRs are still MHC-restricted and therefore require the receiving host to possess the correct human leukocyte antigen allele capable of binding and presenting the peptide, limiting the pool of applicable patients. Many cancers also exhibit MHC I downregulation[95] and antigen processing defects[159, 160] which negatively impact TCR-based recognition. In addition to these limitations, there are inherent issues with TCRα- and TCRβ-chain mispairing between the endogenous and the transgenic sequences that require gene editing or other strategies[156].

ii. Synthetic antigen receptors

Synthetic receptors can be purpose-built to address the constraints placed on TCR-binding. Described first by Eshhar[161, 162], chimeric antigen receptors (CARs) are the most common format of synthetic receptor used to engineer anti-tumor T cells, known as CAR T cells. Synthetic antigen receptors are recombinant proteins constructed by stitching together **i) an antigen-binding domain**, **ii) hinge and transmembrane region**, and **iii) one or more intracellular signaling domains** necessary for cellular activation. Thus, activation and effector function are tied to ligation of the synthetic receptor with the chosen antigen (Figure 1-2).

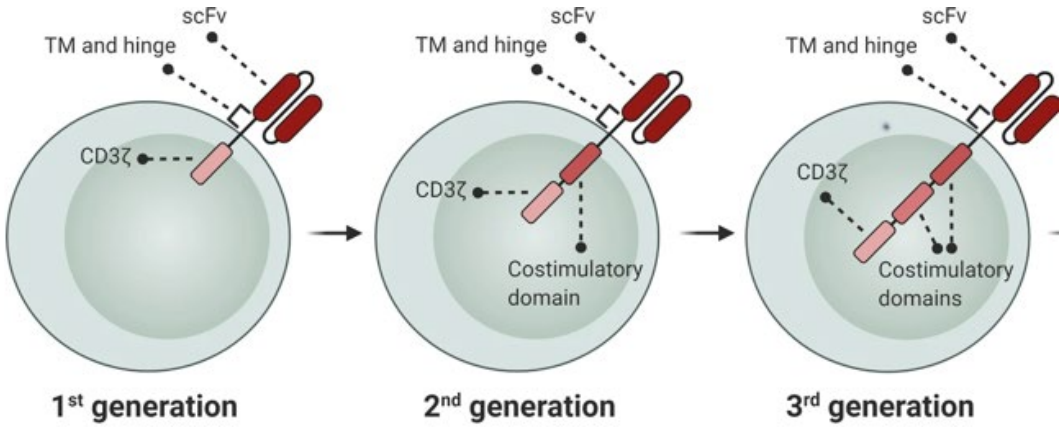

Figure 1-2. The design of first, second, and third generation chimeric antigen receptors; adapted from[163].

Synthetic antigen receptor composition

i. Antigen-binding domain (ABD)

A variety of binding moieties have been utilized in synthetic antigen receptor T cell approaches, including single chain antibody fragments (scFv)[164], nanobodies[165, 166], natural cytotoxicity receptors such as NKG2D[167-169], tandem repeat proteins such as DARPins[170], adhesion molecules and natural ligands such as LFA-1 or APRIL[171, 172], or tethered peptides or cytokines[173, 174]. In fact, TCR-like binding to pMHC is possible using TCR-mimetics or the variable regions of TCR chains[175-178]. Of these choices, scFvs are the dominant ABD used in the engineered T cell field, likely due to the maturity of the monoclonal antibody field.

Unlike TCRs, synthetic receptors are not confined to peptide antigens and MHC-restriction, and can bind native, surface-expressed antigens of protein, carbohydrate, or glycolipid origin. This avenue allows access to antigens previously invisible to T cells.

ii. Hinge and transmembrane domain

A hinge and transmembrane domain are necessary to anchor the synthetic receptor to the membrane.

The hinge is an unstructured linker that gives the ABD flexible access to the antigen. Frequently, the hinge domain is derived from CD8α, IgG, or the TM region utilized in the synthetic receptor[179]. The importance and impact of the hinge region was originally underappreciated. It is now clear that length and composition influences the spacing between the T cell and target cell, and the stericity aspects of the ABD-target interaction, which can impact anti-tumor effectiveness, the sensitivity of the receptor to activation, and/or off-target effects[180-190].

The transmembrane region is typically dependent on the choice of hinge or signaling domains. For example, intracellular signaling domains that have membrane proximal motifs, such as CD28, require their transmembrane region for functionality. Additionally, the transmembrane region can be utilized to promote homodimers of the synthetic receptor, or heterodimers between the synthetic receptor and other membrane associated proteins involved in signaling, such as DAP10 or DAP12[191-194].

iii. Intracellular signaling domains and the need for costimulation

The cytoplasmic portion of synthetic antigen receptors contain domains necessary for downstream signaling and T cell activation. *First generation* CAR designs incorporated either the FcγRIIIA γ-chain or the CD3 ζ-chain because they contain immunoreceptor tyrosine-based activation motifs (ITAM) capable of initiating T cell activation[161]. Virtually all modern CAR T approaches now utilize the CD3ζ intracellular domain to initiate *signal one*.

Chronic antigenic stimulation and inhibitory signals from the tumor can result in an exhausted phenotype in T cells, limiting their efficacy[195, 196]. In the context of T cells, costimulation provides important secondary signals that not only integrate with *signal one* to reinforce activation, but also maintain survival, persistence, and enhance proliferation and effector functions[197-201]. Many T cell costimulatory receptors exist that activate overlapping, yet distinct, signaling programs – the degree of distinction/redundancy within the costimulatory receptor families is context-dependent and an area of on-going research[202, 203]. For example, the prototypical costimulatory receptor CD28 drives a robust glycolytic program[204], whereas 4-1BB facilitates greater mitochondrial function and oxidative phosphorylation[205, 206], and both support expression of anti-apoptotic factors such as Bcl-xL[202].

Second and *third generation* CAR designs include one, or more, costimulatory signaling domains, respectively, in addition to the CD3ζ domain[207]. Of the signaling domains tested thus far, members of the CD28 and TNFR superfamilies are most common, including: CD28[199, 208], 4-1BB[201, 209-211], OX40[212], CD27[213], or ICOS[211, 214]. Of these costimulatory domains, the CD28 and 4-1BB cytoplasmic domains are the most studied and are the only costimulatory domains utilized in current clinically approved CAR T products. For the remainder of the document, the following nomenclature will be used for CAR T cell designs: CD3ζ, CD28, and 4-1BB will be abbreviated to ζ, 28, and BB, respectively. For example, a second generation CAR with the CD3ζ activation domain and a CD28 domain will be labelled as a 28ζ CAR.

Pre-clinical studies have shown that CAR T cells with the 4-1BB endodomain trend towards greater persistence, whereas the CD28-bearing CAR T cells provide more cytotoxic capacity and cytokine production; however both mediate robust anti-tumor efficacy[205, 215-217]. Few clinical studies have been conducted to assess the impact of costimulatory domains on CAR T cells. Thus far, two small co-infusion studies have been performed to directly address this question. Savoldo et al. co-infused first generation CD3ζ and second generation 28ζ anti-CD19 CAR T cells and confirmed that inclusion of costimulation enhanced persistence and expansion, although the authors noted minimal anti-tumor efficacy in general[199]. A study conducted by Cheng et al. co-infused 28ζ and BBζ anti-CD19 CAR T cells in patients with B cell leukemia. The authors observed a statistically insignificant trend towards greater persistence of 4-1BB-bearing CAR T cells[218]. Given the small cohorts it is impossible to draw meaningful conclusions from these trials, however it is noteworthy that the results reflect some of the findings found in pre-clinical work.

Retrospective studies are an avenue to provide more understanding in this situation. A study comparing patients receiving axicabtagene ciloleucel (axi-cel; anti-CD19 28ζ CAR) or tisagenlecleucel (tisa-cel; anti-CD19 BBζ CAR), both of which utilize the same anti-CD19 scFv (FMC63)[219], discovered greater anti-tumor efficacy with axi-cel that correlated with greater cytokine release syndrome (CRS) and neurotoxicity (CAR T cell toxicities that will be discussed below)[220]. A similar retrospective study comparing patients which received axi-cel, tisa-cel, or JCAR14 (another anti-CD19 BBζ CAR; also utilizing the FMC63 scFv[221]) found a similar observation: patients receiving CAR T products utilizing the BBζ domains exhibited lower toxicity and lower efficacy than those who received axi-cel[222]. However, retrospective studies must be considered carefully as any differences derived may be due to many different factors, of which costimulatory domain is just one.

Does one-size-fit-all?

Ultimately, there is no clear superiority to any particular synthetic antigen receptor component. Given the conflicting evidence on the benefits and shortcomings of different hinges, cytoplasmic domains, and targeting moieties, most reports indicate the choice of individual CAR components is dependent on the target antigen, CAR structure, or indication being treated, and therefore must be determined empirically.

1.7 Current and investigational multiple myeloma immunotherapeutics

Development of immunotherapeutics for multiple myeloma continues to gain pace, accounting for an increasing portion of pre-clinical and clinical investigational treatments.

Checkpoint blockade inhibitors are a class of *antigen-agnostic* antibody therapies that exert their effect not through tumor intrinsic pathways, but rather by blocking suppressive ligand/receptor interactions. These suppressive interactions, such as the PD-1/PD-L1 and CD28/CTLA4 pathways, restrict the anti-tumor activity of the endogenous T cells. Checkpoint blockade monoclonal antibody treatments appear to be more efficacious in tumors that contain high numbers of mutations, and therefore greater neo-antigen frequency and cytotoxic T cell infiltrates[223-225]. The results of these therapies in several cancers has been nothing short of astounding, achieving durable remissions in some patients with previously dismal prognosis and no treatment options[226-233]. Although the PD-1/PD-L1 axis is implicated in multiple myeloma through high expression of PD-L1 on tumor cells, clinical trials utilizing checkpoint blockade inhibitors have not shown beneficial single agent, or combination efficacy with other myeloma treatments[234, 235]. In fact, the FDA intervened in trials combining pembrolizumab (anti-PD-1) plus IMiD (pomalidomide or lenalidomide) due to safety concerns and adverse events in patients[236, 237]. The exception to these findings is an ongoing phase II trial combining nivolumab (anti-PD-1) plus daratumumab (anti-CD38; see below), which has shown promising interim results[238], and pembrolizumab (anti-PD-1) plus belantamab mafodotin (anti-BCMA; see below)[239].

In the case of *antigen-specific* modalities, the lion's share of these immunotherapies are centered on 5 antigens, listed below:

i. CD38

CD38 is a transmembrane glycoprotein present on the majority of myeloma cells, and is used diagnostically in multiple myeloma[240]. CD38 expression is not restricted to plasma cells and is present on other hematopoietic cells such as NK, T, B, myeloid cells, and a small subset of non-hematopoietic cells such as muscle

and prostate cells[241, 242]. Despite this observation, clinical targeting with monoclonal antibodies has not resulted in overt toxicities. The function of CD38 in multiple myeloma is that of an ectoenzyme which catabolizes the production of immunosuppressive adenosine from ATP and NAD+ in the bone marrow niche, facilitating myeloma progression[243, 244].

The first anti-CD38 antibody immunotherapy for multiple myeloma to attain regulatory approval by the FDA was daratumumab. It is now approved for newly diagnosed multiple myeloma as well as relapsed/refractory disease, either as monotherapy or in combination with PI, IMiD, and/or dexamethasone. As a monotherapy daratumumab achieved an ORR of 31.1% with median PFS and OS of 4 and 20.1 months, respectively, in a pooled study of 2 trials[245]. When combined with PI/dex[246] or IMiD/dex[247] in phase III trials, daratumumab achieved significantly greater PFS and OS compared to control arms, achieving ORR of 92.9%, and is now under investigation in a phase III trial utilizing PI/IMiD/dex + daratumumab (ClinicalTrials.gov Identifier: NCT03710603[248]).

Isatuximab is the second anti-CD38 mAb FDA approved for treatment in myeloma, either alone or in combination with IMiD/dex. In a phase III multicenter trial isatuximab plus IMiD/dex achieved an overall response rate of 63% compared to the 33% in the control group. Overall survival was increased with a hazard ratio (HR) of 0.76 and 6.9 month median increase[249].

These drugs have introduced better prospects with combination treatments. Daratumumab exerts its function primarily through CDC and ADCC by NK cells, and resistance to the drug occurs through inhibition of complement pathways or off-tumor cytotoxicity of NK cells by daratumumab[250-252]. Isatuximab functions similarly, although it is also capable of inducing direct apoptosis on myeloma cells[253, 254].

CD38-specific CAR T cells are also under pre-clinical and clinical investigation. Clinically, two trials have explored bispecific anti-CD38/BCMA CAR T cells[255, 256]. These CAR T cells achieve bi-specificity through the use of two scFv in serial configuration on a single CAR, rather than two separate CAR each with one scFv. Both trials recorded high efficacy of 87.5% (81.25% sCR) and 87% (52% sCR), and both reported high incidence of CRS with 75% (31.3% serious grade=>3, 6% grade5/fatal) and 87% (17% grade 3), without neurotoxicity.

ii. BCMA

BCMA is a TNFR superfamily member that is critical for the survival of LLPCs and in turn, multiple myeloma cells[35, 257, 258]. BCMA is overexpressed on multiple myeloma cells and is otherwise restricted to the plasma cell compartment[259-262]. Overexpression and activation of BCMA by its ligands APRIL

or BAFF results in activation/expression of genes and signaling involved in multiple myeloma proliferation and survival such as canonical and non-canonical NF-κB pathways, BCL-2, cIAP2, AKT, ERK1/2, and MAPK[257, 263, 264].

The reliance of multiple myeloma cells on BCMA-mediated survival and growth signals, and its limited expression outside of myeloma, makes it an ideal target. Thus far, BCMA has been the leading candidate for many investigational immunotherapies. Described below are a subset that have shown promise or substantial clinical investigation.

Belantamab mafodotin is an ADC consisting of an afucosylated humanized anti-BCMA antibody conjugated to monomethyl auristatin-F, a microtubule-disrupting agent with potent anti-myeloma properties[265]. The DREAMM-2 trial of single agent belantamab mafodotin had favourable results with an ORR of 52% and median PFS of 5.7 months. This led to the accelerated approval by the FDA, however data as of Nov 2022 from DREAMM-3 comparing belantamab mafodotin versus pomalidomide with dex did not meet the primary endpoint and had worse PFS with a HR of 1.03, leading to market withdrawal[266, 267]. Despite this, the median survival was longer (11.2 vs 7 months), ORR was 41% vs 36% in control, and duration of response at 12 months were 76.8% vs 48.4%, signaling overall potential for this drug. However, it should be noted that these results have yet to be published in a peer reviewed source. Additional trials are underway to further evaluate the drug (ClinicalTrials.gov Identifier: NCT04126200, NCT03544281, NCT04246047, NCT04484623, NCT04091126, NCT05064358).

Idecabtagene vicleucel (ide-cel) was the first CAR T cell product approved for the treatment of RRMM in 2021 and is currently indicated in patients who have undergone 4 or more prior lines of therapy. Phase II trial data showed an ORR of 73% in 128 patients, with 33% having a complete response or better. Median PFS was 8.8 months, and OS was not reached yet with a 15-month event-free rate of 71%. CRS was present in 84% of patients, although only 5% experienced grade 3 or higher. Neurotoxicity, another serious complication of CAR T therapy, occurred in 18% of patients with 3% being grade 3.[268]

Ciltacabtagene autoleucel (cilta-cel) was the second CAR T cell product approved for RRMM, acquiring FDA approval in 2022. Four-year follow up data from a phase I trial showed an ORR of 87.8% in 74 patients with complete response in 54 (73%). Median duration of response was 23.26 months, OS was not yet reached, and 24-month OS rate was 63.4% for all patients. CRS occurred in 91.9% of patients (9.5% grade 3 or higher). Only 1 patient experienced neurotoxicity[269]. A follow up phase Ib/II trial with 97 patients achieved an ORR of 97% with 65 patients (67%) having stringent complete response. Median duration of response

and PFS was not reached, and OS was 89% at 12-months. CRS occurred in 95% of patients (4% grade 3 or 4) and neurotoxicity occurred in 21% of patients (9% grade 3 or 4). Two patients unfortunately experienced grade 5/fatal adverse events, one due to CRS and one due to neurotoxicity[270].

In addition to cilta-cel and ide-cel, there are several other BCMA-specific CAR T cell products in various stages of pre-clinical and clinical development. Several of these have shown promising results thus far and are well summarized in ref[271].

iii. SLAMF7/CS1

Similar to CD38, SLAMF7 or CS1 is a glycosylated surface receptor highly expressed on multiple myeloma cells which also promotes myeloma growth[272, 273]. Expression is also present on other blood cells such as NK, CD8+ T, activated B, and dendritic cells. The exact role of SLAMF7 in multiple myeloma is less clear, however current data points towards facilitating adhesion to the bone marrow niche and acting as a growth signal through interactions with soluble SLAMF7, which also correlates with disease severity[274, 275].

Elotuzumab is the only clinically approved mAb against SLAMF7 and has undergone several clinical trials. Thus far the inclusion of elotuzumab alongside PI or IMiD has achieved the greatest results[276], whereas single agent use did not show efficacy[277]. In combination with IMiD/dex, elotuzumab significantly enhanced overall survival in RRMM[278, 279], but surprisingly did not improve treatment efficacy in newly diagnosed multiple myeloma[280, 281].

SLAMF7/CS1 is also a clinical target for anti-myeloma CAR T cell therapies. Thus far, CAR T cells against SLAMF7 have not demonstrated notable efficacy in early phase studies. A phase I dose-escalation trial with SLAMF7 CAR T cells (NCT03958656, results posted online) in 10 patients did not achieve any complete responses, with 1 partial response and 6 patients with stable disease. Despite the low efficacy, toxicities were minimal, and CRS only occurred in 1 patient at the highest dose group. Another ongoing trial investigating anti-SLAMF7 CAR T cells is CARAMBA-1 (NCT04499339), which utilizes the *Sleeping Beauty* transposon system as the method of T cell engineering rather than viral integration[282]. Data is not yet available from CARMBA-1.

Bispecific anti-CS1/BCMA CAR T cells featuring tandem scFv moieties in a phase I trial of 16 RRMM patients achieved an ORR of 81% (37.5 sCR) with an OS of 83.9% and PFS of 55.2% at 12-months follow-up. 6 patients (38%) experienced CRS, with 1 grade 3, and no neurotoxicity was recorded[283]. While these results are encouraging, they are in-line with outcomes from clinical studies of CAR T cells

that target BCMA alone[271]. Thus, it is unclear whether the CS1-targeting had any influence on clinical outcome.

iv. CD138

CD138 is a well-known myeloma antigen with elevated expression in myeloma cells compared to healthy plasma cells[284]. Although expression of CD138 is broader than the previously discussed targets, appearing within the GI tract and liver in addition to some NKT cells[285], it is tied to myeloma proliferation and survival, therefore also making it a valuable target as an anti-myeloma target.

CD138-directed therapies are comparatively less explored in clinical settings. Indatuximab ravtansine is an ADC which uses ravtansine (maytansinoid drug DM4), a drug shown to inhibit myeloma growth in pre-clinical studies[286]. In a phase I/IIa trial, indatuximab ravtansine had an ORR of 71.7% and 70.6% when combined with lenalidomide or pomalidomide, respectively, however adverse events of grades 3 or 4 lead to discontinuation of treatment in 55% of patients, highlighting potential risks to the drug.

A phase I trial of anti-CD138 CAR T cells showed permissible adverse events, but poor response with 4 of 5 patients achieving stable disease[287]. A single case report of a patient receiving anti-CD138 CAR T cells resulted in a partial response[288]. Other CD138 immunotherapies under testing include pre-clinical studies of a naked anti-CD138 mAb[289], CAR T, and CAR NK cells[290, 291].

v. GPRC5D

GPRC5D is an orphan receptor with little information on its function. Similar to BCMA, expression levels of GPRC5D in multiple myeloma are high and generally believed to be well-restricted to the plasma cell compartment, and expression correlates with poor prognosis[292-294]. GPRC5D is an attractive target due to its independent expression to other myeloma antigens, possibly providing use as a secondary target to in the case of relapse.

Talquetamab, a CPRC5D BiTE[295] displayed good tolerability in a phase I trial (MonumenTAL-1; ClinicalTrials.gov NCT03399799) of heavily pretreated patients, including those relapsed after BCMA CAR T therapy[296]. An ORR of 70% and median duration of 10.2 months were observed when delivered subcutaneously. CRS and skin-related adverse events were the primary toxicities observed and were not limiting.

MCARH109[294], an anti-CPRC5D CAR T cell therapy (ClinicalTrials.gov NCT04555551, NCT04674813) recently completed a phase I dose-escalation trial in heavily pretreated multiple myeloma patients, including those relapsed after BCMA CAR T therapy[297]. Overall response rate (ORR) achieved was 71% at all doses

and 58% at doses with acceptable toxicity profile. The highest dose range of $450x10^6$ CAR T cells resulted in unacceptable high-grade ongoing neurological toxicities in two patients likely due unexpected presence of antigen in the brain, and grade 4 CRS, neurotoxicity, and macrophage activation syndrome in a third. Nevertheless, at tolerable doses the results are promising as a treatment in the case of BCMA-treatment relapse.

The potential of engineered T cell therapies

Despite the impressive strides by immunotherapeutics to achieve deeper remissions, greater minimal residual disease rates, and reach those at greater risk, multiple myeloma remains relapsing and further research is required for this unmet need.

It is the opinion of this writer that engineered T cell therapies hold the greatest potential owing to not only the myriad of ways that genetic tools can augment the product itself, but by the combinatorial potential between these living drugs and other treatment modalities. Indeed, retrospective comparison of both cilta-cel and ide-cel versus real-world standard of care showed significantly greater ORR, OS, and PFS, highlighting the efficacy of these treatments[298, 299].

1.8 Limitations and next generation engineered T cells

Engineered T cell therapies have displayed impressive clinical outcomes, however there are several hurdles that limit the utility and potential of these treatments that must be overcome before greater adoption can occur. Next-generation engineering strategies and T cell extrinsic tools under investigation show promise for safer and more accessible treatment options.

Toxicities associated with engineered T cell therapies

The potency of engineered TCR or CAR T cells has been well-correlated with their adverse effects, a double-edged sword. Although clinicians are better aware and equipped to handle adverse events related to these therapies, the magnitude and extent of treatment-related toxicities is still a major issue. Toxicities can be classified as **i) off-target**, **ii) on-target, off-tumor**, and/or **iii) on-tumor**.

i. Off-target toxicities

Off-target toxicities occur when the antigen-binding domain has previously unknown cross-reactivity. Cross-reactivity has not been observed with synthetic antigen receptors utilizing mAb-based moieties, which may be due to the high affinity and specificity these molecules.

ii. On-target, off-tumor toxicities

The phenotypic heterogeneity of tumor cells presents a difficult challenge in antigen selection. With the exception of few tumor-restricted targets, most utilized tumor antigens are either highly overexpressed in cancers or are differentiation antigens on tissues deemed non-essential for survival. Thus, engineered T cell therapies often exhibit a manageable degree of off-tumor reactivity.

Many CAR T cell therapies under investigation have displayed on-target, off-tumor toxicities. The most prevalent is *B cell aplasia* seen in CD19 CAR T cells for B cell malignancies. Although a highly effective treatment, the consequence of using CD19 (a pan-B cell marker) as a target means all B cells will be targeted and patients may experience hypogammaglobulinemia[300, 301]. Fortunately, pooled i.v. immunoglobulin can be used effectively as treatment, and LLPCs maintaining humoral immunity can persist in these patients[302]. However, B cell recovery can take upwards of years and correlates with the persistence of CAR T cells[300].

Strategies utilizing inducible suicide genes or incorporating targetable sequences into the CAR design to eliminate engineered T cells by antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity are under clinical investigation to remove T cells in cases of severe toxicity[303, 304]. Another approach under investigation is to affinity tune CAR T cells to prevent high affinity interactions that may bind low target antigen expression on healthy tissues[305-308].

iii. On-tumor toxicities – cytokine release syndrome and neurotoxicity

Cytokine release syndrome and neurotoxicity (also known as immune effector cell associated neurotoxicity syndrome; ICANS[309]) are the most common CAR T cell-related adverse events and can lead to serious or fatal outcomes in patients.

CRS is a systemic inflammatory process that cascades from mass CAR T cell activation; GM-CSF, IFNγ, and TNFα released by CAR T cells activate other immune effectors such as macrophages, which leads to production of other inflammatory cytokines including IL-1, IL-6 and IL-8. Eventually high serum concentrations of these mediators lead to high grade fever, multi-organ toxicity, and even death[301, 310]. Guidelines for CRS management have been developed and clinicians expect and treat CRS accordingly[310]. Treatment consists of supportive care for lower grade CRS. Steroids, anti-IL-6R (tocilizumab)/anti-IL-6 (siltuximab), and transfer into the ICU are necessary for management of higher-grade CRS.

Neurotoxicity is comparatively less understood in both mechanism and management. Disruption of the blood brain barrier, increased permeability, and

transmission of cytokines into the cerebrospinal fluid are one postulated mechanism[309]. Neurotoxicity can manifest as encephalopathy, expressive aphasia, headache, seizures, motor-function disturbances, or other neurological complications[311]. As neurotoxicity tends to occur concurrent to or after CRS onset, treatment is primarily supportive with the use of steroids or immunosuppressants at higher grades.

These adverse effects are directly linked to CAR T cell function and efficacy[312], some studies have used dasatanib, a tyrosine kinase inhibitor that limits T cell activation, as a new pharmacological intervention for limiting toxicities[313-315].

Limitations and barriers to therapy with CAR T cells

Engineered T cells represent the cutting edge in cancer immunotherapy and like most new technologies there are logistical and systemic process challenges preventing access to treatment.

CAR T cell manufacture requires both personnel with expertise to generate the T cell products and GMP-facilities suitable for their production. These facilities must be within reasonable distance of the treatment center for timely transfer of materials and products. As one can imagine there are also substantial costs associated with CAR T cell therapy. In addition to the cost of the CAR T product itself, additional costs to the health care system are present for adverse event management, ICU stays, and the necessity of patients being within proximity of the treatment center (and thus accommodation costs).

In the US, mean costs for CAR T cell treatment have been estimated at greater than $500,000 USD[316]. Despite the cost, there are more multiple myeloma patients who would benefit from CAR T cell treatment than available capacity for CAR T cell manufacturing, which presents the ethical dilemma of who should receive treatment first. Unfortunately, a considerable number of patients progress, lose eligibility, or die before treatment slots are available[317].

Strategies to address some of these issues include the development of self-contained all-in-one bioreactor systems capable of producing engineered T cell products with minimal input from users, such as the Lonza Octane Cocoon or the Miltenyi CliniMACS Prodigy systems[318]. In Canada, CAR T cell treatment costs remain high despite greater bargaining power with single payer provincial systems. To provide greater access to patients, academic centers have trialed a program to generate and utilize non-commercial CAR T cells for treatment of CD19+ hematological cancers[319].

Finally, engineered T cell products currently utilize mostly autologous starting material. Leukapheresis products or peripheral blood are harvested from patients for engineering and *ex vivo* culture. However, the state of cancer patient T cells are often unhealthy and impacted by previous chemotherapy treatments[320]. These contribute to manufacturing fails and products which do not reach patients despite intent-to-treat[270]. Considerable research for allogeneic or "off-the-shelf" engineered T cell therapies is underway[321-323]. These strategies would bypass many issues with manufacture and proximity; however, they present their own issues and are beyond the scope of this document.

Resistance to CAR T cell treatment

Multiple myeloma patients who receive CAR T cell therapy continue to relapse despite deep responses, which include stringent complete responses and minimal residual disease negativity.

Current understanding of the mechanisms underpinning CAR T cell failure in multiple myeloma remain largely unknown. This is not unsurprising given the recency of anti-BCMA CAR T cell trials. Nevertheless, several mechanisms have been postulated and hold merit. Engineered T cell-intrinsic factors such as capacity to expand and persist are necessary for clinical activity and therefore are an active field of research [324-327]. It is not exactly clear why some patients treated with CAR T cells do not show T cell persistence. A leading hypothesis is that the memory state, expression of stem-related genes, and degree of differentiation of the T cells prior to manufacturing play pivotal roles[328-330].

Many CAR T cells utilize xenogeneic sequences, often in the form of animal-derived scFvs or nanobodies, which are immunogenic and can result in a host-mounted responses against the CAR T cell product[327, 331]. This can limit the duration and potential of CAR T cell therapies as they are eliminated from the system, particularly in multi-infusion dosing.

Finally, antigen loss or downregulation represents one of the greatest difficulties in cancer immunotherapy. BCMA downregulation, loss, or cleavage can render anti-BCMA CAR T therapies for multiple myeloma ineffective[332, 333] and can occur due to generation of mutations, selection of myeloma cells with weak BCMA expression, or through induction of γ-secretases which cleave BCMA into circulation. However, even in some cases of relapse with anti-BCMA treatments, there is no indication of BCMA loss[334].

Logic-gates: accounting for toxicity and antigen loss

Logic-gating multiple synthetic receptors is a promising avenue to push cellular engineering to refine anti-tumor responses and minimize relapse or

adverse events. The premise of logic gates is to integrate signaling from multiple synthetic receptors to better guide T cell activation and response[335].

This strategy allows fine tuning of tissue-targeting by selecting multiple antigens and assigning them to an activating or inhibitory role within the engineered T cell. Logic gates in engineered T cells fall under 3 logic purviews: **i) OR**, **ii) AND**, and **iii) NOT systems**.

i. OR-gated systems

In an OR-gated system, multiple synthetic receptors are expressed in parallel on a given T cells and signaling through any of them will lead to activation or downstream function in the engineered T cell *(ie. receptor 1 OR receptor 2 OR receptor 3…)*. The use of OR-gated receptors has been proposed as a method to reduce the risk of relapse by targeting multiple antigenic targets at once, thus antigen loss is less likely to occur. In multiple myeloma, this is under pre-clinical testing with SLAMF7/BCMA[336] or GPRC5D/BCMA[337] bispecific CAR T cells, and under clinical testing with CD38/BCMA bispecific CAR T cells[255].

ii. AND-gated systems

AND-gated systems have been predominantly investigated as a means to reduce toxicities and limit responses against healthy tissues. Using this strategy, signaling through several receptors is required to initiate T cell activation *(ie. receptor 1 AND receptor 2 AND receptor 3…)*. Separation of the CAR signaling domains into a dual receptor systems is one method to achieve this: the CD3ζ activation signal and costimulatory signal are split apart onto 2 receptors, and ligation of both is required to achieve optimal anti-tumor responses. In a proof-of-concept study, splitting the signals to two prostate cancer antigens, PSMA and PSCA, resulted in greater specificity[338]. An alternative approach is to restrict expression of the activating synthetic antigen receptor behind the ligation of one or more other receptors. In this AND-gated systems, recognition of one or more targets is required to express the final activating receptor as another method to prevent off-tumor toxicities[339-341].

iii. NOT-gated systems

NOT-gated approaches similarly focus on restricting activity to tumor tissues. However, in place of multiple receptors required for activation, a suppressive synthetic antigen receptor specific to a healthy tissue antigen transmits inhibitory signaling[342]. This strategy is effective in cases where the target tumor antigen is present on a second tissue type with a second antigen that differentiates it from the tumor cell[343, 344].

1.9 TCR-centric synthetic antigen receptors

CAR T cell therapies have demonstrated the potential that cancer immunotherapy holds, making significant strides in the treatment of relapsed/refractory hematological cancers and improving patient outcomes. However, associated toxicities and propensity for relapse are major problems in this field. While researchers continue to expend considerable work and resources to identify resolutions to these challenges, innovation should occur concurrently to these efforts.

It is hypothesized that the CAR design incorporating signaling domains results in constant signal from the CAR in the absence of antigen, known as tonic signaling, which promotes a state of exhaustion in the CAR T cells[345-347]. Furthermore, combined with the characteristic of CAR T cells to form non-classical synapses that result in rapid cytotoxicity[348], this tonic signaling may be a driving force behind CAR T cell toxicities. We and others believe a paradigm shift in the way we address synthetic antigen receptors is required to move forward from these challenges.

Synthetic antigen receptors that exploit signaling via the endogenous TCR should lead to a more natural regulation of T cell activation and employ canonical signaling pathways. We have developed a novel synthetic receptor, known as a T cell Antigen Coupler (TAC), as a method of engineering T cells with tumor specificity. The TAC is composed of two binding moieties – a tumor antigen-targeting scFv linked to a CD3ε-targeting scFv – which are anchored to the T cell with the CD4 co-receptor. Integration into the TCR/CD3 complex allows the TAC to redirect T cell-specificity while co-opting endogenous signaling machinery to deliver a natural TCR-signal. The TAC has shown notable efficacy in pre-clinical models of solid and hematological cancers[349], including BCMA+ multiple myeloma models[1, 2]. TAC T cells produce less inflammatory cytokines than CAR T cells and do not produce lethal toxicities that comparable 28ζ CAR T cells do in mouse models[349]. TAC T cells for HER2-positive cancers are currently in a phase I/II dose-escalation trial (NCT04727151).

Other groups have developed TCR-centric platforms since our initial publication. The AbTCR platform fuses antibody Fab fragments to the TCRγ and TCRδ chains. The use of the γδ chains prevents mispairing with endogenous TCRαβ chains. Pre-clinical testing of AbTCR T cells shows these cells maintaining similar anti-tumor efficacy compared to a CAR T cell, but remained less exhausted and produced less inflammatory cytokines[350]. In a phase I trial utilizing anti-CD19 AbTCR T cells for relapsed/refractory B cell lymphoma, the investigators observed an ORR of 83% (50% CR) amongst 12 patients who received a median of

2 T cell infusions (range 1-4). Mild CRS (grade 1 or 2) occurred in all 83% of patients (all who responded) after the first, but not any subsequent, T cell infusions. 1 patient (8%) experienced grade 3 neurotoxicity that resolved within 24 hrs of onset with steroid treatment[351].

The TruC platform is a recombinant protein that fuses a tumor-specific scFv to the CD3ε chain. The scFv-CD3ε incorporates into the natural TCR-CD3 complex upon expression and assigns bi-valent binding to the tumor target, conferring robust anti-tumor efficacy in pre-clinical work[352]. An anti-mesothelin TruC T cell product, gavocabtagene autoleucel, is currently under investigation in a phase I trial. Interim results of 7 patients (6 mesothelioma, 1 ovarian cancer) showed a 43% ORR (100% PR) with median OS of 86% at 6 months. Grade 3 or higher CRS occurred in 2 (29%) patients; no neurotoxicity was observed[353].

The STAR[354] and HIT[355] platforms are the latest TCR-centric approaches and both accomplish target-redirection by fusing scFv variable regions to the TCR α and β constant regions, which is similar in concept to the AbTCR. The STAR platform suffers from mispairing between the modified TCR chains and endogenous TCRαβ, therefore the authors utilized murine sequences to bypass this limitation. The HIT platform is noteworthy in that the authors employ CRISPR/Cas9 editing to insert their modified TCRα and TCRβ chains directly into the TRAC (TCRα heavy chain) locus reducing the potential for mispairing, placing the HIT receptor under control of the endogenous TCR promoter, and eliminating surface expression of endogenous TCR. An additional benefit to this approach is that alloreactivity is prevented due to replacement of the TCR, possibly opening the door to allogeneic approaches. Of interest is the observation that HIT and STAR receptors display greater antigen sensitivity than comparable CARs, which may be due to engagement of CD2[356], and can target low antigen-density tumors. While beneficial to capture antigen-low escape variants, there is potential for reactivity against host tissues and the authors note an AND-gate strategy would be required for the HIT receptor.

1.10 Thesis scope and objectives

Genetic tools and synthetic biology have heralded novel immunotherapeutic treatment options and regimes for cancer, including monoclonal antibody and engineered cellular platforms. CAR T cell strategies in particular have brought success to several hematological malignancies and are a point of hope and evolution within the field. Despite their relative infancy, research and development in this space is moving at a rapid pace to search for determinants of efficacy and further innovation.

The focus behind this body of work has been to push the envelope of engineered TAC T cell therapy for multiple myeloma further. Using BCMA-directed TAC T cells, I have investigated pharmacological and genetic strategies to augment the T cells for improved efficacy in pre-clinical studies. To do so, two objectives were pursued:

- Chapter 2 – Exploring the utility of LCL161, a SMAC mimetic, to enhance anti-BCMA TAC T cell functionality and survival by providing "druggable" costimulation of the TAC T cells.
- Chapter 3 – Strengthening anti-myeloma properties of TAC T cells through forced expression of IL-27, a pleiotropic cytokine that can enhance CTL activity and can modulate the myeloma microenvironment to suppress factors that contribute to disease pathogenesis.

Furthermore, two appendices cover work done to explore the biology and engineering of TAC T cells:

- Appendix I – CRISPR/Cas9 optimization and protocol generation to validate the requirement of TCR for TAC T cell activation. This method was used to address the roles of CD30 and Fas in Chapter 2.
- Appendix II – Exploring mRNA-engineering as an alternative to permanent T cell engineering by lentiviral vector. This work proved the futility of mRNA-engineering for the production of BCMA-specific TAC T cells and this method was not incorporated into either Chapter 2 or Chapter 3.

Chapter 2 – Utilizing LCL161, a SMAC mimetic, to enhance anti-BCMA TAC T cells

Introduction to Chapter 2

Traditional engineered T cells designs have incorporated costimulation directly into the synthetic antigen receptor (e.g. second generation CARs). Our lab's work with TAC T cells has suggested that non-canonical signaling via CARs may render CAR T cells hypersensitive to stimulation, leading to off-target activation[347, 349, 357](and unpublished data). Therefore, we have elected not to modify the structure of the receptor and to explore the use of small molecule drugs as remote activators of costimulation. This approach avoids the risk of disrupting the favorable biology of the TAC cells as a result of additional receptor engineering. Additionally, the use of small molecule drugs allows researchers temporal control of desired pathways for which compounds exist and are approved for clinical applications. In theory, with an appropriate toolbox of targeted agents that modulate T cell function, a base T cell product could be tailored to better suit individual indications. Here, we have explored the use of LCL161, a SMAC mimetic, as a tool to provide "on-demand" costimulation to TAC T cells through activation of non-canonical NF-κB signaling.

2.1 SMAC mimetics

Programmed cell death, also known as apoptosis, is a tightly regulated process with several redundant mechanisms and pathways. Inhibitor of Apoptosis (IAP) family members, including the mammalian cellular IAP1 and IAP2 (cIAP1 and cIAP2) and X-linked IAP (XIAP) proteins, are important nodes which serve to suppress activation of apoptosis[358].

IAP proteins repress the intrinsic (mitochondrial) and extrinsic (death receptor) apoptosis pathways by directly inhibiting caspase-3/7/9, and indirectly inhibiting caspase-8, respectively[359]. Direct inhibition of caspase is primarily mediated by XIAP, whereas cIAP1/2 play critical roles in modulating NF-κB and death receptor signaling from TNF superfamily receptors. At steady state, binding of TNFα to TNFR1 initiates a signaling cascade where the cIAP1/2 proteins ubiquitinate signaling intermediates to recruit the IKK complex, allowing translocation of the NF-κB transcription factor complex to the nucleus to initiate canonical NF-κB pro-survival gene expression[360-362]. However, in the absence cIAP1/2, IKK is not recruited and instead signaling through TNFR1 shifts to formation of complex-II which initiates caspase-8 signaling and ultimately leads to cell death[363-365] (Figure 2-1).

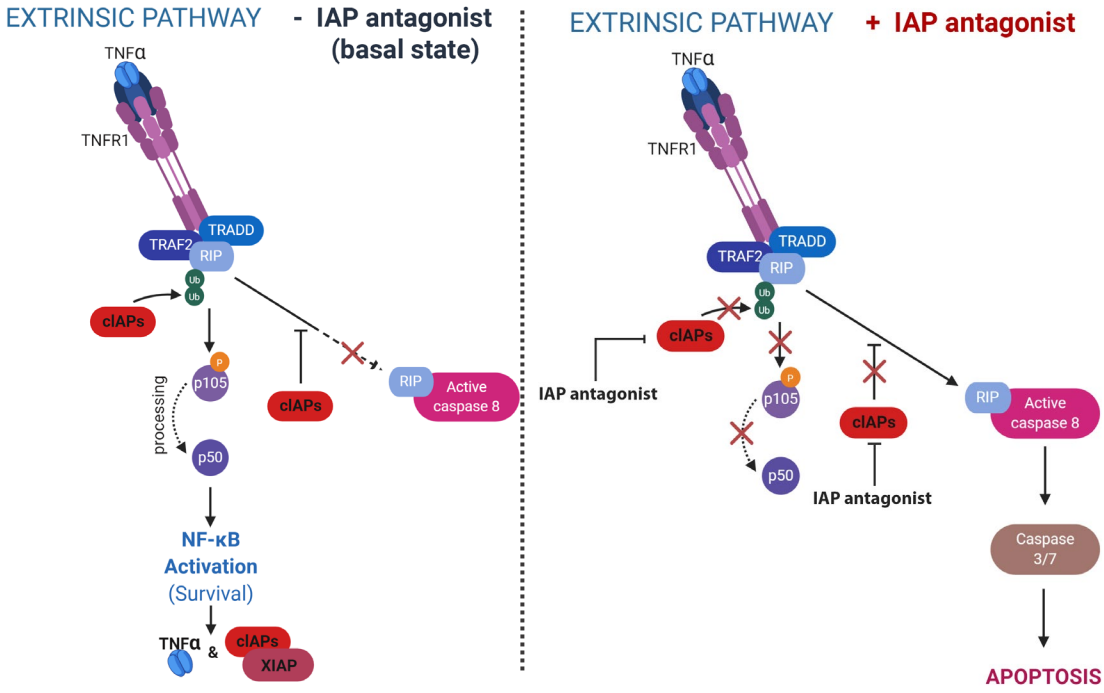

Figure 2-1. Survival and apoptotic signaling through TNF-signaling is affected determined by cIAP1/2 function.

IAP proteins are overexpressed in many cancer cells and function to maintain malignancy and survival[366, 367]. Thus, pharmaceutical suppression of IAP proteins is an area of active investigation which has led to the development of novel anti-cancer drugs. Second Mitochondrial Activator of Caspase (SMAC), also known as DIABLO (direct inhibitor of apoptosis-binding protein with low pI), is an inhibitor of IAP proteins that is released from the mitochondria in response to apoptotic stimuli[368]. SMAC binds to cIAP1 and cIAP2 to promote their autoubiquitination and degradation[369] and competitively binds to XIAP to prevent its interaction with caspase proteins[370]. These properties make SMAC an attractive template for drug development, and has resulted in the creation of drugs that mimic SMAC's biological function: novel small molecule drugs which contain one (monovalent) or two (bivalent) IAP binding motifs to duplicate SMAC function, known as SMAC mimetics[359].

Although SMAC mimetics are primarily being developed for their anti-neoplastic properties, increasing evidence highlights enhancement of anti-tumor immunity by these drugs[371-373]. Several reports have shown beneficial outcomes of combining SMAC mimetics with T cell therapies. As an example, Michie *et al.* recently demonstrated the combination of a SMAC mimetic with murine CAR T cells could sensitize tumor cells to CAR T cell-produced TNFα[374]. In another recent

pre-clinical study, CAR T cell anti-tumor activity was enhanced with birinapant, a bivalent SMAC mimetic, against heterogeneous glioblastoma targets[375]. The authors primarily contextualized the utility of birinapant in augmenting anti-tumor cytotoxicity through TNFα.

SMAC mimetics may also directly enhance T cell-intrinsic parameters such as survival or expansion[375]. At basal state, cIAP1 and 2 also mediate the ubiquitination and degradation of NF-κB-inducing kinase (NIK)[367, 368], which is required for activation of non-canonical NF-κB (ncNF-κB) signaling, an important pro-survival pathway in T cells[361, 366]. This pathway is also activated by TNF superfamily costimulatory receptors, such as OX40 and 4-1BB[376-378]. Inhibition of cIAP1 and cIAP2 relieves the negative regulation on NIK, leading to its accumulation and subsequent activation of the ncNF-κB pathway (Figure 2-2)[379, 380]. Thus, SMAC mimetics, through IAP antagonism, may serve as agents for ncNF-κB activation in T cells[371], providing important costimulatory effects to enhance T cell survival and function.

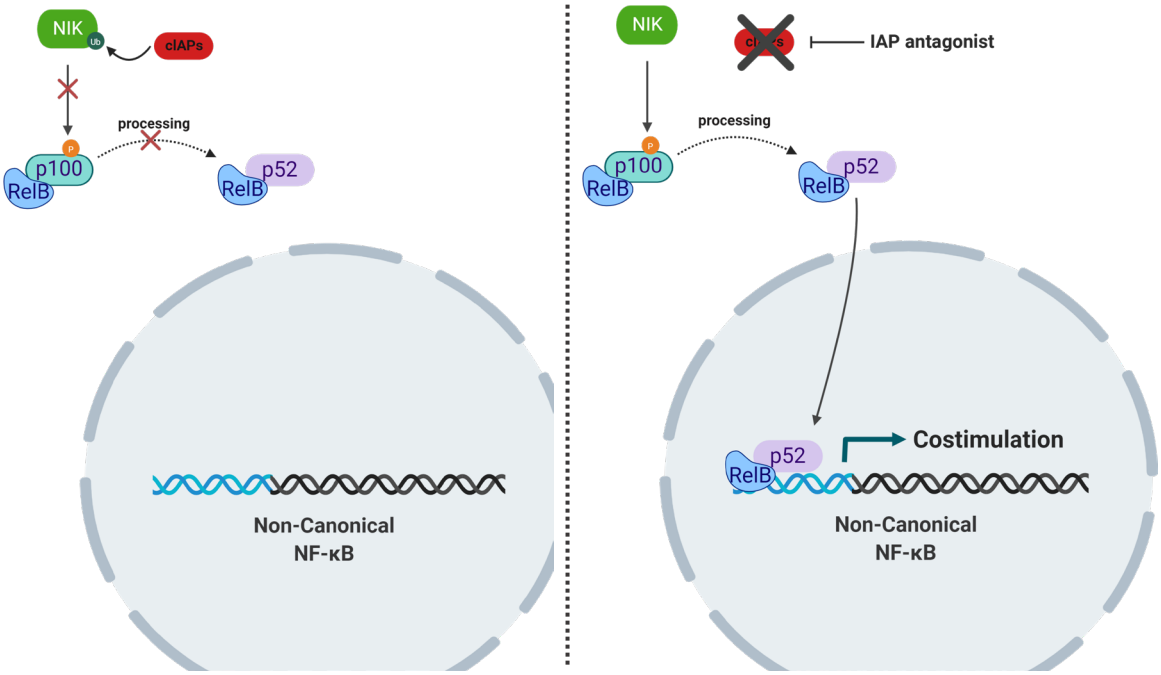

Figure 2-2. cIAP1/2 repress ncNF-κB activation by targeting NIK for degradation.

2.2 LCL161 as a druggable T cell costimulator

There are many SMAC mimetics in the pre-clinical and clinical pipeline[381]. LCL161 is an orally available, monovalent SMAC mimetic with pan-IAP inhibitory activity developed by Novartis. LCL161 has shown anti-tumor activity in pre-clinical studies, in particular when combined with other treatments or drugs[382-386].

As a single agent, the effects of LCL161 are typically reliant on TNFα where the drug can potentiate cell death in cells sensitive to its function[387, 388].

In the case of multiple myeloma, SMAC mimetics show evidence of anti-tumor utility but not through the induction of tumor cell apoptosis, although there is some activity in reducing tumor cell viability *in vitro*[389, 390]. Chesi et al. found the combination of LCL161 and cyclophosphamide sensitized multiple myeloma cells to type I interferon signaling and promoted activation of innate macrophages and dendritic cells for phagocytosis. Further combination with PD-1 blockade could cure mice bearing multiple myeloma [391]. Given the compelling work of Chesi and Bergsagel, which suggests that LCL 161 may be a useful immunostimulatory agent in multiple myeloma, and our interests in delivering transient costimulation to TAC T cells, we elected to explore its utility to impact the costimulatory pathways of engineered T cells as a prelude to possible studies of T cell therapy combined with LCL161. The primary aim of this objective is to investigate and characterize the effects of the SMAC mimetic LCL161 on engineered T cell biology, and their interaction with myeloma cells.

2.3 Manuscript status and citation

Status: Original research article, In review at Journal for the ImmunoTherapy of Cancer.

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LCL161 MANUSCRIPT

The SMAC mimetic, LCL161, provides costimulatory signals to engineered human T cells through CD30 and FAIM3-dependent mechanisms which are enhanced by Fas deletion

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Short Title: LCL161 as transient costimulation for engineered T cells

Immune Cell Therapies and Immune Cell Engineering

Keywords: engineered T cells, SMAC mimetics, LCL161, Multiple myeloma, cancer immunotherapy

DECLARATIONS

Ethics approval and consent to participate

All PBMCs were obtained from healthy and myeloma patient donors who provided informed written consent in accordance with the Hamilton Integrated Research Ethics Board. All animal studies were approved by the McMaster Animal Research Ethics Board.

Consent for publication

All authors reviewed the final manuscript prior to submission and approved the data and conclusions herein.

Availability of data and material

All data relevant to the study are included in the article, uploaded as online supplemental information, or available upon reasonable request from the corresponding author. Materials are available from the authors under a material transfer agreement.

Competing interests

JLB is a co-founder and shareholder of Triumvira Immunologics and is a paid consultant for Triumvira Immunologics. JLB is a co-inventor on patents related to the BCMA TAC described in this manuscript and licensed to Triumvira Immunologics. CLB is paid through a sponsored research agreement with Triumvira Immunologics. ECL is a co-founder, shareholder, and chief scientific officer of Protaxis Therapeutics.

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Authors' contributions

AA, SB, EL, and JLB conceptualized these studies. AA and JLB designed experiments and interpreted results. AA, CMS, AEM, CFG, CLB, MS, and KB generated these data and performed analyses. AA and JLB wrote this manuscript. SB, EL, and CFG edited the manuscript.

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List of abbreviations

SMAC: second mitochondria-derived activator of caspases

IAP: inhibitor of apoptosis

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

cIAP1/2: cellular IAP 1 or 2

TAC: T cell antigen coupler

FAIM3: Fas apoptotic inhibitory molecule 3

ACT: Adoptive cell therapy

TCR: T cell receptor

CAR: Chimeric antigen receptor

TNFRSF8: TNF receptor superfamily member 8

FCMR: Fc Mu receptor

CTV: CellTrace Violet

DMSO: Dimethyl sulfoxide

XIAP: X-linked IAP

ABSTRACT

Background The genesis of SMAC mimetic drugs holds roots in the observation that many cancers amplify IAP proteins to facilitate their survival, and that removal of these pathways would re-sensitize them towards apoptosis. It has become increasingly clear that SMAC mimetics also interface with the immune system in a modulatory manner. Suppression of IAP function by SMAC mimetics activates the non-canonical NF-κB (ncNF-κB) pathway and can augment T cell function, which opens the possibility of using SMAC mimetics to enhance immunotherapeutics.

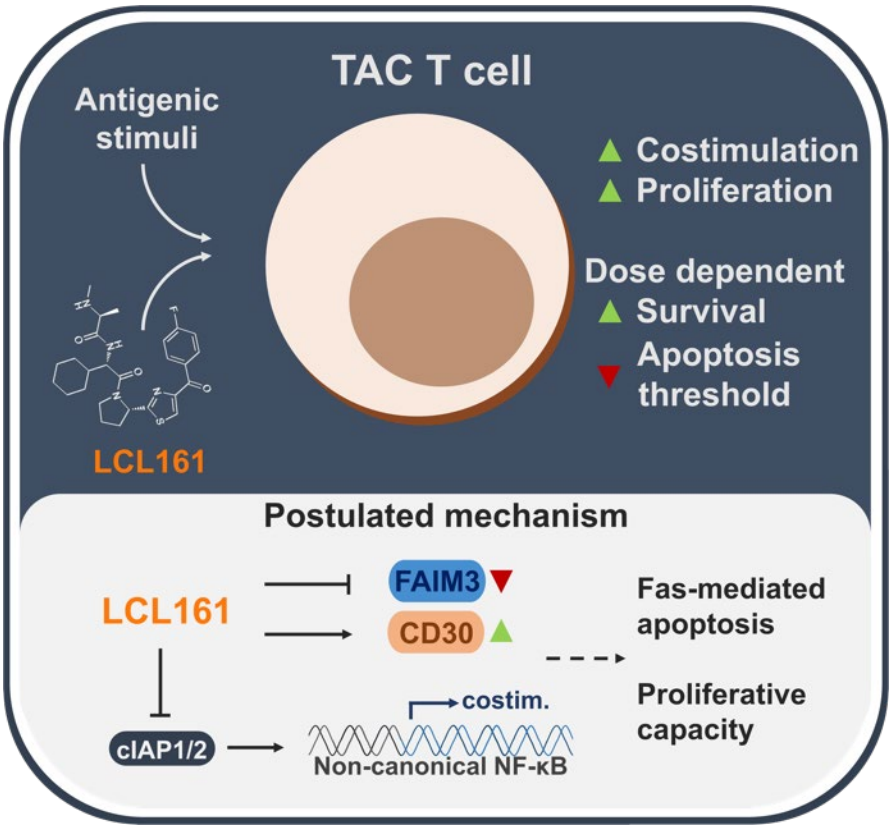
Methods We have investigated the SMAC mimetic LCL161, which promotes degradation of cIAP-1 and cIAP-2, as an agent for delivering transient costimulation to engineered BMCA-specific TAC T cells. In doing so we also sought to understand the cellular and molecular effects of LCL161 on T cell biology.

Results LCL161 activated the ncNF-κB pathway and enhanced antigen-driven TAC T cell proliferation and survival. Transcriptional profiling from TAC T cells treated with LCL161 revealed differential expression of costimulatory and apoptosis-related proteins, namely CD30 and FAIM3. We hypothesized that regulation of these genes by LCL161 may influence the drug's effects on T cells. We reversed the differential expression through genetic engineering and observed impaired costimulation by LCL161, particularly when CD30 was deleted.

While LCL161 can provide a costimulatory signal to TAC T cells following exposure to isolated antigen, we did not observe a similar pattern when TAC T cells were stimulated with myeloma cells expressing the antigen target. We questioned whether FasL expression by myeloma cells may antagonize the costimulatory effects of LCL161. Fas-KO TAC T cells displayed superior expansion following antigen stimulation in the presence of LCL161 suggesting a role of Fas-related T cell death in limiting the magnitude of the T cell response to antigen in the presence of LCL161.

Conclusions Our results demonstrate that LCL161 provides costimulation to TAC T cells exposed to antigen alone, however LCL161 did not enhance TAC T cell anti-tumor function when challenged with myeloma cells and may be limited due to sensitization of T cells towards Fas-mediated apoptosis.

GRAPHICAL ABSTRACT



What is already known on this topic – SMAC mimetics are novel anti-cancer agents known to enhance immune cell function; however, little is understood on how these drugs may impact T cell-based immunotherapies.

What this study adds – LCL161 provides a costimulatory signal to enhance TAC T cell function through a mechanism that involves CD30 and FAIM3, the high doses of drug that are required to augment T cell anti-myeloma activity may be deleterious to T cell survival.

How this study might affect research, practice or policy – As new immunotherapies emerge, our study provide insight into interactions that should be considered in combination therapies that include T cell-based modalities with SMAC mimetics.

BACKGROUND

T lymphocytes manifest robust anti-tumor capabilities, which has established them as a leading cellular candidate for adoptive cell therapy (ACT) of cancer. However, naturally occurring anti-tumor T cells are rare due to immunological tolerance mechanisms that prevent self-reactivity. Synthetic biology has overcome this issue through the development of synthetic antigen receptors that can be engineered into T cells to redirect them towards a chosen antigen. We have developed a TCR-based synthetic receptor designed to co-opt TCR signaling, known as the T cell antigen coupler (TAC), which yields potent anti-tumor T cells with reduced off-tumor toxicity[349]. The activation signal transduced through the TAC receptor results in robust cytotoxicity against tumor cells, cytokine expression, and cellular proliferation.

T cell activation can be enhanced with costimulatory signaling[202]. Many synthetic antigen receptors, like chimeric antigen receptors (CARs), have incorporated costimulation into the receptor for greater benefit[199, 392]. However, it remains unknown whether non-canonical signaling delivered through CARs, where the TCR signaling domain is physically linked to costimulatory signaling components, contributes to the serious toxicities associated with CAR-engineered T cell therapy[393]. The TAC receptor was purpose-built to deliver a canonical T cell activation signal and, hence, does not incorporate costimulatory elements into the receptor design. We recognize that provision of costimulation may enhance TAC T cell function and are pursuing chemical biology strategies to do so via small molecule drugs that would enable temporal and titratable costimulation without compromising the basal T cell state.

Second mitochondria-derived activator of caspases (SMAC) mimetics are relatively novel drugs under investigation as anti-cancer agents, and several have proven tolerability in clinical trials. These compounds regulate both intrinsic and extrinsic apoptosis pathways, which are dysregulated in many cancers[366, 373]. SMAC mimetics have been shown to impact NF-κB signaling pathways, resulting in T cell costimulation through non-canonical NF-κB (ncNF-κB) activation[371], and

subsequent increased T cell survival[210]. Current data indicates that combination of SMAC mimetics with anti-tumor T cells can result in combinatorial tumor cell killing through sensitization of tumor cells to TNFα[374, 375]. However, little research has been conducted on the impact of SMAC mimetics on the engineered T cell product themselves.

Here we have investigated the use of the monovalent SMAC mimetic LCL161 as an agent for delivering transient costimulation to anti-myeloma TAC T cells and to understand what subsequent changes are occurring within the T cells. We confirmed the costimulatory effects of LCL161 under conditions where TAC T cells are stimulated with antigen alone and observed enhanced survival and proliferation of T cells. However, these effects were not tied directly to the magnitude of ncNF-κB activation, and we further characterized the contributions of apoptosis-related proteins, CD30, FAIM3, and Fas, to the costimulatory outcomes. Under conditions where the T cells were co-cultured with myeloma cells, we failed to see an anti-tumor benefit of LCL161 except at doses that potentiate T cell death. Deletion of Fas enhanced the survival of T cells in the presence of LCL161 and lead to enhanced expansion. Thus, although LCL161 can provide costimulation to TAC T cells, these costimulatory properties did not enhance the anti-myeloma effect of non-edited TAC T cells.

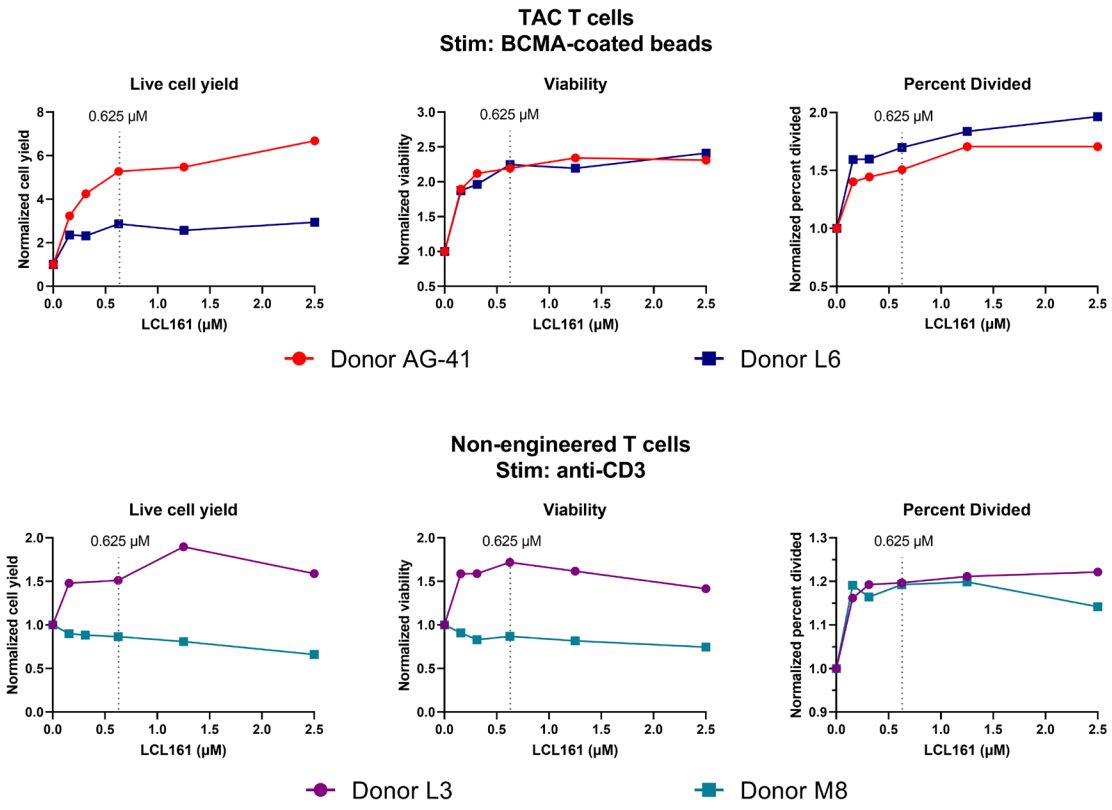
RESULTS

LCL161-mediated costimulation of TAC T cells

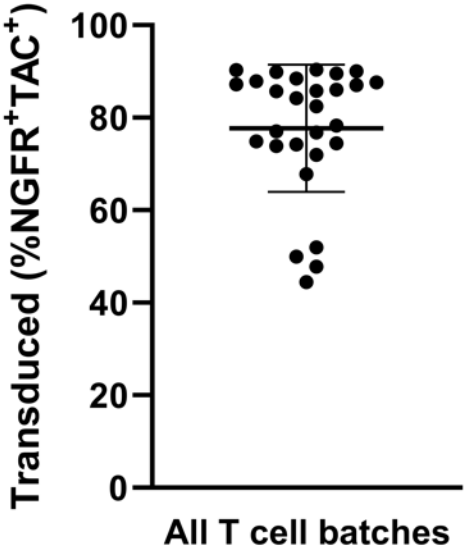
T cell costimulation is multi-faceted and results in several downstream functional changes. Proliferative capacity and survival are two key parameters augmented by costimulation that are positively associated clinically with T cell ACT[394, 395], and thus were used as our primary functional readouts. To characterize the costimulatory properties of LCL161, a dye-dilution proliferation assay using CellTrace Violet (CTV) was used. To determine the dose of LCL161 that provided optimal costimulation, we stimulated non-engineered T cells and engineered BCMA-specific TAC T cells[2] through the TCR/TAC receptor alone using plate-bound agonistic anti-CD3 or 6-7 μm diameter polystyrene microbeads coated with BCMA, respectively. Both non-engineered and TAC-engineered T cells demonstrated increased entry into division and increased cell yield and viability after stimulation in the presence of increasing concentrations of LCL161, which plateaued between 0.5-1 μM (Supplemental Figure 2-1). Therefore, we selected a concentration of 0.625 μM LCL161 for further experiments.

We noted donor variation in the degree of costimulation engendered by LCL161. Therefore, we generated TAC T cells from a series of healthy and myeloma donors to garner broad insights regarding the influence of donor on the

biological outcomes (Supplemental Figure 2-2, Supplemental Table 2-1). Treatment of T cells with LCL161 should initiate ncNF-κB signaling by conversion of NF-KB2 p100 precursor to the active p52 subunit[371]. TAC T cells were stimulated with plate-bound BCMA in the presence of 0.625 μM LCL161 or vehicle for 24 – 72 hours, after which the p52/p100 ratio were determined by western blot. The amount of immobilized BCMA was determined by serial dilution in a dye-dilution proliferation assay and a concentration of 0.05 μg/mL was chosen as it provided relatively weak stimulation to readily visualize LCL161 costimulation (data not shown). Stimulation with antigen and vehicle resulted in a limited increase in the p52/p100 ratio, whereas antigenic stimulation alongside LCL161 resulted in significantly greater conversion of p100 to p52 amongst all donors (Figure 2-3B) indicating activation of the signaling pathway. The relative conversion of p100 to p52 varied among the T cell products tested, indicating donor variability (Figure 2-3C).



Supplemental Figure 2-1. LCL161 enhancement of T cell proliferation plateaus at ~0.5 - 1 µM. $0.5x10^6$ CellTrace Violet-labeled TAC T cells generated from 4 donors were stimulated in the presence of increasing concentration of LCL161 for 96 hrs with either BCMA-coated microbeads or 1 µg/mL plate-bound agonistic anti-CD3 antibody. Cells were then collected and analyzed by flow cytometry and proliferation statistics were modeled using FCS Express software. These data represent 2 independent experiments.



Supplemental Figure 2-2. Overall transduction of engineered T cell batches. TAC T cells generated from healthy or myeloma patient donors were phenotyped for expression of the TAC receptor and tNGFR transduction marker. Data comprises 8 healthy donors and 3 myeloma patient donors. Cells were collected and stained for flow cytometric analysis either post manufacturing or post-thaw from cryopreservation. n=28.

Donor	Multiple myeloma stage
MM-16	3
HN-18	1
AG-41	1

Supplemental Table 2-1. Patient donor multiple myeloma staging. Cancer staging of multiple myeloma donors used for TAC T cell manufacturing at time of PBMC donation.

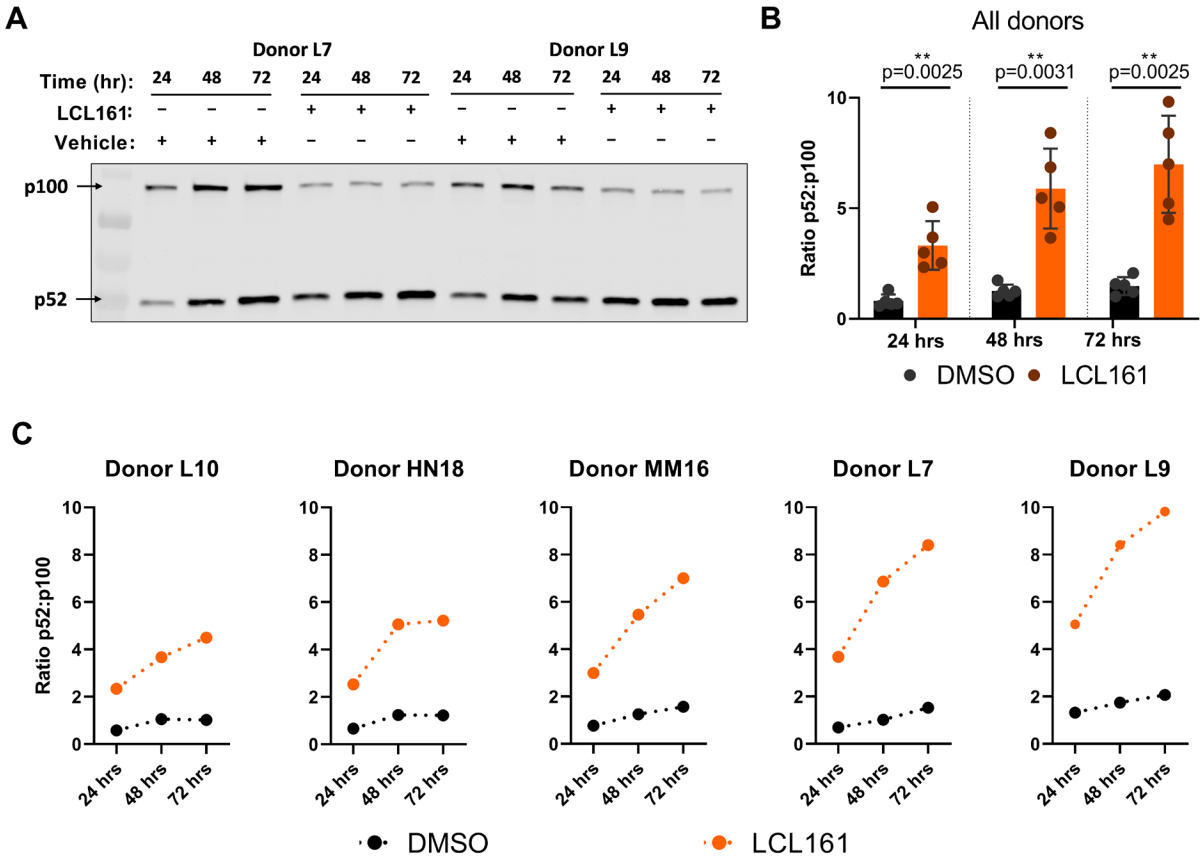


Figure 2-3. LCL161 activates the ncNF-κB signaling pathway in TAC T cells. $0.5x10^6$ TAC T cells were stimulated on plates coated with 0.05 ng/mL BCMA-Fc and either DMSO or 0.625 μM LCL161. Cells were collected and lysates were processed, ran on 4-20% polyacrylamide gels, and immunoblotted for NF-κB2 p100/p52. **A** Representative western blot indicating levels of NF-κB2 p100 and p52. **B-C** Signal quantification and normalization using Li-Cor Empiria software. NF-κB2 p100 and p52 signal was quantified and normalized to total protein loaded per lane and a ratio was calculated. **B** n=5. *= p<0.05, **=<0.01 as calculated by two-tailed paired t test. **C** data from individual donors in B. These data represent 2 independent experiments.

Given the variability in ncNF-κB signaling when TAC T cells from different donors were stimulated with target in the presence of LCL161, we assessed the proliferation and survival of TAC T cells from the donors shown in Figure 2-3. TAC T cells stimulated with BCMA-coated beads and 0.625 μM LCL161 displayed significantly greater viability, total live cell yield, and overall percentage of cells entering division than those stimulated with beads and vehicle alone (Figure 2-4A-B). While all donors displayed some enhancement in these parameters in the presence of LCL161, the effect again varied among the donors (Supplemental Figure 2-3). We examined the relationship between p100 to p52 conversion and the proliferation metrics by creating a simple proliferation composite score (p_c) that averages the three metrics we have examined ($p_c = \frac{viability+divided\%+cell\ yield}{3}$). We then compared this composite score to the area-under-the-curve of the p52:p100 ratio plots and found that there was no relationship between the absolute magnitude of NF-κB2 activation and the level of proliferation and survival enhancement delivered by LCL161 (Figure 2-4C, Supplemental Figure 2-4).

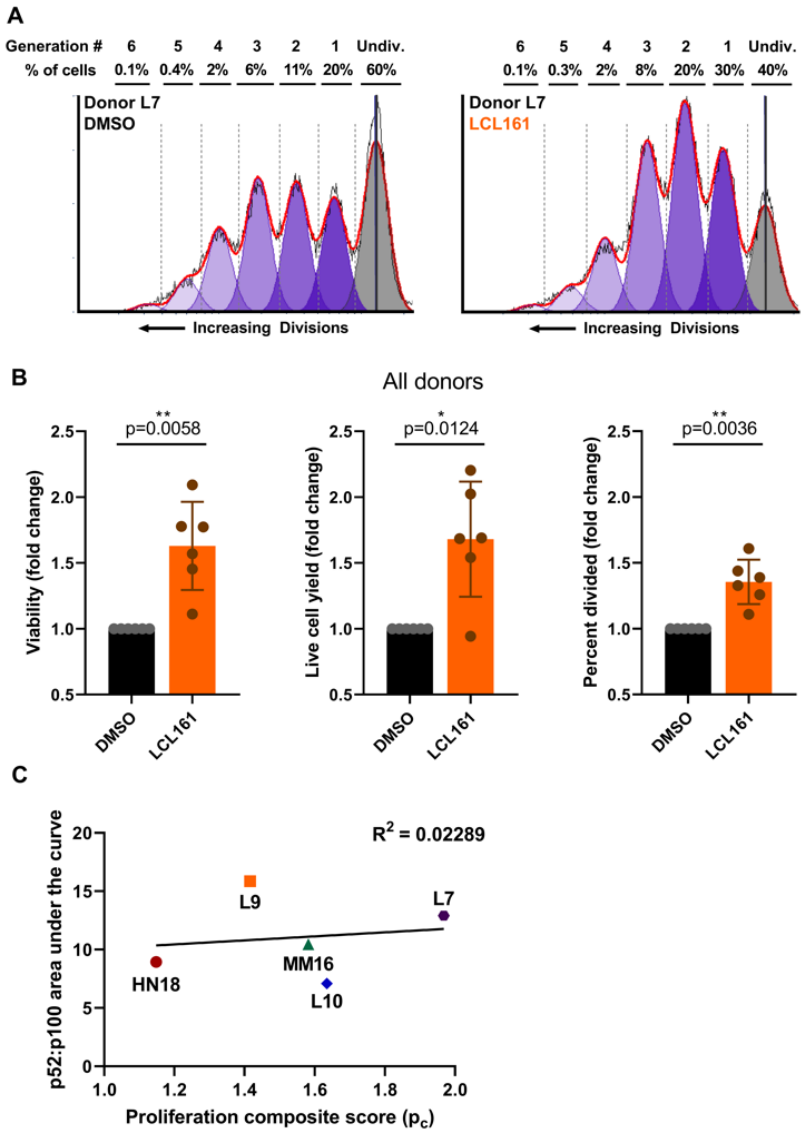
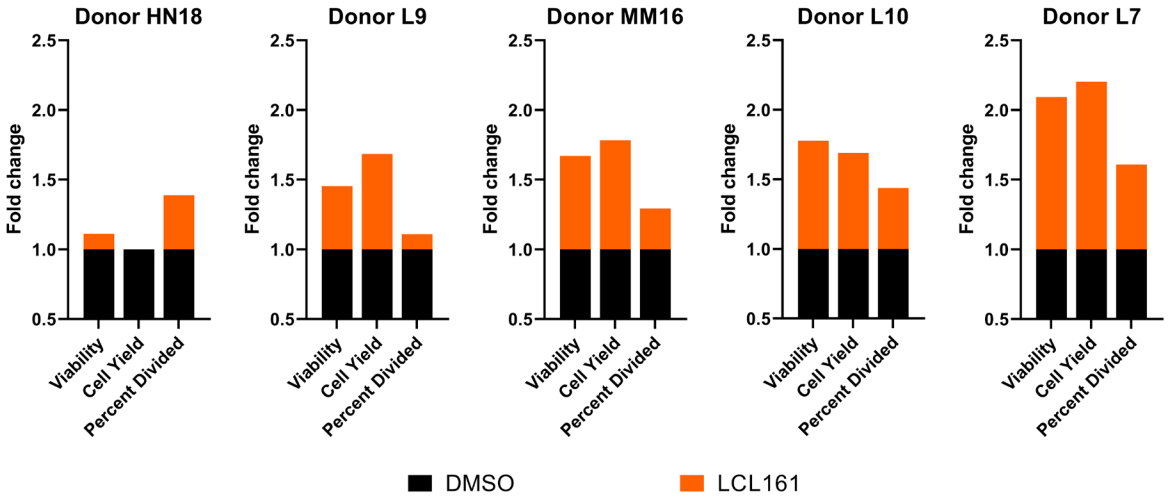
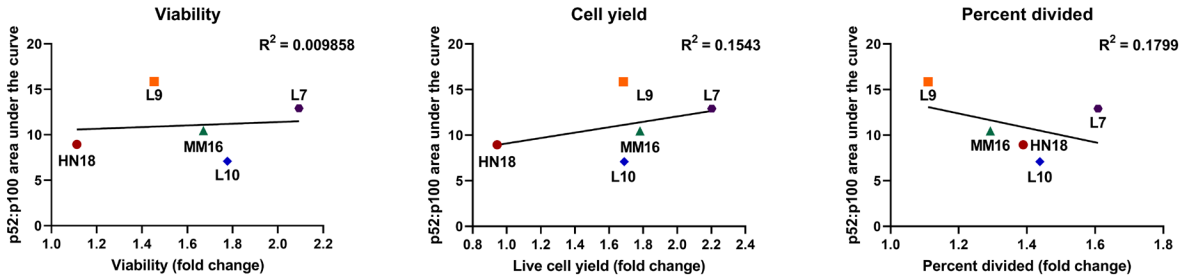


Figure 2-4. LCL161 treatment amplifies proliferation and enhanced survival of antigen-stimulated TAC T cells. CTV-labelled TAC T cells were stimulated with Protein G polystyrene beads loaded with 50 ng BCMA-Fc/million beads at an E:T of 1:1 for 96 hrs in the presence of 0.625 μM LCL161 or DMSO. T cells were harvested and stained for flow cytometric analysis. **A** representative flow plots with proliferation modeling, and **B** analyzed proliferation data from 5 donors, n=6. These data represent 3 independent experiments. C Composite proliferation score was calculated as the average of the three proliferation parameters and compared to the area under the curve of the p52:p100 ratio curves in Figure 2-3C. A simple regression was calculated to determine linear fit.



Supplemental Figure 2-3. Proliferation metrics in response to stimulation with antigen and LCL161 amongst donors. Protein G polystyrene beads were loaded with 50 ng BCMA-Fc/million beads overnight at 4°C while mixing. CTV-labelled TAC T cells were cultured with loaded beads at an E:T of 1:1 for 96 hrs. LCL161 was utilized at a concentration of 0.625 µM. T cells were harvested and stained for flow cytometric analysis. These data represent 3 independent experiments.



Supplemental Figure 2-4. There is no relationship between individual proliferation parameters to p52:p100 ratio area-under-the-curve. Area-under-the-curve values of the p52:p100 curves in Figure 2-3C were compared to the individual proliferation parameters calculated in Supplemental Figure 2-3.

CD30 and FAIM3 are implicated in the costimulatory role of LCL161

To gain insight into the mechanism(s) by which LCL161 influences T cell biology we performed transcriptional profiling on three donor (HN18, MM16, and L10) TAC T cells stimulated with BCMA-coated microbeads alongside LCL161 or vehicle for 24 or 72 hrs. After stimulation, RNA was collected and analyzed using the Clariom-S Pico human genechip, which covers >20,500 genes. We primarily observed differential expression of genes related to immune function and activation (Supplemental Figure 2-5). Of particular interest to us were two genes highly affected by LCL161: *FCMR* (encoding FAIM3) and *TNFRSF8* (encoding CD30) (Figure 2-5A). FAIM3[396, 397] and CD30[398-400] are both involved in regulation of costimulation and apoptosis pathways in T cells and contribute to a balance of survival and death signaling. We confirmed the transcript data by measuring the surface expression of CD30 and FAIM3 on TAC T cells following stimulation with antigen and increasing concentration of LCL161. Indeed, we observed that antigen-mediated stimulation of TAC T cells in the presence LCL161 resulted in up- and down-regulation of CD30 and FAIM3, respectively, in CD8+ TAC T cells (Figure 2-5B). A similar observation was seen with CD4+ TAC T cells (data not shown).

We sought to investigate the roles of these proteins in LCL161-mediated costimulation. We speculated that reversing the LCL161-mediated repression and induction of FAIM3 and CD30, respectively, would alter the costimulatory effect of the drug. To investigate how these receptors influence the proliferative capacity and survival of T cells during extended stimulation, we used genetic engineering in our BCMA TAC T cells. To force constitutive expression of *FCMR* (FAIM3 OE), we inserted a copy of the cDNA upstream of the TAC receptor in the lentiviral vector used to engineer the T cells (Figure 2-5C). To remove *TNFRSF8* expression (CD30 KO), we deleted the gene using CRISPR/Cas9 with a triple-gRNA approach (Figure 2-5D). *[For an in-depth optimization of CRISPR/Cas9 editing in human T cells using electroporation of sgRNA:Cas9 ribonucleoprotein (RNP), refer to* ***Appendix I****].* As a control, we exposed BCMA TAC T cells to a non-targeting gRNA/Cas9 RNP to account for any effect of gRNA/Cas9 or electroporation on the T cells. We used genotyping to determine CD30 gene-disruption (Figure 2-5E) and phenotypic analysis to determine CD30 absence and FAIM3 forced surface expression (Figure 2-5F-G). To do so, we stimulated TAC T cells with anti-CD3 and high dose LCL161 to maximally induce CD30 and repress FAIM3. Under the effects of LCL161 we noted a marked reduction in CD30 expression on the cell surface of the gene-edited TAC T cells compared to controls (Figure 2-5F), although we were unable to achieve complete knock down of CD30 despite extensive optimization of the knock-out protocol. Similarly, forced expression of FAIM3 was successful

and surface expression was maintained in the presence of LCL161 during stimulation (Figure 2-5G). Next, we evaluated the effects of CD3O knockout and/or constitutive FAIM3 on TAC T cell proliferation and survival following stimulation with antigen-coated microbeads in the presence of LCL161. Disruption of CD30 had a profound negative impact on the proliferative capacity of TAC T cells in multiple donors, even though we did not achieve complete disruption of the CD30 gene (Figure 2-5H). The effect of FAIM3 was variable; constitutive expression of FAIM3 impaired cell expansion of TAC T cells generated from donors L10 and L3 but had no impact on TAC T cells derived from donor L6. The combination of CD30-disruption and forced expression of FAIM3 also yielded variable outcomes as the combination further impaired the cell yield from Donor L10 TAC T cells, revealed no combinatorial effect with TAC T cells from Donor L3 and, in the case of TAC T cells from Donor L6, overexpression of FAIM3 counteracted the effects of CD30 deletion. These results demonstrate that the costimulatory effects of LCL161 on TAC T cells are CD30-dependent, while the role of FAIM3 in LCL161-mediated costimulation is variable and donor-dependent.

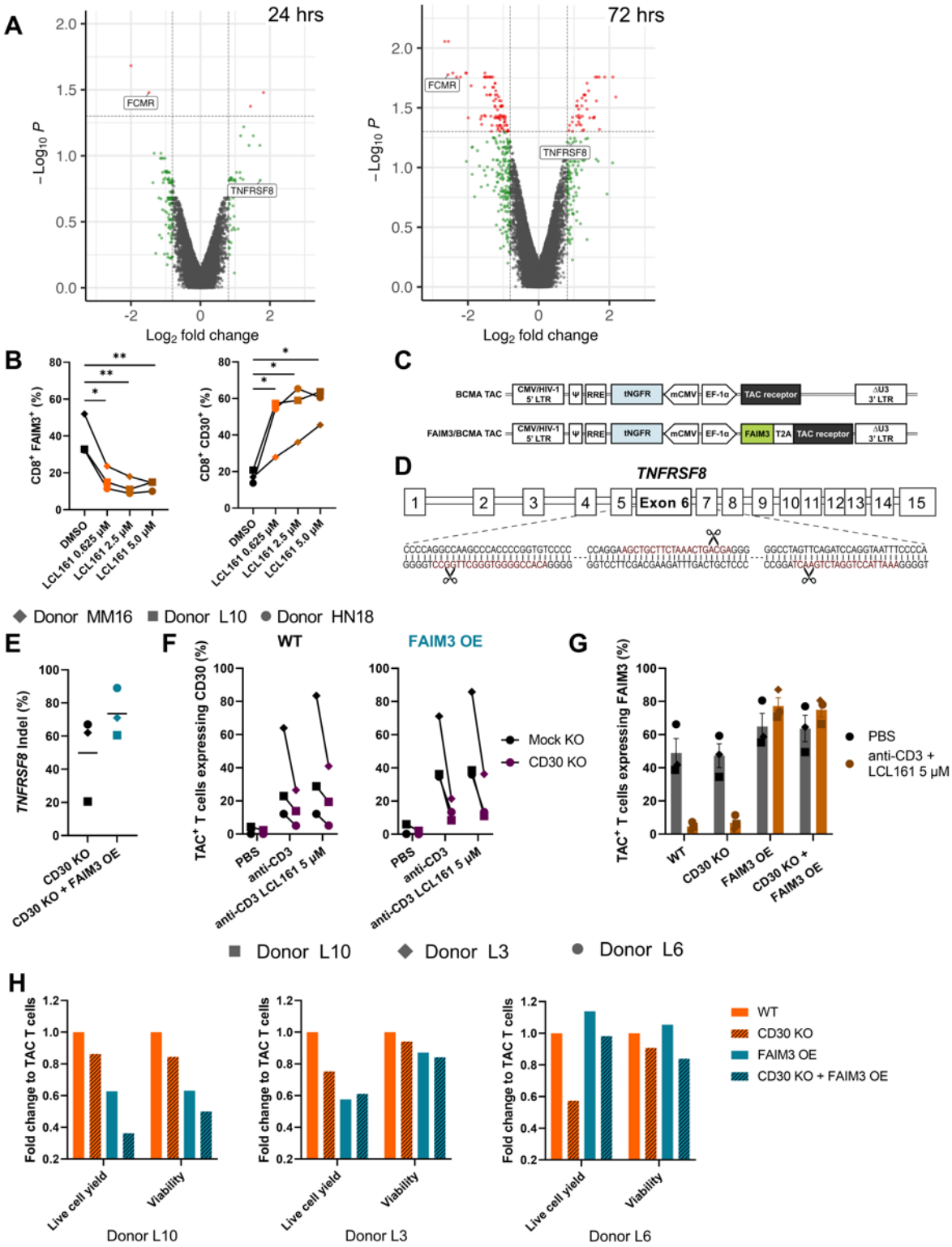
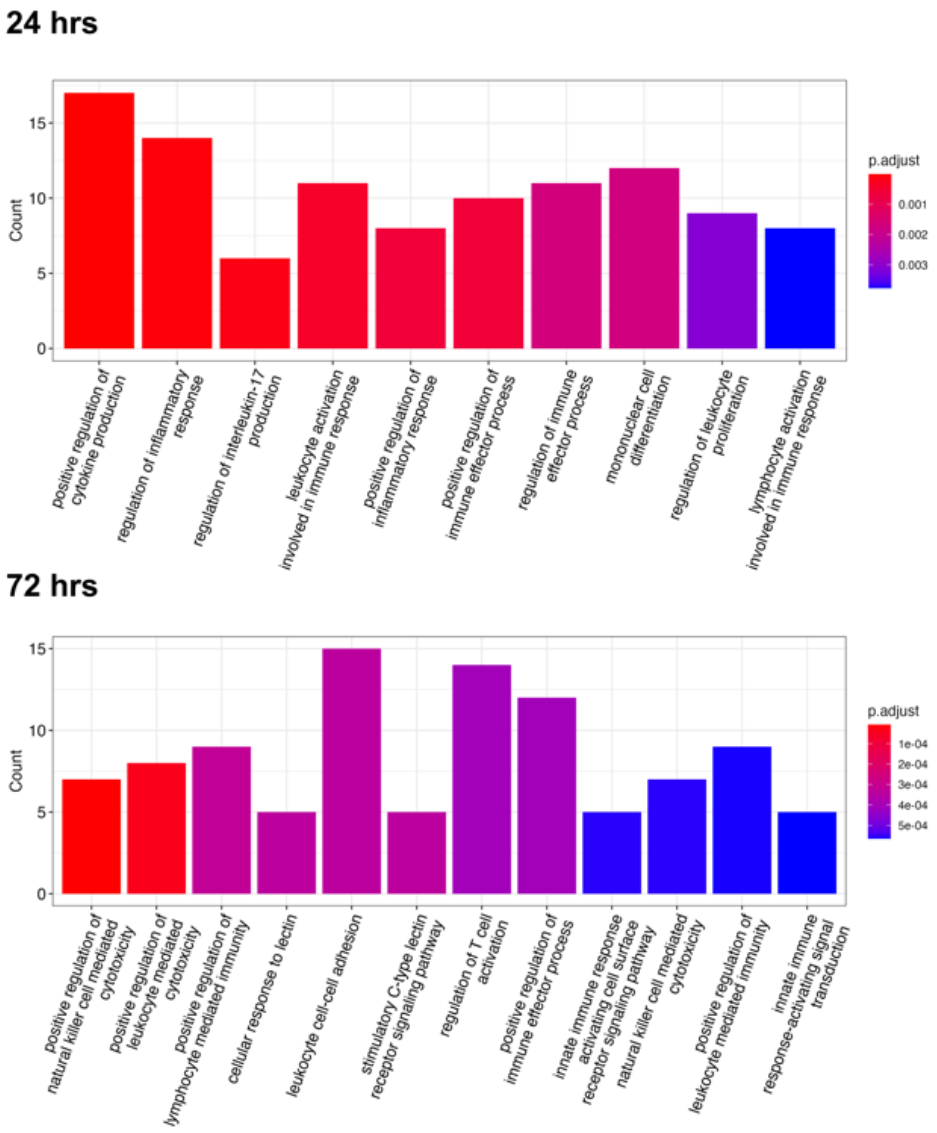

A
24 hrs
72 hrs
FCMR
TNFRSF8
−Log10 P
Log2 fold change
B
CD8+ FAIM3+ (%)
CD8+ CD30+ (%)
DMSO
LCL161 0.625 µM
LCL161 2.5 µM
LCL161 5.0 µM
C
BCMA TAC
FAIM3/BCMA TAC
CMV/HIV-1 5' LTR
Ψ
RRE
tNGFR
mCMV
EF-1α
TAC receptor
FAIM3
T2A
ΔU3 3' LTR
D
TNFRSF8
Exon 6
Donor MM16
Donor L10
Donor HN18
E
TNFRSF8 Indel (%)
CD30 KO
CD30 KO + FAIM3 OE
F
WT
FAIM3 OE
TAC+ T cells expressing CD30 (%)
Mock KO
CD30 KO
PBS
anti-CD3
anti-CD3 LCL161 5 µM
G
TAC+ T cells expressing FAIM3 (%)
PBS
anti-CD3 + LCL161 5 µM
WT
CD30 KO
FAIM3 OE
CD30 KO + FAIM3 OE
Donor L10
Donor L3
Donor L6
H
Fold change to TAC T cells
Live cell yield
Viability
Donor L10
Donor L3
Donor L6
WT
CD30 KO
FAIM3 OE
CD30 KO + FAIM3 OE

Figure 2-5. FAIM3 and CD30 mediate, in part, the costimulatory mechanisms of LCL161 in enhancing TAC T cell proliferation. A Cryopreserved TAC T cells were thawed into complete media, rested for 24 hrs, then $5x10^5$ T cells were stimulated by 0.05 µg/mL plate-bound BCMA antigen for 24 or 72 hrs in the presence of DMSO or LCL161. After stimulation, cells were collected, homogenized, and RNA was collected. Transcripts were assessed using a Clariom-S Pico human genechip. n=3, 1 per donor (HN18, L10, MM16). Volcano plots of the 24- and 72-hour Clariom data were generated using a p-value threshold of 0.05 (red dots) and fold change of 1.75 (red or green dots). **B** Thawed and rested cryopreserved TAC T cells were stimulated for 72 hrs with BCMA-coated microbeads and LCL161 or vehicle. Cells were collected, stained, and analyzed by flow cytometry for FAIM3 and CD30 expression. **C** Schematic diagram of FAIM3 cDNA separated by a T2A sequence upstream of the TAC receptor within the lentiviral vector. **D** *TNFRSF8* was deleted by triple gRNA editing with CRISPR/Cas9, schematic indicates the sequence of gRNA (in red) and cut sites on the targeted exon. **E-H** BCMA TAC T cells ± FAIM3 overexpression (OE) were cultured as normal and edited on day 3 with CD30 multi-guide or negative control gRNA/Cas9 RNP by Neon electroporation. T cells were then cultured until day 14-15 and utilized fresh in assays. **E** TAC T cell genomic DNA was collected on day 13-14 of culture by extraction with Lucigen DNA extraction solution. The *TNFRSF8* exon 6 locus was amplified by PCR, sequenced by sanger sequencing, and indel frequency was computed using Synthego ICE. **F-G** BCMA TAC T cells ± FAIM3 OE were stimulated using 1 µg/mL plate-bound agonistic anti-CD3 antibody ± 5 µM LCL161 for 24 hrs (donor L10) or 72 hrs (donors L3 and L6). After stimulation T cells were collected and stained for **F** CD30 and **G** FAIM3 expression and analyzed by flow cytometry. **H** CTV-labelled TAC T cells were stimulated with 0.625 µM LCL161 and BCMA-coated microbeads at an E:T 1:1 for 96 hrs then analyzed by flow cytometry. Data was modeled using FCS Express 7 and values were normalized to wildtype TAC T cells. n=3. The data in panel B represent 1 independent experiment. The data in panels E – H represent 3 independent experiments.



Supplemental Figure 2-5. Gene-signature enrichment analysis of LCL161-differentially expressed genes. Bar plots were generated from the results of an over-representation analysis using the gene ontology (GO) database with the 24- and 72-hour results. The GO analysis was done using the biological processes (BP) ontology with the Benjamin Hochberg (FDR) p-value adjustment. An adjusted p-value threshold of 0.01 was used to identify significant GO terms.

LCL161-mediated costimulation impairs TAC T cell expansion in co-culture with myeloma cells.

To assess whether LCL161 could provide costimulatory benefit when TAC T cells were co-cultured with myeloma cells, we stimulated T cells with BCMA+ MM.1S myeloma tumor cells in the presence or absence of 0.625 μM LCL161. The absolute proliferative response of the TAC T cells to the myeloma cell line was much more robust than the antigen-coated beads (Figure 2-6A; data from Figure 2-4 is included as a point of reference). Although MM.1S stimulation gave an overall stronger proliferative signal, the combination of LCL161 and MM.1S stimulation resulted in reduced expansion of the TAC T cells, which manifest as a decrease in TAC T cell numbers at the end of the co-culture, and there was no augmentation of survival or cells entering division as previously observed with bead stimulation (Figure 2-6B). These data reveal that while LCL161 costimulation was important when TAC T cells were stimulated with antigen alone, there may be opposing signals delivered by the tumor cells which mitigate the beneficial impacts of the LCL161.

T cell expansion can be limited by Fas-mediated death[401], and the reduced apoptosis threshold as a result of LCL161 targeting of IAPs may underpin the negative effects of LCL161 in the context of the T cell myeloma cell cultures. Indeed, we confirmed that the tumor lines used in this study uniformly expressed FasL (Figure 2-6C). To address the possibility that Fas signaling mitigates the beneficial effects of LCL161, we removed Fas from the TAC T cells by CRISPR-mediated gene-editing. Here, we reproducibly achieved a high efficiency KO at >80% in all cases (Figure 2-6D). The removal of Fas in the TAC T cells enhanced total cell yield, viability, and cells entering division of TAC T cells in the presence of 0.625 μM LCL161 when T cells were stimulated with antigen alone (“Beads”; Figure 2-6E). This effect was greater in cells treated with LCL161 rather than DMSO. Furthermore, we observed that the incidence of apoptotic T cells was reduced following stimulation with antigen alone in the presence of LCL161 when Fas was disrupted, supporting a role for Fas in limiting the costimulatory effects of LCL161, presumably through fratricide (“Beads”; Figure 2-6F). Removal of Fas from the TAC T cells did improve cell yield following stimulation with MM.1S, but did not impact viability, cell division or incidence of apoptosis in LCL161 treated T cells compared to DMSO (“MM.1S”; Figure 2-6E-F).

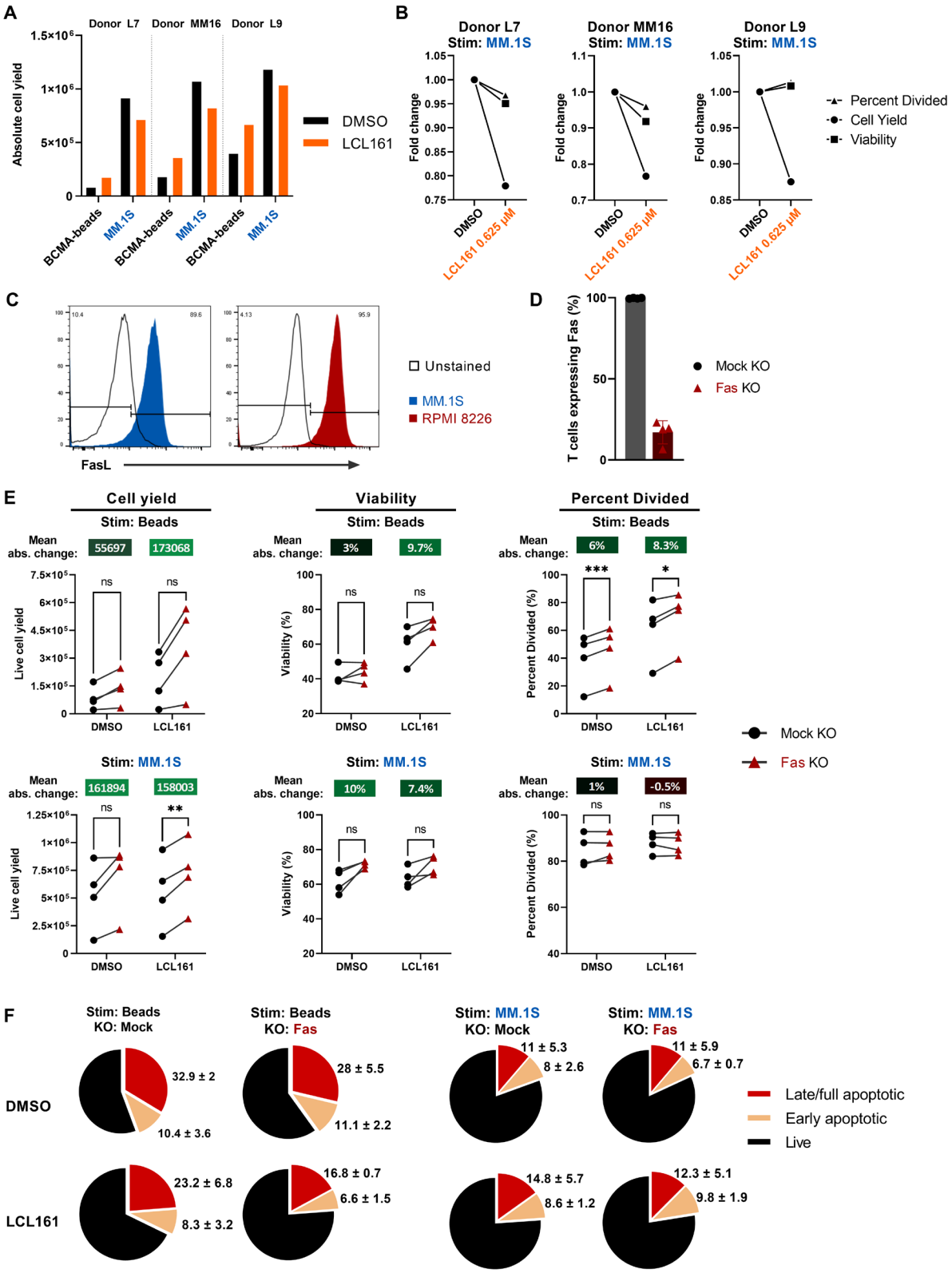

A
Donor L7
Donor MM16
Donor L9
Absolute cell yield
DMSO
LCL161
BCMA-beads
MM.1S
B
Donor L7
Stim: MM.1S
Donor MM16
Stim: MM.1S
Donor L9
Stim: MM.1S
Fold change
DMSO
LCL161 0.625 µM
Percent Divided
Cell Yield
Viability
C
Unstained
MM.1S
RPMI 8226
FasL
D
T cells expressing Fas (%)
Mock KO
Fas KO
E
Cell yield
Viability
Percent Divided
Stim: Beads
Mean abs. change:
55697
173068
3%
9.7%
6%
8.3%
Live cell yield
Viability (%)
Percent Divided (%)
ns
***
*
Stim: MM.1S
161894
158003
10%
7.4%
1%
-0.5%
**
F
Stim: Beads
KO: Mock
Stim: Beads
KO: Fas
Stim: MM.1S
KO: Mock
Stim: MM.1S
KO: Fas
DMSO
LCL161
32.9 ± 2
10.4 ± 3.6
28 ± 5.5
11.1 ± 2.2
11 ± 5.3
8 ± 2.6
11 ± 5.9
6.7 ± 0.7
23.2 ± 6.8
8.3 ± 3.2
16.8 ± 0.7
6.6 ± 1.5
14.8 ± 5.7
8.6 ± 1.2
12.3 ± 5.1
9.8 ± 1.9
Late/full apoptotic
Early apoptotic
Live

Figure 2-6. LCL161 does not enhance TAC T cell survival and proliferation when co-cultured with myeloma cells *in vitro* and is independent of Fas. A-B Thawed and rested cryopreserved BCMA TAC T cells were stimulated 1:1 with MM.1S tumor cells or BCMA-coated microbeads in the presence of 0.625 μM LCL161 for 96 hrs. Absolute cell count was enumerated using counting beads during flow cytometric analysis. The bead proliferation data displayed in *A* is shared in Figure 2-2B. **B** Proliferation metrics from TAC T cells stimulated with MM.1S cells were normalized to the vehicle control. **C** Cultured MM.1S and RPMI 8226 myeloma cells were stained for FasL expression and analyzed by flow cytometry. **D-F** BCMA TAC T cells were cultured as normal and edited on day 3 with Fas multi-guide or negative control gRNA/Cas9 RNP by Neon electroporation. T cells were then cultured until day 14-15 and utilized fresh in assays. n=4. **D** BCMA TAC T cells edited with mock or Fas RNP were stained for Fas expression on day 13-14 and analyzed by flow cytometry. **E** Mock or Fas-edited TAC T cells were stimulated 1:1 with MM.1S tumor cells or BCMA-coated microbeads and in the presence of 0.625 μM LCL161 or vehicle for 96 hrs. After stimulation, samples were stained, mixed with counting beads, and analyzed by flow cytometry. Proliferation metrics were modeled using FCS Express 7 software. Mean absolute change between mock and Fas KO is reported above each condition. **F** Mock or Fas-edited TAC T cells were stimulated 1:1 with MM.1S tumor cells or BCMA-coated microbeads and in the presence of 0.625 μM LCL161 or vehicle for 72 hrs. After stimulation, cells were stained for live/dead, fixed, and finally stained for phosphatidylserine (PS) on the outer membrane. Cells were analyzed by flow cytometry. Early apoptotic cells were defined as live/dead$^-$, PS+, late/full apoptotic cells were defined as live/dead+, PS+. Pie charts represent mean values, with mean + standard deviation written outside the pie slice. n=3. These data represent 3 independent experiments.

LCL161 enhancement of TAC T cell anti-tumor cytotoxicity is accompanied by T cell toxicity

Although we did not observe enhanced proliferation or survival on TAC T cells stimulated with myeloma tumor cell lines *in vitro*, we wondered if LCL161 would augment T cell-mediated cytotoxicity against myeloma cells. To this end, TAC T cells were co-cultured with myeloma cells in the presence of LCL161 and tumor viability was assessed. Enhanced killing of the tumor cells was only observed with high concentrations of LCL161 tested (5 μM; Figure 2-7A). In fact, a small change in concentration to 2.5 μM failed to provide enhanced killing (Figure 2-7B). Inhibition of the IAPs in tumor cells is predicted to result in increased sensitivity to TNF-mediated cell death by switching TNFα signaling from activation of the classical NF-κB pathway towards caspase-8 mediated apoptosis[365, 402], and we have observed that TAC T cell produce large amounts of TNFα upon stimulation by myeloma cell lines (Figure 2-7C). To address whether LCL161 augments TAC T cell killing through sensitization of the multiple myeloma tumor cells to TNFα, we cultured MM.1S or RPMI 8226 cell lines with 5 μM LCL161 +/- recombinant human TNF. MM.1S cells displayed a modest increase to cell death when treated with both LCL161 and TNF, whereas RPMI 8226 cells remained largely unaffected (Figure 2-7D). This was not entirely unexpected as there is considerable heterogeneity in susceptibility of MM cell lines to LCL161[389], which may be in part be due to deletions of cIAP1/2 or other NF-κB-associated factors that occur in some multiple myelomas[391, 403]. Investigating these two cell lines further showed a complete basal absence of cIAP2 and p100 in the RPMI 8226 cells (Supplemental Figure 2-6), which may explain the lack of sensitization to TNFα. Thus, the increased killing of the myeloma cell lines in the presence of higher concentration of LCL161 (5 μM) is likely due to something other than sensitization of TNFα-mediated killing.

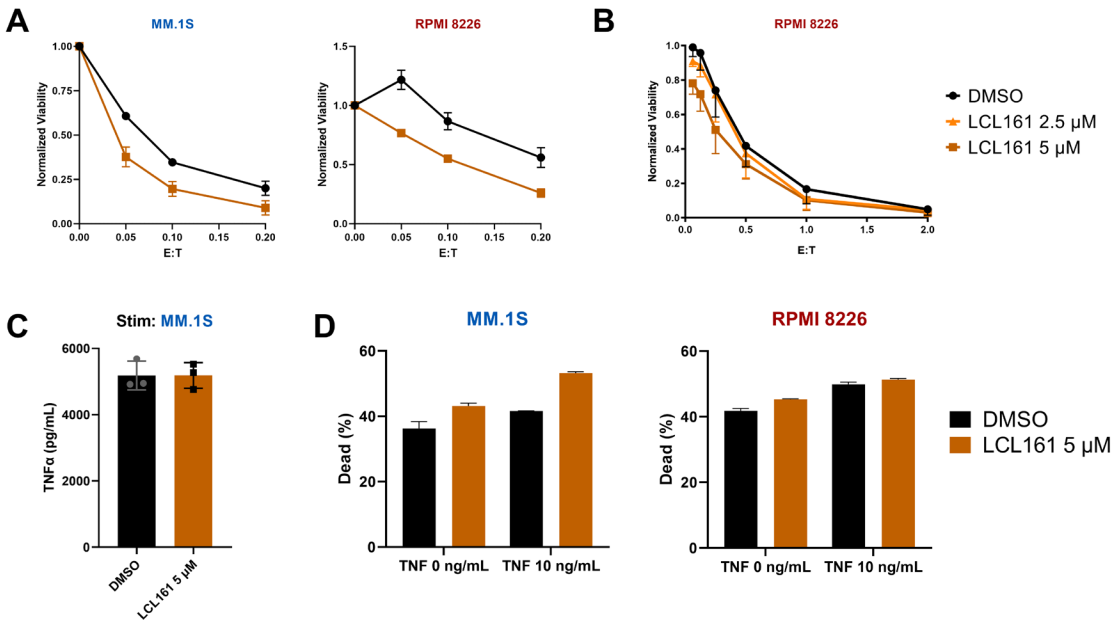
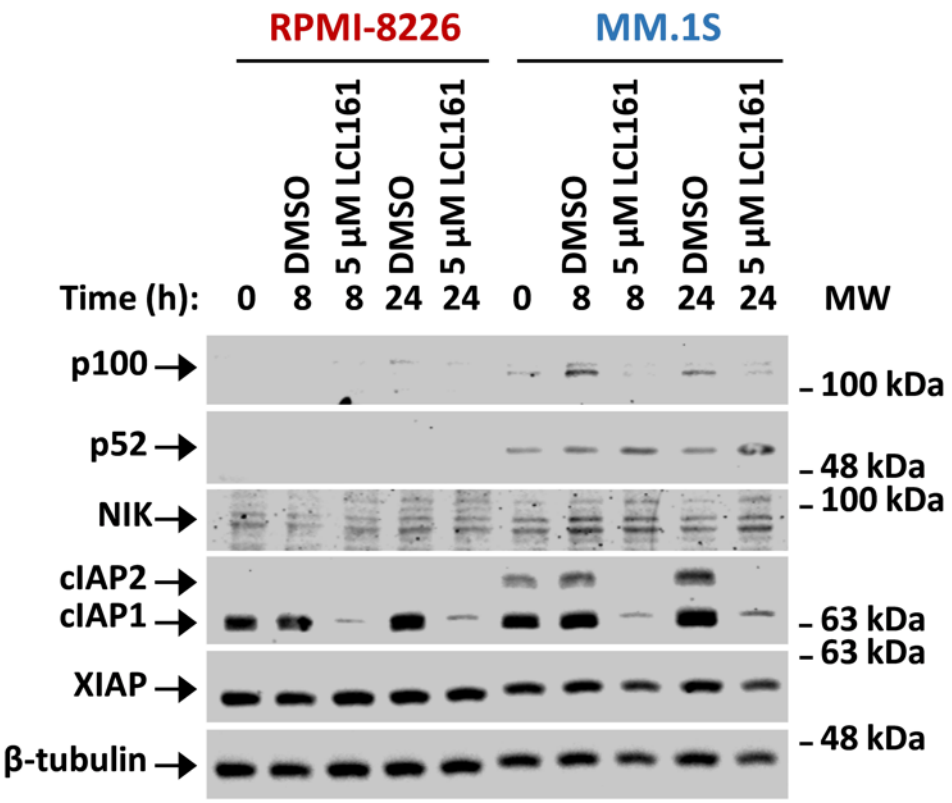


Figure 2-7. LCL161 sensitizes multiple myeloma cells to T cell cytotoxicity at higher doses. **A** 5x10^5 tumor cells were co-cultured with TAC T cells and 5 μM LCL161 or vehicle, in biological triplicate at various E:T for 24 hrs. After co-culture, cytotoxicity was measured through flow analysis and normalized to tumor-only controls; data shown as mean and SD. Data were generated with T cells from 3 different donors (L5, L6, L9) run independently in 1 experiment **B.** Luciferase-based cytotoxicity assay of TAC T cells co-cultured with effLuc+ myeloma cell lines for 48 hrs in the presence of vehicle or 5 μM LCL161. Samples were run in triplicate and normalized to tumor-only cell controls; data shown as mean and SEM. Data were generated with T cells from 3 different donors (L6, L7, L9) run independently in 1 experiment. **C** TAC T cells were stimulated with MM.1S cells at an E:T ratio of 1:1 in the presence of either DMSO or LCL161 for 24 hrs and TNFα levels were measured in the supernatant. The data reflects technical triplicates **D** Multiple myeloma cell lines were incubated with DMSO or LCL161, in the presence of TNFα for 48 hrs. Cells were harvested, stained for viability, and analyzed by flow cytometry. The data reflects technical triplicates.



Supplemental Figure 2-6. Expression of cIAP1/2 and ncNF-κB signaling proteins in the multiple myeloma cell lines RPMI 8226 and MM.1S. Cell lines were cultured from thaw for 1 week prior to analysis. Cells were pelleted and cellular lysates were utilized for western blot analysis.

The results in Figure 2-4 indicated that maximal costimulation of the T cells is achieved with an LCL161 concentration below 1 μM, however the results in Figure 2-7 indicated that concentrations of 5 μM were required to observe enhanced killing of myeloma tumor cells. We, therefore, explored the effect of 5 μM LCL161 on TAC T cells. While we observed that this high concentration of LCL161 could significantly enhance the percent of T cells that entered division (Figure 2-8A), we also noted a concomitant increase in activation induced cell death (AICD). We excluded strictly dead/necrotic cells and measured the portion of live TAC T cells that were sensitized by 5 μM LCL161 towards apoptosis by measuring active caspase-3 expression and the presence of phosphatidylserine on the outer membrane leaflet of TAC T cells stimulated with antigen-loaded beads for 48 hrs. We observed a trend towards increased proportion of stimulated TAC T cells entering apoptosis and decreased viability (Figure 2-8B). We questioned whether this observation might be explained by a second IAP target of LCL161: X-linked IAP (XIAP). As XIAP is a regulator of caspase-3, -7, and -9 related apoptotic pathways[404], depression of caspase-inhibition by XIAP at high concentrations of LCL161 may further potentiate T cells towards death[405]. We stimulated T cells from two healthy donors (M12 and L15) with plate-bound BCMA-Fc and 5 μM LCL161 for 72 hours and examined the levels of XIAP within these cells, but we did not observe any difference in XIAP levels (Supplemental Figure 2-7). We questioned if the impact of Fas is more pronounced with higher dose LCL161 due to the propensity of sensitization of TAC T cells towards death at the 5 μM dose. Fas KO-TAC T cells were stimulated with antigen-coated beads or MM.1S myeloma cells and we assessed proliferative capacity, viability, and susceptibility to apoptosis in the presence of 5 μM LCL161 (Figure 2-8C-D). The deletion of Fas again manifested greater enhancement to proliferation and viability, and reduction to apoptosis in bead-stimulated T cells than MM.1S stimulated T cells. The mean absolute change in proliferation metrics and viability of Fas-deleted TAC T cells stimulated with bead-based antigen in the presence of 5 μM LCL161 was greater than previously measured at the lower 0.625 μM dose, suggesting that Fas limits T cell expansion under these conditions. Similar to the observation with 0.625 μM LCL161, TAC T cells stimulated with myeloma cells in the presence of 5 μM displayed enhanced accumulation of TAC T cell at the end of the culture period but no significant improvement to viability, which points to a mechanism unrelated to Fas.

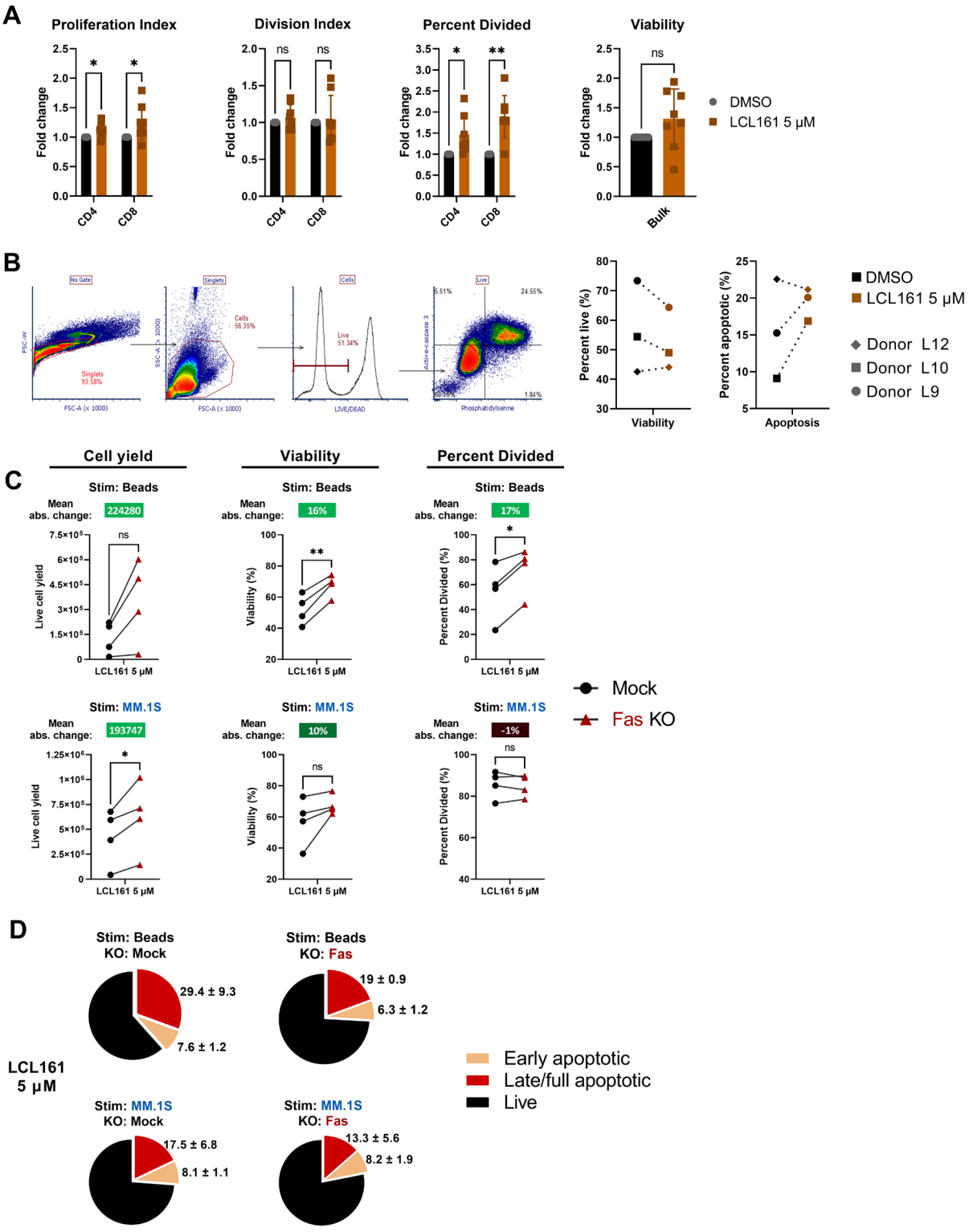
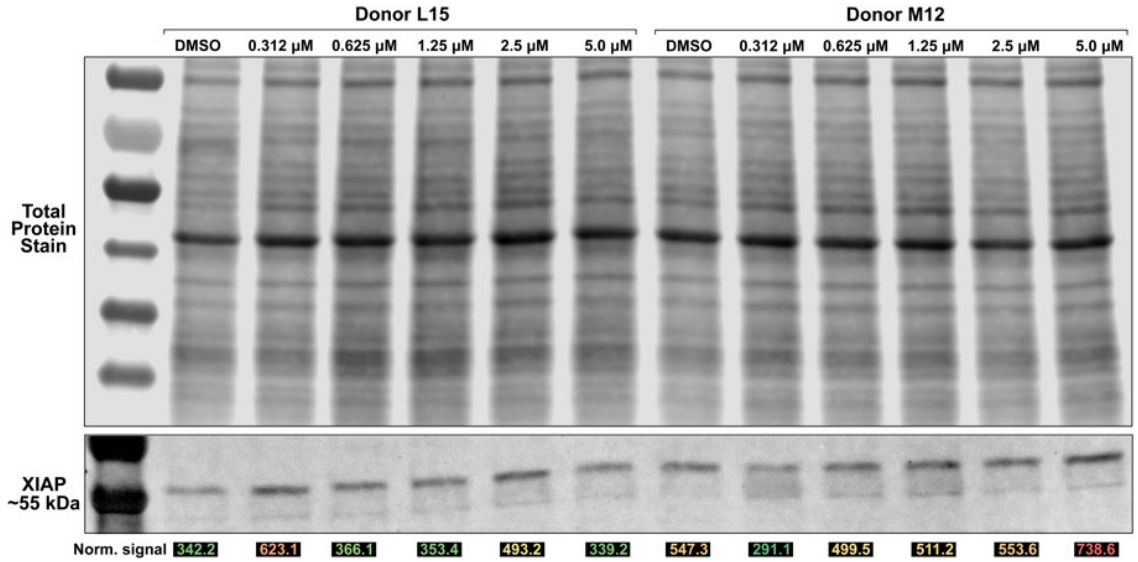

A
Proliferation Index
Division Index
Percent Divided
Viability
Fold change
CD4
CD8
Bulk
ns
DMSO
LCL161 5 µM
B
No Gate
Singlets
Cells
Live
Percent live (%)
Percent apoptotic (%)
Viability
Apoptosis
Donor L12
Donor L10
Donor L9
C
Cell yield
Viability
Percent Divided
Stim: Beads
Stim: MM.1S
Mean abs. change:
224280
16%
17%
193747
10%
-1%
Live cell yield
Viability (%)
Percent Divided (%)
LCL161 5 µM
Mock
Fas KO
D
Stim: Beads
KO: Mock
KO: Fas
Stim: MM.1S
LCL161 5 µM
29.4 ± 9.3
7.6 ± 1.2
19 ± 0.9
6.3 ± 1.2
17.5 ± 6.8
8.1 ± 1.1
13.3 ± 5.6
8.2 ± 1.9
Early apoptotic
Late/full apoptotic
Live

Figure 2-8. High dose LCL161 initiates activation induced cell death in stimulated TAC T cells that is responsive to Fas KO only in the case of antigen-alone stimulation. A TAC T cells labeled with CellTrace Violet were cultured with loaded beads at an E:T of 1:1 for 96 hrs. T cells were harvested and stained for flow cytometric analysis. Proliferation metrics were modeled with FCS Express 7. Proliferation and division indices are a measure of the average number of cells any or a dividing cell generates, respectively. Paired and unpaired parametric t tests with Holm-Sidak correction was used to calculate statistical significance; ns: P>0.05, *: P<=0.05, **: P<= 0.01. n=8, comprising 5 different donors generated in 7 independent experiments **B** TAC T cells were stimulated with protein G polystyrene beads coated with antigen-loaded microbeads for 48 hrs. Viability and proportion of apoptotic cells as defined by live/dead, phosphatidylserine, and active-caspase 3 staining. Viable cells are defined as live/dead negative, and apoptotic cells defined as active caspase3+ and PS+. Data represent T cell products from 3 donors produced in 3 independent experiments. **C** CTV-labeled mock or Fas-edited TAC T cells stimulated 1:1 with MM.1S tumor cells or BCMA-coated microbeads and in the presence of 5 μM LCL161 or vehicle for 96 hrs. After stimulation, samples were stained, mixed with counting beads, and analyzed by flow cytometry. Proliferation metrics were modeled using FCS Express 7 software. Mean absolute change between mock and Fas KO is reported above each condition. Data were generated with T cells from 4 donors (L15, M12, M28, M46) produced in 3 independent experiments. **D** Mock or Fas-edited TAC T cells were stimulated 1:1 with MM.1S tumor cells or BCMA-coated microbeads and in the presence of 5 μM LCL161 or vehicle for 72 hrs. After stimulation, cells were stained for live/dead, fixed, and finally stained for phosphatidylserine. Cells were analyzed by flow cytometry. Early apoptotic cells were defined as live/dead$^-$, PS+, late/full apoptotic cells were defined as live/dead+, PS+. Pie charts represent mean values, with mean + SD written outside the pie slice. Data were generated with T cells from 3 donors (M12, M28, M46) produced in 2 independent experiments.



Supplemental Figure 2-7. Measurement of XIAP protein levels in TAC T cells stimulated with antigen-alone in the presence of LCL161. TAC T cells were stimulated for 72 hrs with plate-bound BCMA with increasing concentration of LCL161. After stimulation, cells were collected, fractured, and processed for protein lysates. 12.5 µg of protein was loaded onto a 4-20% TGX polyacrylamide gel with denaturing conditions and transferred to a nitrocellulose membrane. Total protein signal was measured by Li-Cor REVERT stain for normalization and then washed prior to immunoblotting for XIAP 1:1000 (Cell Signaling Technologies). Normalized XIAP signal was calculated using Li-Cor Empiria software.

DISCUSSION

Unlike chimeric antigen receptors which require the inclusion of costimulation domains for optimal function[199], TAC receptors provide robust anti-tumor immunity without the need for engineered costimulation. Indeed, other synthetic receptor strategies that utilize TCR-centric designs also show potent function in the absence of costimulation[350, 352]. These properties notwithstanding, costimulation promotes survival, greater proliferative capacity[406], and memory formation/persistence[407] – important traits for anti-tumor engineered T cell products[330]. Herein we investigated a strategy for controlled and transient druggable delivery of costimulation using the clinical candidate, LCL161.

Our study employed engineered TAC T cells from both healthy donors and multiple myeloma patient donors to broaden the applicability and relevance of our findings. A reductionist antigen-on-bead system allowed us to fine tune signaling events and stringently study the effects of LCL161 on our engineered TAC T cells. Costimulation from IAP antagonism is mediated through the ncNF-κB pathway[371], an observation we corroborated. Low dose LCL161 potentiates processing of NF-κB2 p100 to p52 that increases for at least 72 hrs following TAC-mediated stimulation. Functionally, low doses of LCL161 provides functional improvements to T cells measured by enhanced survival and expansion. We noted that the extent to which the LCL161 enhances survival and expansion did not appear to correlate with the ratio of p100 to p52 conversion; for example, donor HN18 displayed little enhancement of proliferation with LCL161 yet strongly activated the ncNF-κB pathway. While the exact mechanism underpinning this donor-to-donor variability remains unclear, genechip transcriptional profiling identified numerous targets which led us to investigate in more detail the roles of CD30 and FAIM3 in LCL161-mediated enhancement of TAC T cell proliferation. How these receptors function is not fully elucidated within the field of T cell biology; research is mixed with some studies showing a pro-apoptotic capacity[398, 399], and others showing a costimulatory or survival property[396, 397, 400]. CD30 is a well-known TNFRSF member that plays a pivotal role in T cell activation as well as providing survival signals for lymphomas that is cIAP-dependent[408]. Indeed, SMAC mimetic treatment has been shown to provide similar cellular signals as CD30, intersecting on canonical and ncNF-κB signaling[409, 410]. Our data with CD30 KO clearly demonstrated its requirement in enhancing proliferation and survival in the context of LCL161 treatment. The impact of FAIM3 overexpression was less clear due to donor variation, but the data indicated a trend towards restricting proliferation when it is constitutively expressed in TAC T cells. A recent report suggested that costimulation via FAIM3 was dependent on the availability of its ligand, IgM[397]. We reasoned that bovine IgM present in our culture medium may

not provide an appropriate ligand, so we added exogenous human IgM to our culture medium but found no additive effects with the FAIM3 overexpressing cells (data not shown).

Although we observed a slight enhancement to TAC T cell-mediated killing of multiple myeloma cells *in vitro* at very high doses of LCL161 (5 μM), this effect did not persist with a 2-fold reduction in concentration. Indeed, we expanded our investigation to *in vivo* studies and similarly observed no benefit in anti-tumor activity of TAC T cells against system MM.1S xenografts (described in our recent publication[2]) when mice were concomitantly treated with either 25 or 75 mg/kg LCL161 (data not shown). Our data indicate that the negative effects of the drug on the T cell population need to be carefully considered. A balance between the costimulatory effects of LCL161 and unwanted induction of AICD in the T cells became evident as we titrated the dose of drug on activated T cells, an observation previously seen with non-engineered T cells at high doses of LCL161[411]. We suspected that knockdown of both the cIAP1/2 as well as XIAP at higher doses of LCL161 could render the T cells more susceptible to full caspase-3 activation and subsequent cell death[405]. This may be exacerbated by TNFα produced by the TAC T cells, which can further potentiate death signaling in the absence of cIAP1/2[365, 412]. We did not observe increased knockdown of XIAP at increasing LCL161 doses 72 hrs after antigenic stimulation. However, it is possible that XIAP is competitively inhibited rather than degraded, and thus is not suppressing caspase-activation or death signaling. Indeed, the apoptotic sensitization in the T cells may be transduced through death-receptor pathways as XIAP is known to restrict Fas-mediated apoptosis[413]. As activated T cells express Fas and FasL for regulation and immune homeostasis[414], we speculated that expression of FasL on the tumor cells contributed to the restricted ability of LCL161 to enhance TAC T cell survival and proliferation in the context of myeloma cell stimulation. In addition to this, T cell fratricide through Fas-FasL interactions with neighboring T cells would compound this effect. To address this, we knocked out expression of Fas on our TAC T cells and showed a reduction to T cell toxicity at the low and high doses of LCL161 with bead stimulation, supporting our hypothesis that LCL161 does reduce the threshold to apoptosis in activated T cells through Fas-driven fratricide. However, this effect did not translate as clearly to MM.1S cell-based stimulation which indicates that other factors or mediators are likely involved in the restriction of LCL161-mediated proliferative enhancement with myeloma cell lines. Fas-deletion recovers the loss of TAC T cell yield observed following MM.1S stimulation in the presence of 5 μM LCL161, which supports the hypothesis that the decline in T cell yield following stimulation with MM.1S is mediated, at least in part, through Fas signaling. Nevertheless, even in the absence of Fas, we do not observe an increase in TAC T cell viability or

proliferation in the presence of LCL161, which suggests that LCL161-mediated costimulation is inconsequential in the presence of the full complement of adhesion molecules and costimulatory ligands (such as LFA-3[415]) found on the myeloma cells. Indeed, we have detected CD137L and CD86 on RPMI 8226 and MM.1S cells by flow cytometry (data not shown).

Given that the myeloma lines RPMI 8226 and MM1S were resistant to LCL161[389] and TNFα, we were able to tune our investigations to draw conclusions under the lens of a TAC T cell-centric observation and understand the impact on T cell biology. Our study highlights several pathways and mechanisms by which LCL161 integrates into T cell proliferation and survival, expanding our understanding of SMAC mimetics as enhancers of anti-cancer immnuity[416]. Additional refinement to SMAC mimetic small molecule drugs through medicinal chemistry, or development of direct cIAP1/2 antagonists, could increase the therapeutic threshold between costimulatory effects and unwanted sensitization to AICD. Beyond their use as drugs to activate costimulation in the context of cell therapy, the evidence that LCL161 can enhance T cell proliferation *ex vivo* in response to a reductionist stimulation suggests that small-molecule IAP antagonists may be of use during the manufacturing of engineered T cells through enhancement of proliferation.

METHODS

Cell lines

Human myeloma cell lines RPMI-8226 (CCL-115; purchased from ATCC (Manassas, VA) and MM.1S (kindly provided by Dr. Kelvin Lee, Roswell Park Cancer Institute, NY) were cultured in RPMI 1640 (Gibco (Thermo Fisher Scientific), Waltham, MA), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop Canada Inc., Burlington, ON, Canada), 10 mM HEPES (Roche Diagnostics, Laval, QC, Canada), 1 mM sodium pyruvate (Sigma-Aldrich Canada Co. (Millipore Sigma), Oakville, ON, Canada), 1 mM nonessential amino acids (Gibco), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). To generate luciferase-expressing cell lines, parental RPMI 8226 or MM.1S cell lines were transduced with lentivirus encoding enhanced firefly luciferase (effLuc) as well as puromycin N-acetyltransferase at an MOI 10 and selected in culture media supplemented with 8 µg/mL puromycin (InvivoGen, San Diego, CA). HEK293T cells (kindly provided by Dr. Megan Levings, University of British Columbia, Canada) were cultured in DMEM (Gibco), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche Diagnostics), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco), or 0.1 mg/mL normocin (InvivoGen). All cells were cultured at 37 °C, 95% ambient air, 5% CO2, and

routinely tested for mycoplasma contamination using the PlasmoTest detection kit (InvivoGen, San Diego, CA, USA).

Lentivirus production

Lentivirus encoding the BCMA-specific TAC receptor[2] with or without FAIM3 was produced as previously described[170] using a third generation packaging system[154]. Briefly, 12 x 10^6 HEK293T cells plated on a 15-cm dish (NUNC (Thermo Fisher Scientific)) were transfected with plasmids pRSV-REV (6.25 µg), pMD2.G (9 µg), pMDLg-pRRE (12.5 µg), and pCCL BCMA TAC (32 µg), using Opti-MEM (Gibco) and Lipofectamine 2000 (Thermo Fisher Scientific). 12-16 hours post-transfection, media were exchanged to media supplemented with 1 mM sodium butyrate (Sigma-Aldrich). 24-36 hours later, supernatants were harvested, and viral particles were concentrated using an Amicon Ultra 15 100 kDa centrifugal filter (Millipore Sigma) and stored at -80 °C. Viral titer (TU/mL) was determined post-thaw by serial dilution, transduction of HEK293T cells, and enumeration of percent NGFR+ cells by flow cytometry.

Generation of engineered human T cell products

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy or myeloma patient donors who provided informed written consent in accordance with the Hamilton Integrated Research Ethics Board, or were collected from commercial leukapheresis products (HemaCare, Northridge, CA and STEMCELL Technologies, Vancouver, BC, Canada). In the case of leukapheresis products, samples were transported at room temperature and processed within 24 hrs of collection. Whole blood was collected from donors using BD CPT sodium heparin collection tubes (BD Biosciences). PBMCs were isolated from blood or leukapheresis by Ficoll-Paque Plus gradient centrifugation (Cytiva, Vancouver, BC, Canada) and cryopreserved in inactivated human AB serum (Corning, Corning, NY) containing 10% DMSO (Sigma-Aldrich), or in CryoStor10 (STEMCELL Technologies) for healthy donors, and RPMI (Gibco) containing 12.5% HSA (Sigma-Aldrich) and 10% DMSO (Sigma-Aldrich) for myeloma patient donors. Samples were cryopreserved in an isopropanol controlled-rate freezer (Thermo Fisher Scientific) at -80°C for 24-72 hrs prior to long term storage in liquid nitrogen. Post-thaw, PBMCs were activated with ImmunoCult soluble anti-CD3/28/2 tetrameric complexes (STEMCELL Technologies), and cultured in RPMI 1640 (Gibco) containing 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche), 1 mM sodium pyruvate (Sigma-Aldrich), 1 mM non-essential amino acids (Gibco), 55 µM β-mercaptoethanol (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 1.5 ng/mL rhL-2 and 10 ng/mL rhIL-7 (PeproTech, Cranbury, NJ). 16-24 hours later, activated T cells were transduced

with lentivirus at an MOI of 1-2. T cells edited by CRISPR/Cas9 were washed with 1x PBS on day 3 to remove soluble activator, otherwise T cells were washed on day 4. Transduced cells were enriched with the EasySep Human CD271 Positive Selection Kit II (STEMCELL Technologies) on day 7-10 of culture. Culture yields were enumerated every 2-3d and supplemented with cytokine-containing media to dilute cultures to ~ 1.0×10^6 cells/mL. Engineered T cell products were expanded for a total culture period of 14-15 days prior to use. In some cases, TAC T cell products were cryopreserved prior to use. In short, after culture cells were cryopreserved in CryoStor10 according to manufacturer's directions at 20×10^6 cells/mL (for downstream *in vitro* assays). Prior to use of cryopreserved TAC T cell products in any *in vitro* assay, cells were thawed according to manufacturer's directions and rested for 24 hrs in cytokine-containing media (as above). Mean viability and recovery were 85.5% (±7.2% std. dev.; 95% CI [83.2, 87.7]) and 57.2% (±16.4% std. dev.; 95% CI [52.1, 62.3]), n=42.

All assays were performed in T cell medium without cytokines; FBS lots were assessed compared to previous lots to ensure similar T cell manufacturing (expansion, viability, cryopreservation) and functionality (proliferation, cytotoxicity, and cytokine production).

CRISPR/Cas9 Editing of T cells

T cell gene-editing was accomplished by electroporation of complexed gRNA/Cas9 ribonucleoprotein (RNP). To generate RNP, a triple sgRNA pool (Synthego, Redwood City, CA, USA) or negative control gRNA (IDT, Newark, NJ, USA) was complexed with 20 pmol Alt-R HiFi Cas9 V3 (IDT) at a 3:1 sgRNA:Cas9 molar ratio for 10-15 min. Prior to electroporation activated T cells were pooled and washed with 1x PBS. $5x10^5$ T cells per electroporation were resuspended in buffer T (Thermo Fisher Scientific) and mixed with complexed RNP (20 pmol on a Cas9 basis) and shocked using a NEON electroporation system (Thermo Fisher Scientific) set to 1600 V, 10 ms pulse width, and 3 pulses. Immediately after electroporation, T cells were dispensed into antibiotic-free T cell medium. To edit the *TNFRSF8* gene, exon 6 was targeted with sgRNAs with sequences (5' – 3'): AAATTACCTGGATCTGAACT, AGCTGCTTCTAAACTGACGA, and ACACCGGGGTGGGCTTGGCC. To edit the *FAS* gene, exon 2 was targeted with sgRNAs with sequence (5' – 3'): CACUUGGGCAUUAACACUUU, UACAGUUGAGACUCAGAACU, and GUGUAACAUACCUGGAGGAC. Genomic DNA was collected from T cells on d14/15 with QuickExtract DNA extraction solution 1.0 (Lucigen, Hoddesdon, UK) following manufacturer's instructions, and targeted exons were amplified by PCR and sequenced by sanger sequencing. Indel analysis was performed by Synthego ICE[417] with PCR/sequencing primers (5' – 3') for *TNFRSF8* exon 6: CTCCCCCTCATCTCAAGAGCTATC and

TGAGCCTCAAACCAAAGCAAGA; *FAS* exon 2 TGAAGAACCTGAGATCCAAACTGCT and TGGTAGATCCTAATCAGTTTTGACATGA.

Flow cytometry

Flow cytometry data were collected with BD LSR II (V/B/R or V/B/YG/R laser configuration), BD LSR Fortessa (V/B/R laser configuration) or Beckman Coulter CytoFLEX LX (NUV/V/YG/B/R laser configuration) and analyzed using FCS Express v7 Software (DeNovo Software, Pasadena, CA).

Phenotypic characterization of T cell products

Surface expression of BCMA TAC constructs was determined by staining with recombinant human BCMA-Fc protein (R&D Systems, Minneapolis, MN), followed by goat anti-human IgG and antibodies against markers CD4, CD8α, CD30, FAIM3, and/or NGFR. Unless otherwise stated, all stains were done at room temperature for 30 minutes in PBS + 2% BSA + 2.5 mM EDTA. Staining was assessed by flow cytometry.

Cytokine quantification

Supernatants from TAC T cells stimulated 1:1 with MM.1S tumor cells in the presence of DMSO or LCL161 were collected after 24 hrs following centrifugation of the plate to pellet cells and other debris. Supernatants were frozen to -80°C and shipped to EveTechnologies for multiplex analysis of cytokines using the HDF15 panel.

In vitro cytotoxicity assay

For luciferase-based cytotoxicity assays TAC T cells were co-cultured in triplicate with $2.5x10^4$ luciferase-expressing RPMI 8226 or MM.1S cells in the presence of DMSO or LCL161 at indicated effector:target ratios for 48 hours at 37°C. Following co-culture, 0.15 mg/mL D-Luciferin (Perkin Elmer, Waltham, MA) was added, and luminescence was measured with an open filter using a SpectraMax i3 (Molecular Devices, San Jose, CA) plate reader. Tumor cell viability was calculated as $\frac{(Emission-background)}{(Tumor\ alone-background)} \times 100\%$. For flow-based cytotoxicity assays, engineered T cells were co-cultured with $2.5x10^4$ RPMI 8226 or MM.1S cells with DMSO or LCL161 at indicated effector:target ratios for 24 hrs at 37°C. Following co-culture, samples were stained for NGFR, CD138, and viability and CD138+NGFR$^-$ cell viability was utilized as a measure of tumor cell survival.

Microbead antigen loading

Protein G-conjugated ~6-7 μm polystyrene beads (Spherotech, Lake Forest, IL, USA) were coated with 50 ng BCMA-Fc chimera protein (R&D Systems, Minneapolis, MN, USA) per 10^6 beads in PBS + 0.1% BSA at a concentration of $5x10^6$

beads/mL and incubated rotating overnight at 4°C. Immediately prior to use, beads were resuspended in T cell medium without cytokines to achieve the desired effector:target ratio.

Proliferation assay

Engineered T cells were labelled with CellTrace Violet dye (Thermo Fisher Scientific) and stimulated with MM.1S tumor targets or antigen-coated microbeads at a 1:1 effector:target ratio (non-stimulated control wells were also plated). After 4d at 37°C, co-cultures were stained with Live/Dead Fixable stain and antibodies against markers CD8α, CD4, NGFR, CD3, and/or CD138, and mixed with absolute counting beads (Thermo Fisher Scientific). Flow cytometry data were acquired as indicated above. Results were analyzed using Proliferation Plots in FCS Express 7. In short, non-stimulated control T cells were used to identify and fix a Starting Generation within Proliferation Fit Options. Proliferation Fit Statistics were used to calculate the percent of original cells that divided (percent divided). Absolute cell yield was calculated as $cell\ count = \frac{\#\ CD3+\ live\ events}{\#\ bead\ events} \times \#\ beads\ loaded\ into\ sample$.

Clariom-S RNA transcriptome analysis

$5x10^5$ TAC T cells were stimulated with 0.05 µg/mL plate-bound BCMA-Fc antigen in a 24-well plate in the presence of 0.625 µM LCL161 or DMSO for 24 or 72 hrs. After stimulation, T cells were collected, washed with 1x PBS to remove media, then processed to collect total RNA. Briefly, T cells were homogenized by centrifugation through QIAshredder columns (QIAGEN, Hilden, Germany), and RNA was purified from the resultant lysates by RNeasy Mini Plus (QIAGEN) RNA purification kit. Purified RNA was assessed for quality above RIN>7 by NanoDrop OneC spectrophotometry (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Total RNA was hybridized using a Clariom-S Pico genechip, and resultant data was analyzed using the Transcriptome Analysis Console (Thermo Fisher Scientific) to determine differential gene analysis (DGA) between DMSO and LCL161 group with donor as a repeated measure. To generate volcano plots, the differentially expressed gene were input using the EnhancedVolcano() package in R[418]. Genes were identified as significant with a p-value below 0.05 and a fold change >1.75/<-1.75. Gene ontology (GO) over-representation analysis was conducted using the clusterProfiler() package in R[419-421]. Specifically, the biological processes (BP) ontology was used to identify enriched pathways. A Benjamin & Hochberg[422] (BH) correction was applied post-hoc and gene sets were identified as significant with an adjusted p-value below 0.05. All analyses were performed using R Statistical Software (v4.2.1[421]).

Western blotting

T cell NF-KB2 p100/p52 and XIAP analysis was accomplished by first stimulating $5x10^5$ TAC T cells on 0.05 µg/mL plate-bound BCMA-Fc antigen in a 24-well plate in the presence of 0.625 µM LCL161 or DMSO for 24, 48, or 72 hrs. After stimulation, T cells were collected, washed twice in 1x PBS, then frozen as cell pellets at -20°C. Protein lysates were generated from cell pellets by lysis with RIPA lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (Roche, Basel, Switzerland) for 15 min on ice, followed by 15 min centrifugation. Lysates were quantified by BCA assay (Thermo Fisher Scientific), and 8.5 (NKFB2) or 10 (XIAP) µg protein was loaded onto a 4-20% gradient gel (Bio-Rad). Proteins were then transferred to a nitrocellulose membrane, stained for total protein (Li-COR Biosciences, Lincoln, NE, USA), and then blotted for NF-KB2 (Cell Signaling Technologies) or XIAP (Cell Signaling Technologies). All imaging was accomplished using an Odyssey Lx Imaging System (Li-COR Biosciences) set to auto mode. Blots were analyzed using Empiria Software (Li-COR Biosciences) where NF-KB2 p100/p52, or XIAP, signals were normalized to total protein signal of their respective wells. For analysis of tumor cells, MM.1S and RPMI 8226 cells were pelleted and lysed in an SDS-RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5% deoxycholic acid, 0.5% Tween 20, 0.5% NP-40, 0.1% SDS) with protease and phosphate inhibitor cocktail (MiliporeSigma) for 20 minutes on ice. Cells were then sonicated for 10 seconds at 20% amplitude before centrifugation and protein quantification by DC protein assay (Bio-Rad). Equal amounts of protein (25 µg) were loaded onto 10% SDS-PAGE gels. Immunoblotting was performed overnight at 4°C with the primary antibodies, p100/p52 (Cell Signaling Technologies), NIK (Abcam, Cambridge, UK), β-Tubulin (DSHB, Iowa city, IA, USA), cIAP1/2 (MBL International, Woburn, MA, USA), and XIAP (MBL International). The next day, bound primary antibodies were probed with secondary antibodies conjugated to either Alexa Fluor680 (Thermo Fisher Scientific) or IRDye 800CW (Li-COR Biosciences) at room temperature for 1 hour, then blots were scanned on the Odyssey Infrared Imaging System (Li-COR Biosciences).

T cell apoptosis and AICD assay

T cell apoptosis was investigated by stimulating $5x10^5$ engineered T cells 1:1 with antigen-loaded microbeads or MM.1S tumor cells for 24, 48, or 72 hrs in the presence of DMSO or LCL161. Afterwards, cells were immediately stained with live/dead fixable viability dye at RT, fixed with Cytofix, and stained for phosphatidylserine and CD3 surface expression on ice for 60 min. Cells were analyzed by flow cytometry and populations were defined as live (PS^- live/$dead^-$), early apoptotic (PS+ live/$dead^-$), and late apoptotic (PS+ live/dead+). To investigate

AICD in TAC T cells, cells were stimulated as above with antigen-loaded microbeads for 48 hrs in the presence of DMSO or LCL161. Cells were stained with live/dead fixable viability dye at RT, fixed with Cytofix, stained for phosphatidylserine on ice for 60 min, permeabilized with Phosflow perm/wash I as per manufacturers guidelines, then stained intracellularly for active caspase 3. Percent apoptotic in live cells was defined as live/dead$^-$ PS+ active-caspse3+.

Tumor cell TNF-sensitivity assay

$2x10^5$ MM.1S or RPMI 8226 cells were incubated with LCL161 or DMSO and in the presence of rhTNFα (BD Biosciences) for 48 hrs. After incubation, cells were collected and stained using a fixable live/dead dye. Samples were analyzed by flow cytometry to determine viability.

Statistics

All statistical analyses were performed in GraphPad Prism version 9.4 for Windows (San Diego, CA). Paired two tailed t tests, or multiple paired t tests using the Holm-Šídák method, were utilized to compare between two samples. An ordinary one-way ANOVA using the Dunnett multiple comparison test was used to compare the means of three or more unmatched groups. * = $p > 0.05$, ** = $p > 0.01$, *** = $p > 0.001$, and N.S. = not significant.

Reagent	**Source**	**Identifier**
Goat anti-human IgG (H+L)-PE	Jackson ImmunoResearch	Cat# 109-115-098, RRID:AB_2337675
Anti-human CD4-Pacific Blue	BD Biosciences	Cat# 558116, RRID:AB_397037
Anti-human CD4-APC-H7	BD Biosciences	Cat# 560158, RRID:AB_1645478
Anti-human CD4-AlexaFluor 700	Thermo Fisher Scientific	Cat# 56-0048-82, RRID:AB_657741
Anti-human CD8α-AlexaFluor 700	Thermo Fisher Scientific	Cat# 56-0086-82, RRID:AB_657756
Anti-human CD8α-PerCP-Cyanine5.5	Thermo Fisher Scientific	Cat# 45-0088-42, RRID:AB_1582255
Anti-human CD30-PE	Miltenyi Biotec	Cat# 130-120-783, RRID:AB_2784118

Anti-human FAIM3-APC	BioLegend	Cat# 398103, RRID:AB_2876722
Anti-human NGFR-VioBright FITC	Miltenyi Biotec	Cat# 130-104-893, RRID:AB_2661084 Cat# 130-113-985, RRID:AB_2734063
Anti-human NGFR-Brilliant Violet 421	BD Biosciences	Cat# 562562, RRID:AB_2737657
Anti-human CD138-APC	BioLegend	Cat# 356505, RRID:AB_2561879
Anti-human CD3-Brilliant Violet 605	BioLegend	Cat# 300460, RRID:AB_2564380
Anti-human FasR-APC	BioLegend	Cat# 305612, RRID:AB_314550
Functional grade anti-human CD3	Thermo Fisher Scientific	Cat# 16-0037-85, RRID:AB_468855
Anti-NF-κB2 P100/p52	Cell Signaling Technologies	Cat# 4882, RRID:AB_10695537
Anti-human NIK	Abcam	Cat# ab7204, RRID:AB_2139765
Anti-cIAP1/2	MBL International	Cat# CY-P1041, RRID:AB_10950764
Anti-XIAP	MBL International	Cat# M044-3, RRID:AB_592998
Anti-XIAP	Cell Signaling Technologies	Cat# 2045, RRID:AB_2214866
Anti-β-Tubulin	Developmental Studies Hybridoma Bank	Cat# E7, RRID:AB_528499
IRDye 800CW goat anti-rabbit	Li-COR Biosciences	Cat# 926-32211, RRID:AB_621843
Anti-human FasL-PE	BD Biosciences	Cat# 564261, RRID:AB_2738713

Anti-phosphatidylserine-AlexaFluor 488	MiliporeSigma	Cat# 16-256, RRID:AB_492616
Anti-human active caspase 3-Alexa Fluor 647	BD Bioscience	Cat# 560626, RRID:AB_1727414
LIVE/DEAD fixable Near-IR dead cell stain	Thermo Fisher Scientific	Cat# L10119
Cytofix buffer	BD Biosciences	Cat# 554655
Phosflow perm/wash I	BD Bioscience	Cat# 557885
123count eBeads	Thermo Fisher Scientific	Cat# 01-1234-42
Protein G-conjugated ~6 µm polystyrene beads	Spherotech	Cat# PGP-60-5
Alt-R S.p. HiFi Cas9 Nuclease V3	Integrated DNA Technologies	Cat# 1081061
Pierce RIPA Lysis buffer	Thermo Fisher Scientific	Cat# 89901
cOmplete, Mini, EDTA-free protease inhibitor tablets	Roche	Cat# 04 693 159 001
Protease and Phosphatase Inhibitor Cocktail	MiliporeSigma	Cat# PPC1010
DC Protein Assay Kit	Bio-Rad	Cat# 5000111
Pierce BCA assay	Thermo Fisher Scientific	Cat# 23227
4-20% TGX pre-cast gel, 15 lanes	Bio-Rad	Cat# 4561096
0.2 µm nitrocellulose membrane	Bio-Rad	Cat# 1620112
REVERT 700 Total protein stain kit	Li-COR Biosciences	Cat# 926-11010
Intercept blocking buffer (TBS)	Li-COR Biosciences	Cat# 927-60001

Intercept Antibody Diluent (T20 TBS)	Li-COR Biosciences	Cat# 927-65001
CellTrace Violet	Thermo Fisher Scientific	Cat# C34557
D-Luciferin	Perkin Elmer	Cat# 122799
rhBCMA-Fc chimera	R&D Systems	Cat# 193-BC
rhTNFα	BD Biosciences	Cat# 554618
LCL161	Novartis	N/A

Chapter 3 – Strengthening anti-myeloma properties of TAC T cells through forced expression of IL-27

Introduction to Chapter 3.

Cytokines are small protein messengers that modulate bodily functions and immune response trajectory. For T cells, cytokines provide contextual stimuli and signals to guide differentiation and lineage commitment, survival, and function. In classical T cell biology this is known as *signal three* because of the necessity for cytokines in optimal activation of naïve CD8+ T cells and polarization of CD4+ T cells[423, 424]. For example, IL-12 and type I IFN have been identified as important in preventing anergy and supporting cytolytic function and memory response of CD8+ T cells[425, 426]. As another example, IL-12 promotes CD4+ T cell differentiation to a T_H1 phenotype, which may be useful to support anti-tumor immunity, whereas IL-4 promotes CD4+ T cell differentiation to T_H2 phenotype, which may be more relevant to protection from helminth infection.

Outside of T cell activation and differentiation, several cytokines sustain homeostatic survival of T cells and maintenance of memory programs at resting states. These are namely members of the cytokine receptor common γ-chain family, such as IL-2, IL-7, and IL-15[427, 428]. Signaling by these cytokines facilitates survival through expression of pro-survival factors such as BCL-2 and MCL-1, PI3K-AKT signaling, and inhibition of apoptosis inducers BIM and BAD[429, 430].

3.1 Engineered cytokine expression in T cells

Given the utility of cytokines to support T cell function, numerous groups have engineered T cells to express specific cytokines to enhance T cell function and/or interfere with the tumor environment[431, 432]; such cytokine-producing engineered T cells have been referred to as "*armored*", or TRUCKs (T cells Redirected towards Universal Cytokine Killing). Several cytokines have been assessed in this regard, including IL-12, IL-15, and IL-18.

IL-12 is a heterodimeric cytokine with anti-tumor immunomodulatory properties. In addition to T_H1 polarization, IL-12 induces IFNγ production, potentiates NK cell and CTL cytotoxicity, and inhibits angiogenesis in conjunction with IFNγ[433, 434435]. In a lymphoreplete murine model, IL-12 expressing CAR T cells were capable of promoting epitope-spreading and potentiating endogenous anti-tumor immune responses *in vivo* that persisted beyond the CAR T cells[435]. However, in human clinical trials extended IL-12 release from engineered T cells resulted in limiting toxicities that ultimately required trial termination[436], highlighting the risks involved with systemic use of this cytokine. Another study placed the IL-12 transgene downstream of the NFAT (Nuclear Factor of Activated T cells) promoter to link IL-12 expression with T cell activation, effectively gating IL-12 within antigen-positive tissues (predominately the tumor). This inducible IL-12 system strives to limit systemic toxicities, and was capable of recruiting

macrophages that controlled antigen-negative tumor cells in a pre-clinical model[437].

Similar to IL-12, IL-18 in concert with IL-12 or IL-15 can activate a T_H1 phenotype, induce expression of IFNγ, and augment NK and CTL cytolytic activity as well[438, 439]. Unlike IL-12, IL-18 administration in cancer patients has shown a much more favorable and manageable toxicity profile of primarily grade 1-3 adverse events[438]. In pre-clinical models engineered anti-tumor T cells using inducible IL-18 showed superior tumor control and toxicity profile when directly compared to T cells using IL-12[440, 441].

Immune cells engineered to secrete IL-15 are also under heavy investigation. While rhIL-15 therapy in humans did not have as favorable a safety profile as IL-18, pre-clinical studies of CAR T cells expressing either a membrane-tethered IL-15[442] or CAR T cells expressing soluble IL-15 with a inducible suicide switch[443] have both shown improved anti-tumor efficacy that is in part due to a robust memory phenotype driven by the IL-15. In the clinic, administration of rhIL-15 in a first-in-human phase I trial had mixed results. The investigators ultimately concluded that systemic IL-15 administration proved challenging due to high clinical toxicities that were dose-limiting, and reflected a narrow therapeutic window[444]. However, systemic delivery of high concentration cytokine likely presents differently compared to cellular release. Of note, NK cells engineered to express an anti-CD19 CAR and IL-15 in a phase I/II trial of 11 non-Hodkings lymphoma or chronic lymphocytic leukemia patients did not cause CRS, neurotoxicity or GVHD[445]. These data highlight the safety profile of engineered anti-tumor immune cells expressing IL-15; although one should note that the clinical experience of IL-15-secreting NK cells thus far may not be reflective of IL-15-secreting T cells due to differences in their biology. Clinical trials are underway to explore IL-15 CAR T cells (NCT03721068, NCT04377932).

3.2 IL-27, a pleiotropic cytokine

We wanted to explore other cytokines to armor TAC T cells under the lens of multiple myeloma and investigated IL-27, a pleiotropic member of the IL-12 family of cytokines. Like IL-12, IL-27 is heterodimeric and biases the polarization of CD4+ T cells towards a T_H1 phenotype, an important T cell subset in anti-tumor immunity[446]. IL-27 can enhance NK cell and CTL activity by increasing granzyme B and perforin expression, augment CTL survival, and deplete Tregs – all of which promotes tumor clearance[447-450].

Within the myeloma microenvironment, IL-27 has been shown to suppress osteoclast differentiation and bone resorption, enhance osteoblast proliferation, reduce expression of pro-myeloma factors such as IL-6 and VEGF, and reduce

expression of pro-angiogenic genes in CD138+ myeloma cells leading to weakened vascular branches *in vivo*[450]. Additionally, IL-27 has been shown to mediate endogenous anti-tumor activity in other cancer models. In a murine orthotopic model of primary and metastatic neuroblastoma, the authors observed that mice implanted with IL-27 secreting tumors completely survived and generated far fewer metastases[451]; the surviving mice suppressed tumor growth following rechallenge with parental tumors lacking IL-27 expression, suggesting the development of a *de novo* anti-tumor response in the survivors that required IL-27 during the initial challenge. Using immunodeficient mice or T cell depletion, the authors found that IL-27 enhanced T cell infiltration and function, and rejection of tumors was dependent on the host-immune system, particularly CD8 T cells.

With these properties in mind, we reasoned that IL-27 secretion by BCMA-directed TAC T cell may augment their anti-tumor activity and weaken the myeloma microenvironment, and therefore, investigated forced expression of IL-27 in TAC T cells in the context of multiple myeloma.

3.3 Manuscript status and citation

Status: Short report, under revision at Cytotherapy.

Citation: Afsahi, A., Baker, C.L., Burchett, R., Moore, A.E., and Bramson, J.L. (2023). Constitutive expression of IL-27 diminishes proinflammatory cytokine production without impairing effector function of engineered T cells. In submission, under revision: *Cytotherapy*.

IL-27 MANUSCRIPT

Constitutive expression of IL-27 diminishes proinflammatory cytokine production without impairing effector function of engineered T cells

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ABSTRACT

Immunomodulatory cytokines can alter the tumor microenvironment and promote tumor eradication. IL-27 is a pleiotropic cytokine that has potential to augment anti-tumor immunity while also facilitating anti-myeloma activity. We engineered human T cells to express a recombinant single-chain (sc)IL-27 and a synthetic antigen receptor targeting the myeloma antigen, BCMA, and evaluated the anti-tumor function of T cells bearing scIL-27 *in vitro* and *in vivo*. We discovered that T cells bearing scIL-27 sustained anti-tumor immunity and cytotoxicity, yet manifested a profound reduction in pro-inflammatory cytokines GM-CSF and TNFα. IL-27-expressing T cells therefore present a potential avenue to avert treatment-related toxicities commonly associated with engineered T cell therapy due to the reduced pro-inflammatory cytokine profile.

Key words: Engineered T cell, TRUCK, IL-27, Multiple myeloma, cancer immunotherapy

INTRODUCTION

The tumor microenvironment is an immunosuppressive space that presents a major hurdle for successful adoptive T cell therapy. An attractive approach to counteract inhibitory mechanisms is to arm T cells with cytokines that modulate the microenvironment to enhance survival and function of adoptively transferred T cells, while repressing the tumor-promoting effects of local cells. As an example, T cells engineered to express IL-12[452] or IL-18[440] displayed enhanced anti-tumor activity due to direct effects of the cytokines on the T cells and adjacent tissues.

T cells engineered to express IL-12 have been well studied and display extensive anti-tumor properties in pre-clinical models[452-454]. However, in human clinical trials, the systemic release of IL-12 resulted in severe toxicities that ultimately required trial termination[436]. IL-27, another member of the IL-12 cytokine family, is a heterodimeric protein that has been reported to display multiple anti-tumor functions[455]. Within the tumor microenvironment, IL-27 can enhance CTL activity, augment CTL survival, and deplete Tregs, which serves to promote tumor clearance[447-450]. Furthermore, IL-27 biases the polarization of CD4+ T cells towards a Th1 phenotype, an important subset in directing anti-tumor responses, and away from Th2/Th17 subsets which are comparatively more pro-tumorigenic[446]. Finally, IL-27 has direct impact on tumors through anti-angiogenic effects which are not dependent on IFNγ, in contrast with IL-12[450, 456, 457]. Indeed, forced expression of mIL-27 in murine OT-1 T cells enhanced CD8 T cell proliferation and survival, as well as improved anti-tumor immunity *in vivo* and *in vitro*[458].

We have been exploring the utility of the T cell antigen coupler (TAC) receptor as a tool to direct T cells to attack myeloma[2]. IL-27 is of particular interest in this context because, in addition to the anti-tumor properties listed above, IL-27 has been reported to have direct effects on multiple myeloma through downregulation of pro-angiogenic genes in CD138+ myeloma cells, limiting osteoclast activity and promoting osteoblast formation, and inhibiting tumor growth in the absence of an immune response[450]. We hypothesized that forced expression of IL-27 by B cell maturation antigen (BCMA)-specific engineered T cells would supplement their functionality and enhance efficacy against myeloma and other BCMA-expressing tumors. In this study, we report the characterization of human T cells engineered to simultaneously express a BCMA-specific TAC and

a secreted recombinant IL-27. We addressed our hypothesis in human xenograft models of myeloma and mantle cell lymphoma.

RESULTS

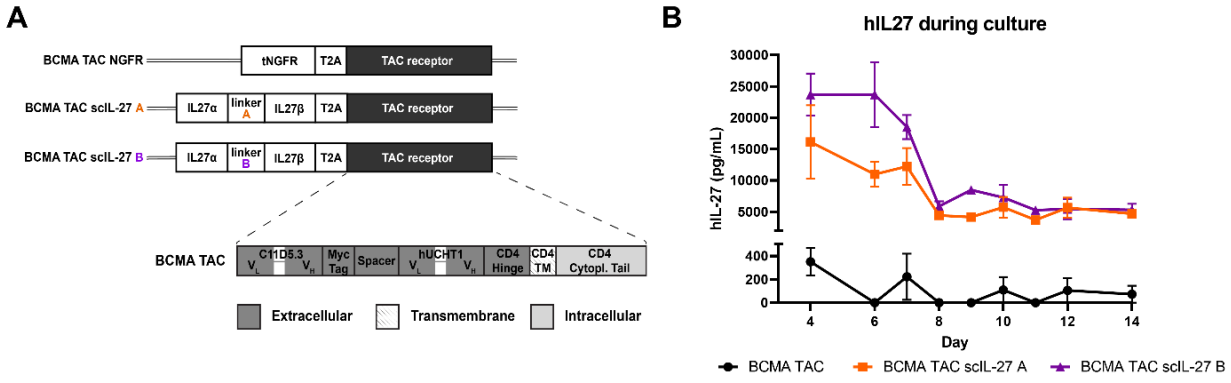
IL-27 expressed as a recombinant single-chain in TAC T cells is biologically active

Natural human IL-27 exists as a heterodimer comprised of Epstein-Barr Virus Induced 3 (EBI3) and IL-27p28 proteins[455]. To facilitate assembly of the heterodimer, we designed two secreted single-chain forms of IL-27 (scIL-27). We used short, flexible peptide linkers to bridge the two subunits together. Two peptide linkers were used, GGGGSGGGGSGGGGSGGGGS (used in the scIL-27 A variant), and GSGSSRGGSGSGGSGGGGSKL (used in the scIL-27 B variant). For our studies, the scIL-27 cDNAs were inserted upstream and in-frame with the TAC cDNA and separated by a 2A viral cleavage sequence (Figure 3-1A). As a negative control, the scIL-27 cDNA was replaced with a non-functional truncated nerve growth factor receptor (tNGFR; Figure 3-1A).

We engineered T cells from 2 healthy donors (L14, M51) and 2 myeloma patient donors (HB14, MM16) with the scIL-27 TAC and control tNGFR TAC vectors. We first confirmed production of the scIL-27 by ELISA using supernatants collected during T cell manufacturing. Both scIL-27 A and B were produced by the engineered T cells, with peak concentrations in the first 7 days of culture before declining to a stable equilibrium of ~5000 pg/mL for the remainder of the culture period (Figure 3-1B). Neither scIL-27 variant affected the CD4:CD8 ratio of the T cells following manufacturing (Figure 3-1C). TAC receptor density was reduced on the cells engineered to co-express scIL-27, possibly due to the larger multi-cistronic genetic sequence rather than a biological influence of the scIL-27 (Figure 3-1D). Overall transduction was somewhat reduced in the T cells engineered with scIL-27 A, but not with scIL-27 B (Figure 3-1E), which may be attributed to differences in viral titre. The presence of scIL-27 A or B during the manufacturing period did not affect the expansion or viability of the TAC T cells during the expansion period (Figure 3-1F-G, individual donor data in Supplemental Figure 3-1).

Suppression of GM-CSF in activated PBMCs is a known consequence of IL-27 signaling[459]. To confirm the scIL-27 was biologically active we stimulated healthy donor PBMCs with anti-CD3/anti-CD28 in the presence of supernatant from TAC T cells engineered to express scIL-27 or tNGFR and assessed the impact on GM-CSF production by ELISA. When compared to medium from tNGFR TAC T cells, the supernatant from scIL-27 TAC T cells suppressed GM-CSF production, demonstrating that our secreted scIL-27 is biologically active (Figure 3-2A). No difference in biological effect was observed between scIL-27 A and scIL-27 B.

Further evidence of biological activity was measured through characterization of signal transduction. IL-27 signaling results in phosphorylation and activation of STAT1 and STAT3[460]. We compared the basal phosphorylation state of STAT1 (pY701) and STAT3 (pY705) in scIL-27 TAC T cells to tNGFR TAC T cells supplemented with commercial recombinant IL-27 or media. Both scIL-27 A and B result in phosphorylation of STAT3 and, to a lesser extent, STAT1 (Figure 3-2B).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IL27α
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IL27β
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TAC receptor
BCMA TAC
C11D5.3 VL
VH
Myc Tag
Spacer
hUCHT1 VL
VH
CD4 Hinge
CD4 TM
CD4 Cytopl. Tail
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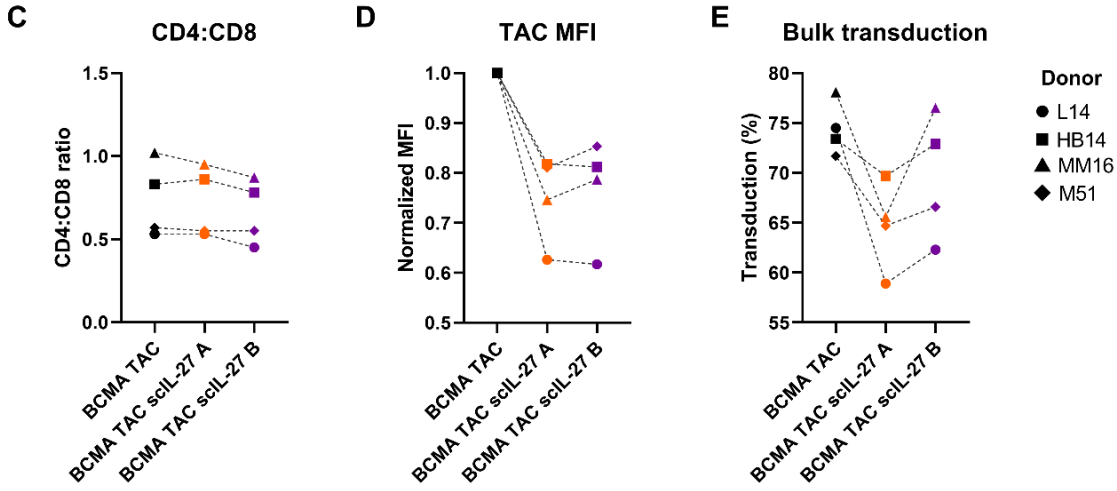
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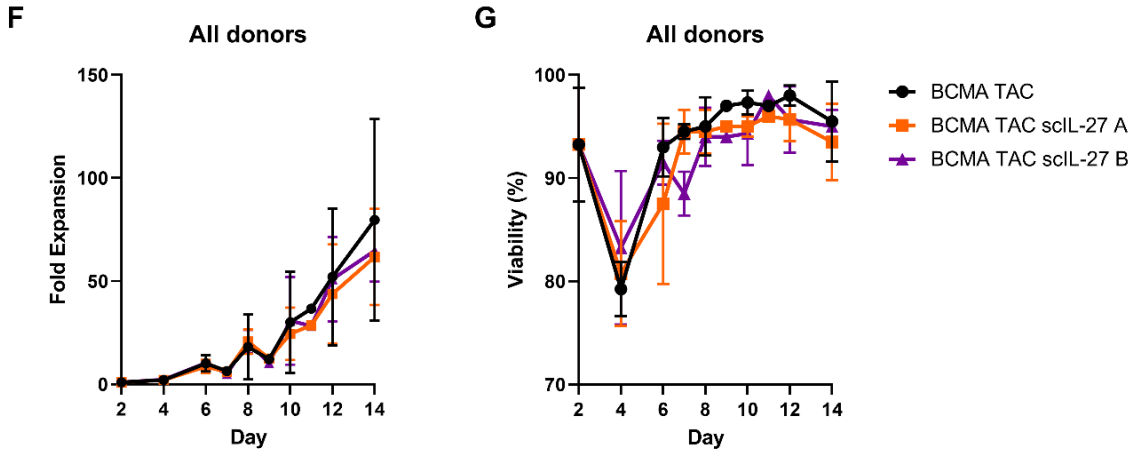
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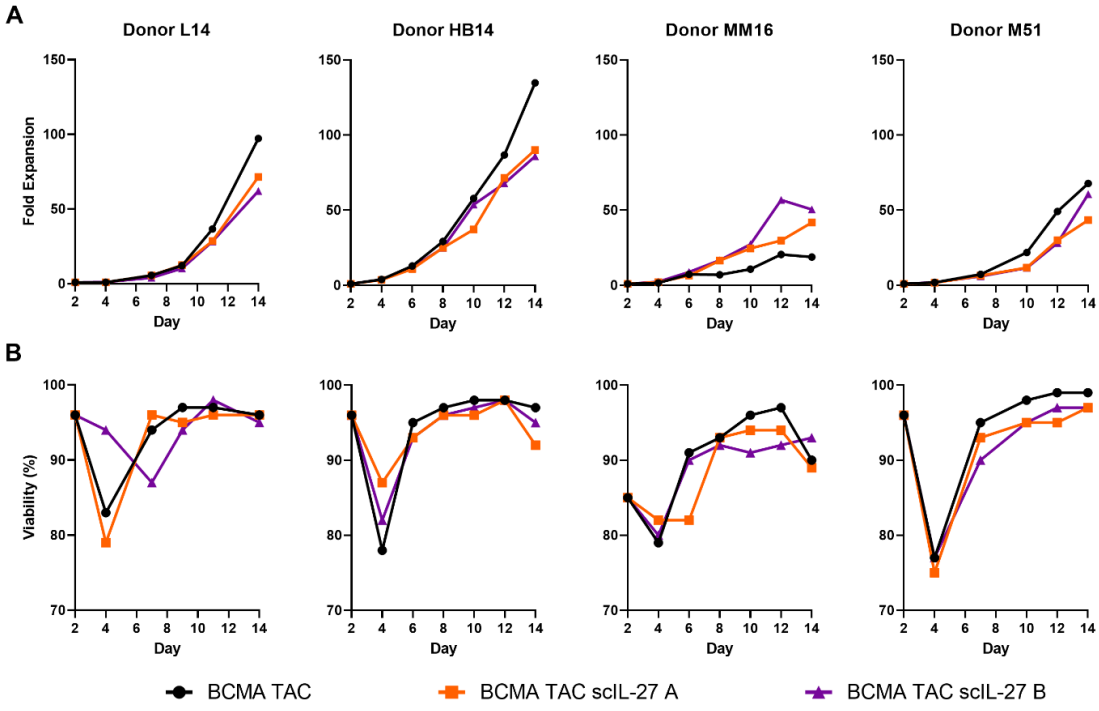
Figure 3-1. Expression of secreted scIL-27 does not affect the growth of TAC T cells. ***A*** Schematic diagram denoting the orientation of the scIL-27 and TAC, and the components of the TAC receptor. ***B*** Supernatants were diluted between 1:2 and 1:8 and assessed for hIL-27 by ELISA. Phenotype of the engineered T cells was assessed by flow cytometry for ***C*** CD4:CD8 ratio, ***D*** the mean fluorescence intensity of TAC expression, ***E*** and total transduction. ***F*** Growth kinetics and ***G*** viability were monitored during culture by trypan exclusion on a Countess II cell counter. n=4 comprising 4 individual donors.



Supplemental Figure 3-1. Individual donor expansion and viability. Growth kinetics and viability of T cells engineered and expanded from individual donor PBMCs.

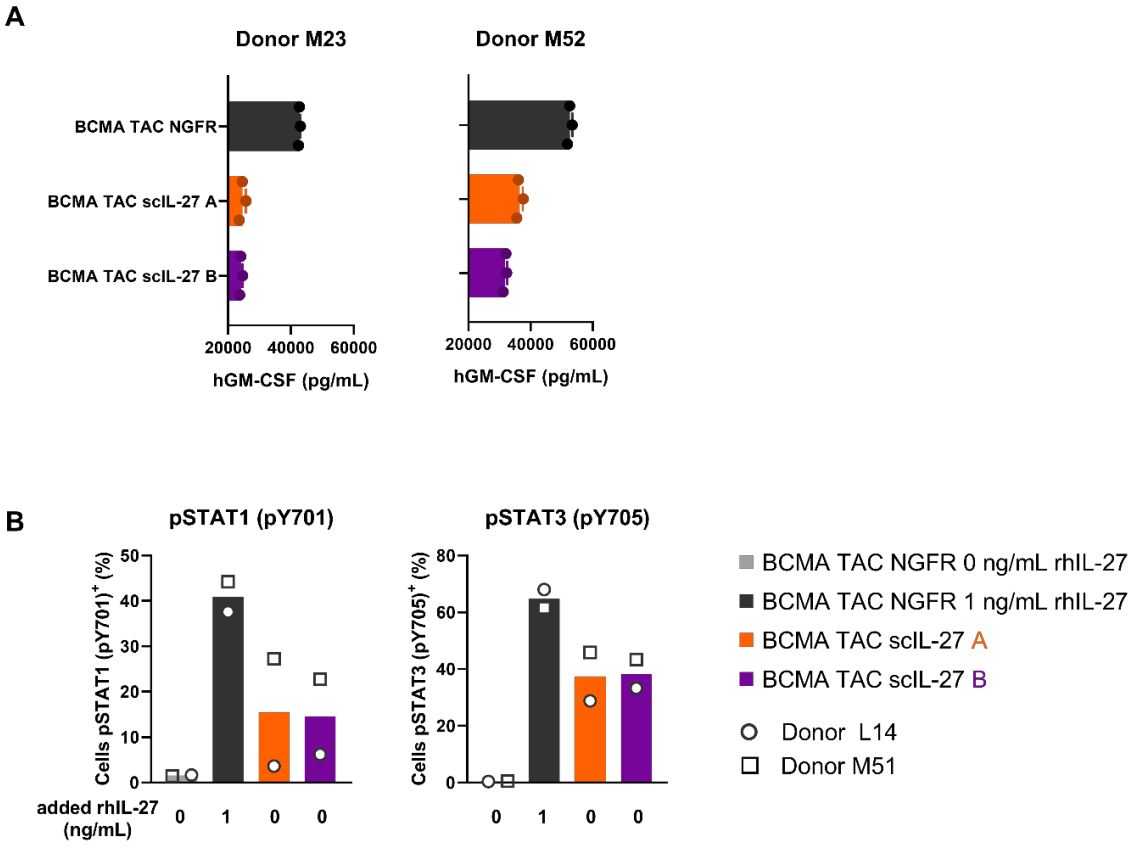
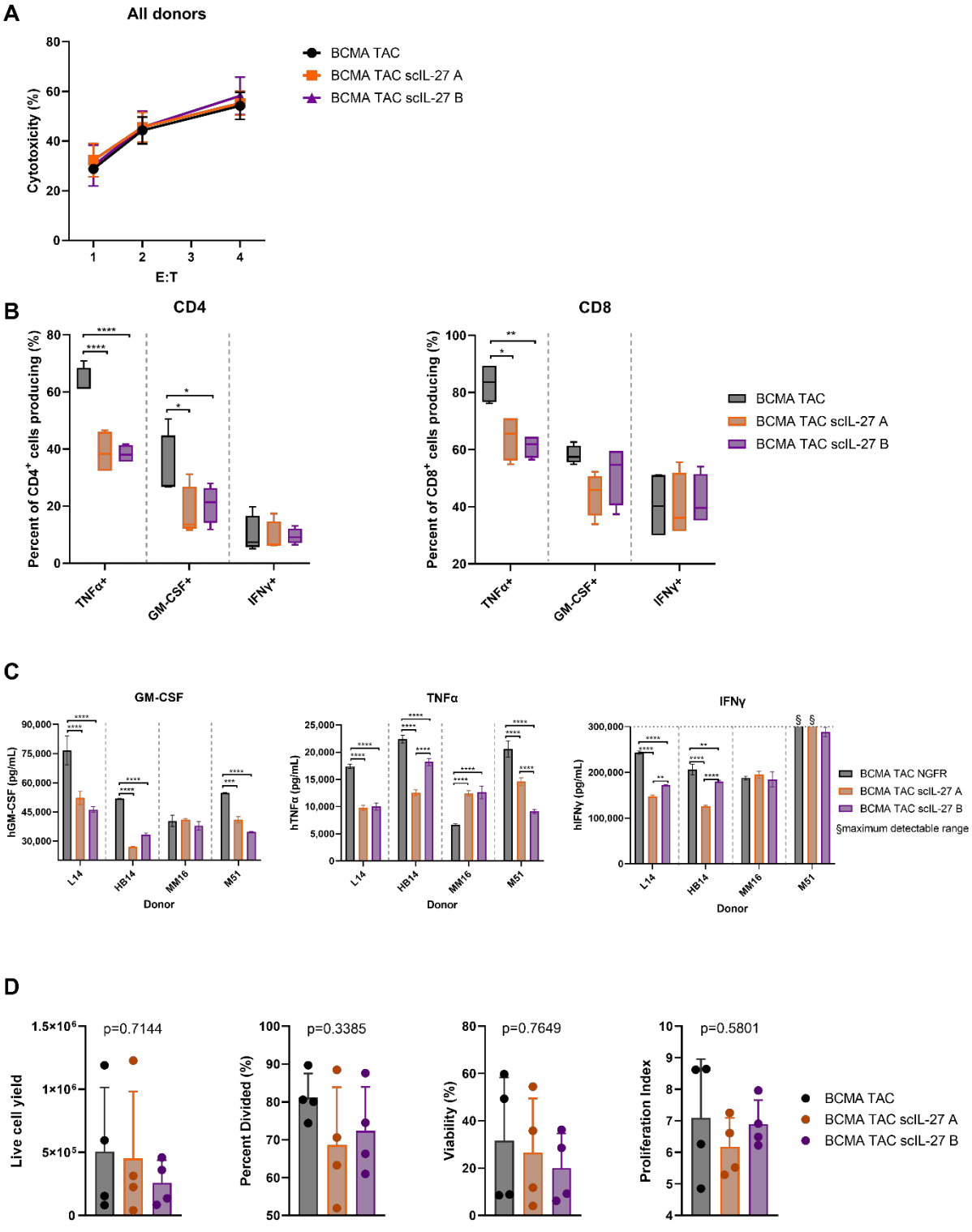


Figure 3-2. scIL-27 are biologically active and facilitate signal transduction through STAT1/3. ***A*** PBMCs were thawed and stimulated for 24 hrs with ImmunoCult soluble anti-CD3/CD28 in the presence of concentrated supernatant from tNGFR TAC T cells or scIL-27 TAC T cells. After stimulation, supernatants were collected and analyzed for GM-CSF by ELISA. n=1 per donor, in technical triplicate. ***B*** T cells were thawed, rested, then starved of cytokine for 24 hrs before stimulation with rhIL-27 or media for 30 minutes. After stimulation, cells were stained for pSTAT1 and pSTAT3, and analyzed by flow cytometry; n=2.

scIL-27 TAC T cells maintain anti-tumor activity with reduced inflammatory cytokine production

Given that IL-27 has been reported to enhance CTL function, we assessed the cytotoxic capabilities of the engineered T cells against SKOV-3 tumor cells which expressed BCMA fused to mCherry using live cell imaging (Figure 3-3A). Across all donors tested, we observed no consistent benefit of scIL-27 on the cytotoxic capabilities of the TAC T cells (pooled results are shown in Figure 3-3A; individual data are provided in Supplemental Figure 3-2). Consistent with the aforementioned suppression of GM-CSF, we noted that following antigen-specific stimulation with BCMA+ MM.1S myeloma cells, significantly fewer scIL-27 TAC T cells produced TNFα (both CD4+ and CD8+ T cells) and GM-CSF (CD4+ only) than control T cells expressing TAC and tNGFR (Figure 3-3B). There was no difference in the frequency of scIL-27 TAC T cells producing IFNγ compared to tNGFR TAC T cells. Similarly, there was marked reduction in the absolute magnitude of GM-CSF and TNFα produced over a 24-hr period and, to a lesser extent, IFNγ (Figure 3-3C). T cells from one of four donors (MM16) demonstrated no difference in the production of inflammatory cytokines in the presence of scIL-27, indicating some degree of donor influence. Finally, we characterized the proliferative capacity of the scIL-27 TAC T cells compared to tNGFR TAC T cells and observed no impact of the scIL-27 on absolute cell yield, viability, and proliferative capacity (Figure 3-3D). Of these differences observed *in vitro*, we noticed a trend towards greater reduction in proinflammatory cytokine production and proliferative capacity with the scIL-27 A variant than the scIL-27 B in most donors.

Using two xenograft tumor models, we further investigated the anti-tumor properties of the scIL-27 TAC T cells using sub-optimal T cell doses to provide an opportunity to resolve any improvements in efficacy due to the scIL-27. NRG mice were implanted with either JeKo-1 cells, a BCMA+ mantle cell lymphoma line, or MM.1S cells, a BCMA+ multiple myeloma line. After tumor establishment, $4x10^6$ engineered T cells were infused into the mice and tumor burden was monitored through bioluminescent imaging. This experiment was performed twice with T cells from different donors. Within the MM.1S model, scIL-27 TAC T cells performed similarly to the control tNGFR TAC T cells (Figure 3-3E-F). Within the JeKo-1 model, we observed a slight, albeit non-significant, increase in survival with the tNGFR TAC T cells compared to the scIL-27 TAC T cells (Figure 3-3H-G). All mice reached endpoint by tumor burden or study completion, other than one mouse which displayed symptoms consistent with GVHD (data not shown).


A
All donors
Cytotoxicity (%)
E:T
BCMA TAC
BCMA TAC scIL-27 A
BCMA TAC scIL-27 B
B
CD4
CD8
Percent of CD4+ cells producing (%)
Percent of CD8+ cells producing (%)
TNFα+
GM-CSF+
IFNγ+
C
GM-CSF
TNFα
IFNγ
hGM-CSF (pg/mL)
hTNFα (pg/mL)
hIFNγ (pg/mL)
L14
HB14
MM16
M51
Donor
BCMA TAC NGFR
BCMA TAC scIL-27 A
BCMA TAC scIL-27 B
§maximum detectable range
D
Live cell yield
Percent Divided (%)
Viability (%)
Proliferation Index
p=0.7144
p=0.3385
p=0.7649
p=0.5801

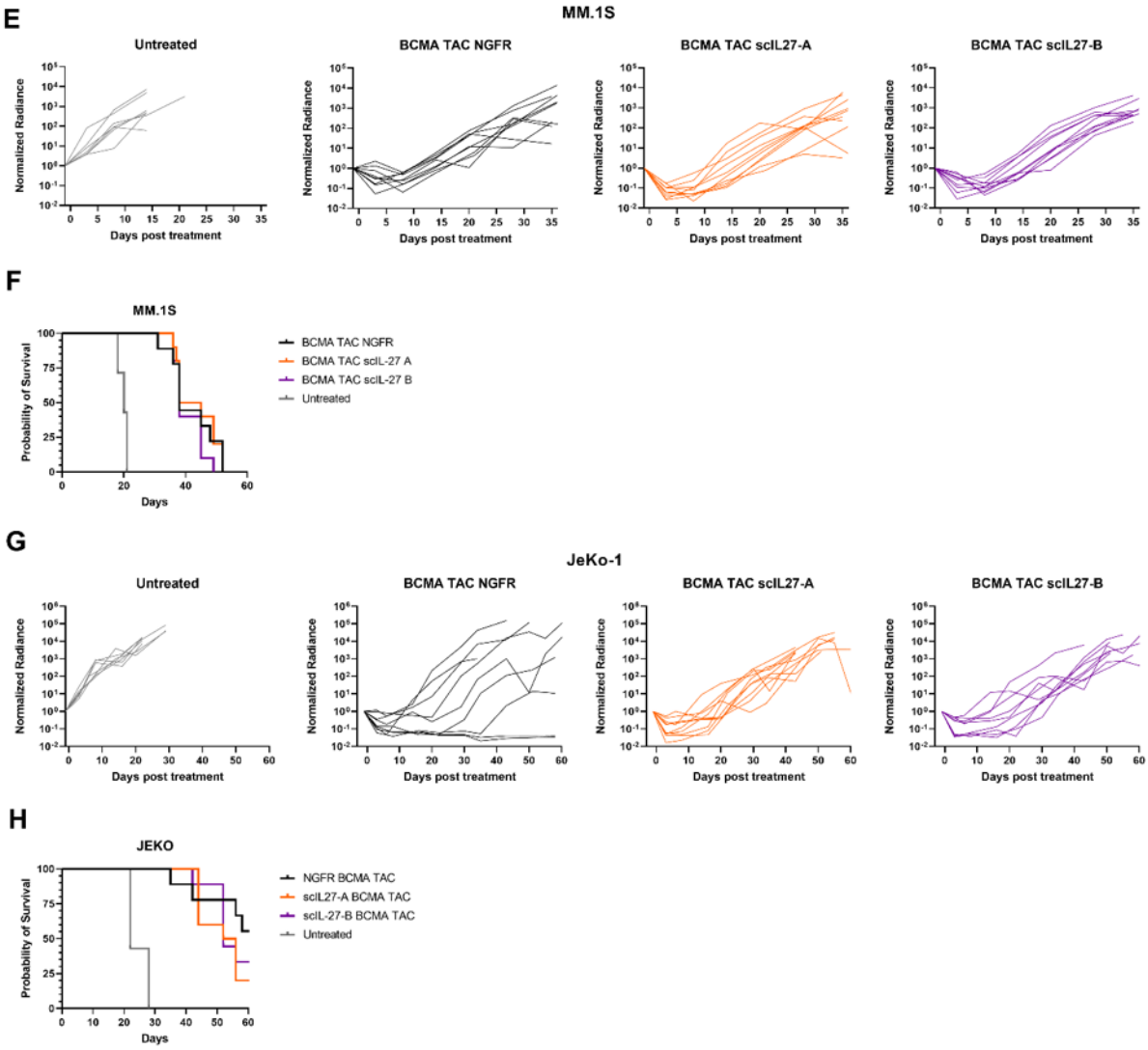
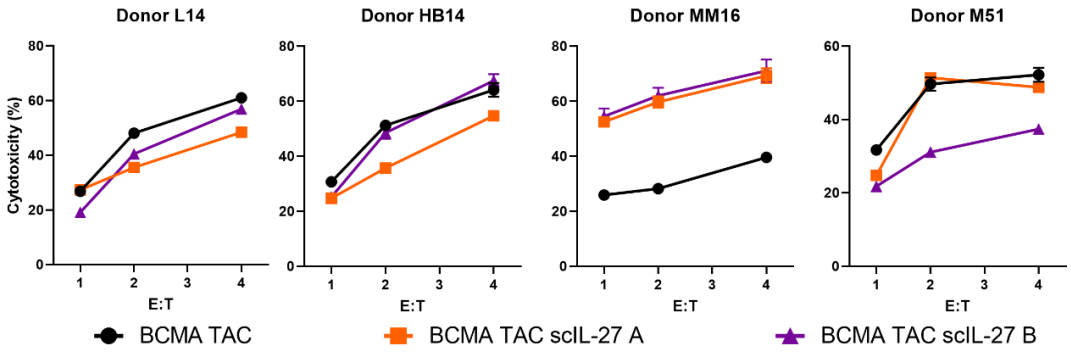

E
MM.1S
Untreated
BCMA TAC NGFR
BCMA TAC scIL27-A
BCMA TAC scIL27-B
Normalized Radiance
Days post treatment
F
MM.1S
Probability of Survival
Days
BCMA TAC NGFR
BCMA TAC scIL-27 A
BCMA TAC scIL-27 B
Untreated
G
JeKo-1
Untreated
BCMA TAC NGFR
BCMA TAC scIL27-A
BCMA TAC scIL27-B
Normalized Radiance
Days post treatment
H
JEKO
Probability of Survival
Days
NGFR BCMA TAC
scIL27-A BCMA TAC
scIL-27-B BCMA TAC
Untreated

Figure 3-3. scIL-27 TAC T cells maintain anti-tumor function with reduced pro-inflammatory cytokine production. ***A*** tNGFR and scIL-27 TAC T cells were co-cultured with BCMA+mCherry+ SKOV3 tumor cells at various effector to target ratios (E:T). Percent cytotoxicity was calculated by comparison of tumor cell only to that of tumor and TAC T cell co-cultured wells. ***B*** TAC T cells were stimulated 1:1 with MM.1S cells and brefeldin A for 4 hrs, then stained intracellularly for cytokines. ***C*** Supernatant collected from TAC T cells and MM.1S cocultures after 24 hr stimulation were assessed for cytokines by ELISA. ***D*** TAC T cells were labelled with CellTrace Yellow and stimulated with MM.1S tumor cells 1:1 for 96 hrs, after which T cells were stained and proliferation metrics were analyzed by flow cytometry and software modeling. Donor HB14 and L14 TAC T cells were assessed *in vivo* against bioluminescent ***E,F*** MM.1S and ***G,H*** JeKo-1 tumor models. Tumors were injected i.v. and established for 7-12 days, then treated with a single dose of $4x10^6$ T cells administered i.v.. A-D n=4 representing 4 donors; E-F n=10 per group in each tumor model, except for untreated where n=6.



Supplemental Figure 3-2. Cytotoxicity profiles of individual donor control or scIL-27 TAC T cells. tNGFR and scIL-27 TAC T cells were co-cultured with BCMA+mCherry+ SKOV3 tumor cells at the indicated E:T ratios for 96-144 hours and imaged every 4-6 hours using an IncuCyte S3 live cell imager.

DISCUSSION

To our knowledge, only one report has examined IL-27 in the context of adoptive T cell therapy of cancer[458]. Ding *et al.* investigated chicken egg ovalbumin-specific OT-1 T cells expressing murine IL-27 in a model where B16 melanoma cells were engineered to express chicken egg ovalbumin. The OT-1 T cells expressing mIL-27 exhibited enhanced tumor control and T cell survival compared to control OT-1 T cells. Here, we have reported that human T cells can be successfully engineered with a synthetic antigen receptor targeting the clinically relevant antigen BCMA and a recombinant single-chain IL-27 without affecting the manufacture and growth of the cells. We noted a slight decline to the transduction and TAC surface expression in the scIL-27 T cells that may be attributed to differences in viral titre or packaging due to increased retroviral genome size, although we cannot dismiss a possible role of IL-27 signaling. Consistent with the known effects of IL-27, the scIL-27 variants suppressed the expression of GM-CSF in activated PBMCs and induced STAT1 and STAT3 signaling in T cells, confirming the bioactivity of the scIL-27.

In contrast to the findings of Ding *et al.* we did not observe any enhanced anti-tumor activity when TAC T cells co-expressed scIL-27. This finding may be in part explained by the relatively lower concentration of hIL-27 produced by the engineered T cells in our study compared to the production of mIL-27 by the OT-1-IL-27 T cells. Ding *et al.* observed concentrations of mIL-27 that reached 50-100 ng/mL *in vitro* and 5-15 ng/mL *in vivo* whereas the concentration of hIL-27 measured in our cultures ranged was on the order of 5ng/ml *in vitro* (Figure 3-1) and undetectable *in vivo* 3d post-infusion (data not shown). It is possible that our T cells did not produce sufficient IL-27 to manifest suppression of myeloma growth *in vivo*. Future work will assess whether improvements to the vector design can enhance production of scIL-27 to establish if higher yields would benefit anti-tumor function as well as T cell expansion and survival.

Although we did not measure any impact of the scIL-27 on TAC T cell cytotoxicity, proliferation, or anti-tumor immunity, we did observe that scIL-27 led to reduced expression of pro-inflammatory cytokines. Cytokine release syndrome (CRS) is a major hurdle towards safe and effective engineered T cell therapy of cancer, often resulting in severe or even fatal toxicities. Macrophages are known to play an important role in potentiating CRS, with GM-CSF acting as a critical mediator in macrophage activation and subsequent adverse events[461]. In fact, GM-CSF inhibition has proven to be effective in mitigating CAR T cell toxicities in pre-clinical[462, 463] and clinical settings[464]. Additionally, TNFα down-modulation has also been demonstrated to alleviate CAR T cell toxicities[465, 466]. In our study we have shown that scIL-27 T cells exhibit significantly reduced

production of these two key pro-inflammatory cytokines while maintaining anti-tumor function and efficacy *in vitro* and *in vivo*, suggesting that this co-engineering strategy may be an effective way to reduce treatment-related adverse events without diminishing anti-tumor T cell potency.

MATERIALS AND METHODS

Cell lines

Human myeloma MM.1S cells (kindly provided by Dr. Kelvin Lee, Roswell Park Cancer Institute, NY) were cultured in RPMI 1640 (Gibco (Thermo Fisher Scientific), Waltham, MA), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop Canada Inc., Burlington, ON, Canada), 10 mM HEPES (Roche Diagnostics, Laval, QC, Canada), 1 mM sodium pyruvate (Sigma-Aldrich Canada Co. (Millipore Sigma), Oakville, ON, Canada), 1 mM nonessential amino acids (Gibco), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). Human JeKo-1 mantle cell lymphoma cells (purchased from ATCC (CCL-86; Manassas, VA)) were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. To generate enhanced firefly luciferase (effLuc)-expressing cell lines, parental MM.1S or JeKo-1 cell lines were transduced with lentivirus encoding enhanced firefly luciferase (effLuc) as well as puromycin N-acetyltransferase at an MOI 10 and selected in culture media supplemented with 8 µg/mL puromycin (InvivoGen, San Diego, CA). PLAT-E cells were cultured in DMEM (Gibco), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche Diagnostics), 10 µg/mL Blasticidin (InvivoGen), 1 µg/mL Puromycin (InvivoGen), and 100 µg/mL Normocin (InvivoGen). During transfection PLAT-E cells were cultured as above but without blasticidin or puromycin. PG13 cells (obtained from ATCC) were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche Diagnostics), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). All cells were cultured at 37 °C, 95% ambient air, 5% CO2.

Generation of scIL-27 transgene

The scIL-27 gene fragments were generated by synthesis (GenScript, Piscataway, NJ). Human IL27α (UniProt ID: Q8NEV9) and EBI3 (UniProt ID: Q14213) main chain subunit sequences were linked together using either a $(G_4S)_4$ (denoted as scIL27 A) or GSGSSRGGSGSGGSGGGGSKL (denoted as scIL27 B) linker. A human consensus Kozak sequence and human CD8α leader sequence were included at the 5' terminus. Restriction cloning was used to insert the scIL-27 into a pDONR transfer vector with NotI/SpeI cut sites. Final gammaretroviral transfer vectors were generated by gateway recombination (Gateway LR Clonase

II; Thermo Fisher Scientific, Waltham, MA) into the pRV100G vector. The scIL27 transgene was inserted 5' of the BCMA TAC[2]. The final vector sequences (from 5' to 3') under the control of the MSCV 5' LTR were composed of a human Kozak and CD8 α leader, scIL27 or tNGFR, T2A skip sequence, C11D5.3 BCMA scFv, $(G_4S)_4$ connector, huUCHT1(Y177T) variant[467], and human CD4 co-receptor domains (continuous from the flexible extracellular membrane-proximal D4-TM junction through transmembrane (TM) and cytoplasmic domains of human CD4), as detailed in Helsen et al. [349]).

Gammaretrovirus production

Amphotropic gammaretrovirus was produced by generating stably expressing PG13 producer lines. Briefly, $5x10^6$ low-passage PLAT-E plated in T75 flasks were transfected with plasmids pRV100G (10 µg) and pCL-Eco (10 µg) using Opti-MEM media (Gibco) and 45 µL Lipofectamine 2000 (Thermo Fisher Scientific). 12-16 hours post-transfection, media were exchanged to media supplemented with 1 mM sodium butyrate (Sigma-Aldrich). Ecotropic RV was collected after 48 hrs, filtered, concentrated using Amicon Ultra-15 100 kDa filters (MiliporeSigma, MA, USA), and stored at -80°C. Next, $1x10^5$ low-passage PG13 cells were plated in T25 flasks, then transduced with the frozen ecotropic RV daily for 3 days to produce amphotropic gRV expressing lines which were cryopreserved. Amphotropic gRV was prepared by thawing, resting, and splitting producer PG13 lines for 5 days, after which supernatant is collected, filtered through a 0.45 µm filter, and cryopreserved at -80 °C until used for T cell transduction.

Engineering human T cell products

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy and myeloma patient donors who provided informed written consent in accordance with the Hamilton Integrated Research Ethics Board. PBMCs were also collected from commercial leukapheresis products (HemaCare, Northridge, CA and STEMCELL Technologies, Vancouver, BC, Canada). PBMCs were isolated by Ficoll-Paque Plus gradient centrifugation (Cytiva, Vancouver, BC, Canada) and cryopreserved in inactivated human AB serum (Corning, Corning, NY), containing 10% DMSO (Sigma-Aldrich) or in CryoStor10 (STEMCELL Technologies). Post-thaw, PBMCs were stimulated with ImmunoCult soluble anti-CD3/28/2 tetrameric complexes (STEMCELL Technologies) and cultured in RPMI 1640 (Gibco) containing 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche), 1 mM sodium pyruvate (Sigma-Aldrich), 1 mM non-essential amino acids (Gibco), 55 µM β-mercaptoethanol (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 1.5 ng/mL rhL-2 and 10 ng/mL rhIL-7 (PeproTech, Cranbury, NJ). Non-tissue culture treated 24-well plates were coated

with 50 μg/mL RetroNectin (Takara Bio, Kusatsu, Shiga, Japan) overnight at 4°C, then loaded with amphotropic gRV supernatant and centrifuged for 2 hrs at 2000 RCF. Leftover supernatant was aspirated and the activated PBMCs loaded onto gRV-coated plates. Culture yields were enumerated every 2-3d and supplemented with cytokine-containing media (1.5 ng/mL rhIL-2 + 10 ng/mL rhIL-7) to dilute cultures to ~ 1.0×10^6 cells/mL. Engineered T cell products were expanded for a total culture period of 14-15 days prior to use or cryopreservation. For cryopreservation, cells were cryopreserved in CryoStor10 according to manufacturer's directions at either 20×10^6 cells/mL (for downstream *in vitro* assays) or such that a desired effective dose would be obtained in 200 μL (for downstream use *in vivo*). Prior to use of cryopreserved TAC T cell products in any *in vitro* assay, cells were thawed and rested for 24 hrs in cytokine-containing media (as above).

Flow cytometry

Flow cytometry data were collected with Beckman Coulter CytoFLEX LX (NUV/V/YG/B/R laser configurations) and analyzed using FCS Express v7 Software (DeNovo Software, Pasadena, CA).

Phenotypic characterization of T cell products

Surface expression of BCMA TAC constructs was determined by staining with recombinant human BCMA-Fc protein (Cat No.193-BC, R&D Systems, Minneapolis, MN), followed by PE-conjugated goat anti-human IgG (Cat No. 109-115-098, Jackson ImmunoResearch, West Grove, PA). Other phenotypic markers were detected with Pacific Blue (Cat No. 558116, BD Biosciences, Mississauga, ON, Canada) or AlexaFluor700 (Cat No. 56-0048-82, eBioscience (Thermo Fisher Scientific))-conjugated mouse anti-human CD4, AlexaFluor700 (Cat No. 56-0086-82, eBioscience) or PerCP-Cyanine5.5 (Cat No. 45-0088-42, eBioscience)-conjugated mouse anti-human CD8α, and VioBright FITC (Cat No. 130-104-893, Miltenyi Biotec)-conjugated mouse anti-human NGFR. Staining was assessed by flow cytometry.

Phospho-STAT signaling characterization of T cell products

Cryopreserved engineered T cells were thawed into T cell media supplemented with rhIL-2 and rhIL-7 and let to rest overnight. Cells were washed and placed into base T cell media without cytokines and rested again overnight. 7.5×10^5 BCMA TAC NGFR T cells were stimulated with 0 or 1 ng/mL rhIL-27 (Cat No. 200-38, PeproTech) for 30 minutes at 37°C. scIL-27 TAC T cells were incubated similarly but without any rhIL-27. Cells were then immediately fixed with an equal volume Cytofix (BD Biosciences), washed, then permeabilized with Phosflow Perm III (BD Biosciences) for 30 minutes on ice as per manufacturer's

instructions. Cells were washed twice with FACS buffer then resuspended in 100 µL FACS per $1x10^6$ cells and stained with PE-conjugated anti-pSTAT1 pY701 (Cat No. 612564, BD Biosciences) and AlexaFluor647-conjugated anti-pSTAT3 pY705 (Cat No. 557815, BD Biosciences) as per manufacturer's instructions. Cells were then analyzed by flow cytometry.

In vitro cytokine quantification by enzyme-linked immunosorbent assay

Supernatants were collected from cell culture or *in vitro* stimulations and centrifuged to remove any particulates. Samples were diluted between 1:2 to 1:500 and analyzed by R&D DuoSet ELISA as per manufacturers guidelines for the following analytes: hIL-27 (Cat No. DY2526), hTNFα (Cat No. DY210), hIFNγ (Cat No. DY285B), and/or hGM-CSF (Cat No. DY215). ELISA were developed using either the R&D ancillary reagent kit (Cat No. DY008), or KPL SureBlue TMB reagent (Cat No. 5120-0075, Seracare, Milford, MA, USA), and analyzed with a SpectraMax i3 (Molecular Devices, San Jose, CA, USA).

Intracellular cytokine staining assay

$5x10^5$ engineered T cells were stimulated 1:1 with MM.1S cells for 4 hours at 37 °C in the presence of brefeldin A (BD GolgiPlug, Cat No. 555029, BD Biosciences). Cells were stained for surface expression of CD4 and CD8 (as above), fixed and permeabilized in Cytofix/Cytoperm buffer (Cat No. 554714, BD Biosciences), and stained with APC-conjugated mouse anti-human IFNγ (Cat No. 554702, BD Biosciences), PE-conjugated anti-human GM-CSF (Cat No. 502306, BioLegend), Brilliant Violet 605-conjugatged mouse anti-CD3(Cat No. 300460, BioLegend), and FITC-conjugated mouse anti-human TNFα (Cat No. 554512, BD Biosciences). Flow cytometry data were acquired and analyzed as indicated above.

In vitro cytotoxicity assay

SKOV-3 cells engineered to express human BCMA and mCherry were plated one day in advance at $2x10^3$ cells per well of a flat-bottom 96-well plate. Engineered T cells were co-cultured in triplicates at 4:1, 2:1 and 1:1 effector:target ratios for 96-144 hours at 37 °C in an IncuCyte S3 live cell imager (Sartorius AG, Göttingen, Germany). Wells were imaged every 4-6 hours. Tumor cells were masked by fluorescence and cell size/elongation, and image red mean over time was calculated by IncuCyte software. Area under the curve (AUC) analysis was completed on the resultant red mean curves and percent cytotoxicity was calculated by comparison of signal to tumor alone control wells: % Cytotoxicity = (Tumor alone AUC mean – Sample AUC mean)/(Tumor alone AUC mean)*100%.

Proliferation assay

Engineered T cells were labelled with CellTrace Yellow dye (Thermo Fisher Scientific, Cat No. C34573) and stimulated with MM.1S tumor targets at a 1:1 effector:target ratio (non-stimulated control wells were also plated). After 4d at 37°C, co-cultures were stained with Live/Dead Fixable Near-IR stain (Thermo Fisher Scientific Cat No. L10119), Alexa Fluor 700-mouse anti-human CD8α (Cat No. 56-0086-82, eBioscience), Pacific Blue-conjugated mouse anti-human CD4 (Cat No. 558116, BD Biosciences), Brilliant Violet 605-conjugated anti-human CD3 (Cat No. 300460, BioLegend), and Brilliant Violet 785-conjugated mouse anti-human CD138 (Cat No. 356537, BioLegend). Flow cytometry data were acquired as indicated above. Results were analyzed using Proliferation Plots modeling in FCS Express 7. In short, non-stimulated control T cells were used to identify and fix a Starting Generation within Proliferation Fit Options. Proliferation Fit Statistics were used to calculate the percent of original cells that divided (% Divided) and proliferation index.

Xenograft tumor models

All animal studies were approved by the McMaster Animal Research Ethics Board. 6-8-week-old NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ (NRG) mice (Strain #007799, The Jackson Laboratory, Bar Harbor, ME) were bred in-house and injected intravenously (i.v.) with 1x10^6 MM.1S-effLuc or 0.5x10^6 JeKo-1-effLuc tumor cells in 200 µL PBS. Mice bearing 12-day-old (MM1.S) or 7-day-old (JeKo-1) tumors were treated i.v. with engineered TAC T cell products. Cryopreserved T cell products were thawed at 37°C in a shaking heat block and immediately injected (200 µL dosing prepared to contain 4×10^6 TAC+ T cells). Tumor burden was monitored by bioluminescent imaging, beginning 1d prior to treatment, and approximately weekly thereafter. Mice were anesthetized, injected intraperitoneally with 150 mg/kg D-luciferin (Perkin Elmer), and imaged for bioluminescence using an IVIS Spectrum (Perkin Elmer) ~14 minutes later. Images were analyzed with Living Image Software v4.2 (Perkin Elmer). Tumor burden was quantified as the sum of whole-body dorsal and ventral average radiance (p/s/cm^2/sr).

Statistics

All statistical analyses were performed in GraphPad Prism version 9.2 for Windows (San Diego, CA). An ordinary one-way ANOVA was used to compare the means of three or more unmatched groups. Two-way ANOVA was used to compare the means of three or more unmatched groups when considering multiple donors. Post hoc pairwise comparisons were made using Tukey's multiple comparisons test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, and ns = not significant.

Chapter 4 – Discussion

CAR T cells have proven to be a particularly efficacious method to treat relapsed/refractory multiple myeloma, albeit with significant challenges. Paramount amongst these difficulties are treatment-associated toxicities which can be severe or fatal, limiting the accessibility of frail patients to these therapies. Reducing the incidence of adverse events will decrease treatment costs and increase the therapeutic index, allowing for more widespread utility outside of specialist centers.

CAR T cell related toxicities may be driven in part by non-canonical signaling originating from the CAR, which provides potentially unregulated and tonic stimulation. TCR-centric synthetic antigen receptors, such as the TAC receptor, activate T cells through natural signaling pathways and maintain anti-tumor efficacy with reduced potential for toxicity[347]. Pre-clinical work from our lab comparing anti-HER2 TAC T cells to CAR T cells bearing the same antigen-binding domain showed proof-of-concept in reducing toxicities[349].

The design of the TAC receptor limits potential toxicities; however, TAC T cells may still benefit from costimulation. My thesis work sought to augment the anti-tumor activity of TAC T cells without introducing further modifications to the TAC receptor itself. To this end, I investigated two separate strategies using exogenous mediators to enhance T cell function and suppress myeloma fitness: **i) small molecule drugs to deliver critical costimulatory effects to mimic *signal two* in T cells**, and **ii) forced expression of IL-27 to deliver *signal three* to T cells and reshape the myeloma microenvironment**.

4.1 LCL161 for transient T cell costimulation

The discovery of IAPs and their overexpression in cancer led to the development of SMAC mimetic IAP antagonist drugs[470]. While their utility as chemotherapeutics is under substantial clinical investigation, less research has been conducted on how SMAC mimetics impact immune-related functions and immunotherapeutics. There is reasonable rationale for combining SMAC mimetics with engineered T cell therapy to modulate anti-tumor efficacy. IAP antagonism can:

- sensitize cancer cells to TNFα/death-receptor-mediated cytotoxicity[374, 375],
- activate ncNF-κB signaling in T cells[371],
- and promote anti-tumor effects in local immune cells such as macrophages[391].

Indeed, though an unbiased exploration of pathways that enhance CAR T cell killing, a combined drug/genome CRISPR screen identified SMAC mimetics and their effects on death-receptor signaling (specifically TRAIL-2) as enhancers of

CAR T cell cytotoxicity through a RIPK1- and FADD-mediated mechanism in B cell acute lymphoblastic leukemia cell lines[468]. These results further support the rationale for combining engineered T cell therapies with SMAC mimetics.

I investigated whether the SMAC mimetic LCL161 can be used to provide transient costimulation to TAC T cells and supplement their expansion and survival following interaction with BCMA, a key multiple myeloma target. Additionally, given that TAC T cells produce high levels of TNFα following ligation of BCMA, the combination with SMAC mimetics could sensitize myeloma cells to TNFα-mediated cytotoxicity.

Using a set of healthy and myeloma donor-derived TAC T cells I confirmed that LCL161 enhanced survival and expansion when TAC T cells were stimulated with antigen-coated beads. These beads were labeled with a concentration of BCMA-Fc chimeric antigen that triggered low levels of proliferation (on average ~10-40% of TAC T cells enter division). However, I did not observe a similar enhancement when LCL161 was included with myeloma cell stimulation. Stimulation with myeloma cells is considerably more robust, on average ~75-100% of TAC T cells entered division when stimulated with myeloma cells alone. We determined that the myeloma lines used expressed multiple costimulatory ligands, which may supersede the benefits from LCL161 and achieve near-maximal stimulation.

Perhaps unsurprisingly, a non-linear dose-dependent effect became apparent when higher concentrations of LCL161 were combined with bead-stimulated TAC T cells. Beyond an undetermined threshold, LCL161 no longer augmented viability and instead increased the fraction of apoptotic T cells. Collectively, two factors may have contributed to this phenotype: i) TAC T cells express high levels of the death receptor Fas, and often also co-express its ligand FasL, and ii) SMAC mimetics reduce the apoptotic threshold. Combined, these effects may result in fratricide (from adjacent T cells with FasL) and/or T cell cytotoxicity (from FasL expressed on myeloma cells). Therefore, we elected to genetically delete Fas in TAC T cells and investigate outcomes at both low and high concentrations of LCL161. Fas deletion reduced the apoptotic propensity of low or high dose LCL161 when included in the context of the bead-based stimulation, bringing T cell apoptosis values in line with MM.1S + LCL161 stimulated TAC T cells. These data suggest that fratricide was occurring between the T cells, and the more robust stimulus from myeloma cells containing costimulatory ligands may compensate for the effects of Fas/FasL interaction. There was no apparent change in the frequency of apoptosis when Fas KO TAC T cells were stimulated with myeloma cells and LCL161, which again may be due to the presence of costimulatory ligands. Alternatively, as LCL161 converges on multiple pathways

involved in cellular death – inhibition of XIAP, and TNF-related death receptor signaling – targeting a single node such as Fas may not account for redundant death signaling facilitated by LCL161.

Transcriptional profiling of LCL161-treated bead-stimulated TAC T cells revealed several differentially expressed genes, two of which were of particular interest to me: TNFRSF8 and FCMR. Both proteins encoded by these genes have known impacts on costimulatory and apoptotic pathways in T cells. FCMR encodes the Fc receptor for IgM molecules (FcμR), which is also known as FAIM3 (Fas apoptosis inhibitory molecule 3) or Toso, which has not been associated with SMAC mimetics before. It is interesting that FAIM3 was one of the most highly downregulated transcripts given it was originally discovered and presumed to function as an antiapoptotic inhibitor of Fas through its interactions with RIPK1[469-471]. This function was contested in the early 2010s with some evidence for no protective effect in murine T cells, while others showed protective effect in human PBMCs and T cell lines[472-474]. Currently, it is commonly identified as the Fcμ receptor[475], although some recent evidence also suggests that FAIM3 has a costimulatory function in human T cells[397]. My observations with FAIM3 overexpression did not serve to elaborate on its biology; the effects were variable amongst the 3 donors tested and likely would require a greater breadth of experimentation to elucidate its role with LCL161. Given its potential association with degradation of RIPK1, combining Fas KO and forced expression of FAIM3 may provide more information.

I also discovered upregulation of *TNFRSF8* 24 hrs after stimulation with LCL161 that dropped by 72 hrs. Weak upregulation (~1.3 fold) of *TNFRSF8* by SMAC mimetics has been shown before, albeit with the bivalent SM-164 on the large T cell lymphoma line Karpas 299 after 3 hrs of stimulation[410]. Similar to FAIM3, the reported effects of CD30 activation are somewhat conflicting, and there are few specific data for primary T cells. CD30 signaling activates both canonical and non-canonical NF-κB pathways[476], which may explain the high expression of CD30 in several lymphomas. Like SMAC mimetics, CD30 activation leads to the degradation of cIAP1/2 and ncNF-κB activation[409, 410], although this is accomplished through a mechanism that does not involve autoubiquitination and instead requires TRAF2. The effects of CD30 agonism are still not well understood, with some studies indicating CD30 signaling leading to apoptosis and cell cycle arrest[476, 477]. It should be noted that the majority of CD30 studies to-date have used tumor cell lines and their conclusions may not apply to primary T cells, although an early study reported CD30-mediated inhibition of T cell proliferation[478]. The effects of CD30 on CAR T cells have been evaluated in two studies: work by Hombach found that antagonism or depletion of CD30+ T cells improved the

cytotoxic function of anti-CEA CAR T cells[399]; conversely, a meeting report utilizing the CD30 signaling domain within the CAR design in αβ and γδT cells found these performed similarly to CAR T cell designs utilizing conventional costimulatory domains[479]. Given the above information and that both CD30 and its ligand CD30L (CD153) are expressed on activated T cell subsets[480], we had predicted that deletion of CD30 would enhance TAC T cell function. However, my work showed that, in comparison to non-edited TAC T cells, CD30 KO TAC T cells displayed impaired proliferation following stimulation with antigen-coated beads and LCL161. Given that these findings contrast with other reports on the role of CD30 on T cells, there is merit to investigate further on manipulating CD30 signaling in TAC T cells. Deletion of CD30 from the beginning of T cell manufacture is confounded by whether CD30 signaling after activation shapes the T cell population. An alternate strategy to address this question could be to include recombinant CD30L on the stimulatory beads or include antagonistic CD30 blocking antibodies during stimulation.

I did not observe any enhancement to T cell cytotoxicity of myeloma cell lines by LCL161 *in vitro*, except at the highest dose. Furthermore, our *in vivo* evaluations also produced no change in anti-tumor efficacy. As I have employed a xenograft system in immunodeficient mice, the immune repertoire is altered and may not reflect the potential of LCL161 with TAC T cells to recruit other immune cells towards anti-myeloma immunity.

LCL161 has undergone several phase I and II clinical trials[381]. Early data indicated a 1800 mg dose was generally well tolerated and higher doses induced dose-limiting adverse events including CRS[481]. Follow-up studies have shown greater serious adverse events in mono- and combination therapy[482, 483]. Overall clinical studies of LCL161 have not shown much efficacy as monotherapy or in combination therapy (NCT02890069; results posted online). However, there is some indication of moderate efficacy in combination with paclitaxel in specific indications such as myelofibrosis[484], or TNFα gene signature-positive triple-negative breast cancer[482]. In multiple myeloma, a phase II study (NCT01955434) of LCL161 therapy with or without cyclophosphamide in 25 RRMM patients did not show single agent activity with an ORR of 0% as monotherapy and a median PFS of 10-months[391]. Novartis has recently terminated 2 trials (NCT02649673 and NCT01968915) with LCL161 due to a change in their clinical programs, suggesting a change in course for the use of LCL161.

Given the unfavourable clinical results, and the data generated from my study, I do not believe there is value in continued studies of combining LCL161 with TAC T cells for myeloma. The pan-IAP antagonism of LCL161 may limit its utility as an enhancer of T cell function because concurrent sensitization to

apoptosis may offset any costimulatory benefits. Novel approaches for direct and specific degradation of cIAP1/2 using proteolysis-targeting chimera (PROTAC) molecules may overcome this limitation. PROTACs provide highly-specific protein destruction using the ubiquitin-proteasome pathway[485, 486]. For example, a PROTAC specifically designed to degrade cIAP1/2 would initiate non-canonical NF-κB signaling without triggering apoptosis through unwanted effects on XIAP.

4.2 Expression of recombinant IL-27 to supplement anti-myeloma activity

Engraftment, survival, and persistence of adoptively transferred engineered T cells are critical determinants of success in T cell therapies for cancer. Considerable research has been concentrated on bolstering these properties in engineered T cells. Manipulation of pathways involved in T cell homeostasis can be used to impart signals or programs that aid in T cell anti-tumor function. The integration of many signaling networks, both inhibitory and stimulatory, functions as a rheostat to balance immune responses, which allows a certain level of biological "*tuning*". Many of these pathways are key participants found in the induction of a T cell response, which is a highly orchestrated process with well-defined parameters to initiate, maintain, and subsequently contract the response. Cytokines are one such pathway as they can dictate broad changes in immune function by directing T cell responses and function.

Encoding expression of T cell supportive cytokines within the transgene cassette of engineered T cells is an effective tool for protecting ("*armoring*") and augmenting the performance of the engineered T cells. Furthermore, the deployment of a cytokine payload by engineered T cells can modulate surrounding endogenous immune cells and modify the tumor microenvironment leading to enhanced anti-tumor effects[487].

Several cytokines have been investigated as a means to arm engineered T cell therapies, including IL-2, IL-12, IL-15, and IL-18[431]. IL-27 has not been studied to the same extent, but we recognized many features that suggested it would be useful in this application. IL-27 can promote the survival and expansion of CTLs, support the cytolytic function of NK cells and CTLs by inducing perforin and granzyme B expression, inhibit regulatory T cells, and polarize a T_H1 response. Of particular interest, IL-27 has been shown to have anti-tumor effects on multiple myeloma patient samples *in vivo* by downregulating pro-angiogenic genes in CD138+ myeloma cells, limiting osteoclast activity and promoting osteoblast formation, and inhibiting tumor growth in the absence of an immune response by disrupting intratumoral vasculature development[450].

Therefore, I pursued forced expression of IL-27 in anti-BCMA TAC T cells as a proof-of-concept study. In parallel with my research, Ding et al. recently

described engineered murine IL-27 (mIL-27) secretion by mouse T cells. Using murine OT-1 T cells armed with mIL-27 against tumors with transgenic chicken ovalbumin (OVA) expression, they observed greater CD8+ T cell activation (based on expression of CD69 and CD25), proliferation, production of inflammatory cytokines, and cytotoxic capabilities, with reduced apoptosis[458]. These results were encouraging and mIL-27-expressing OT-1 cells mediated superior anti-tumor efficacy *in vivo*.

I designed a recombinant single-chain human IL-27 (schIL-27) molecule and validated its expression and biological activity through functional assays and assessment of STAT1 and STAT3 signaling. However, I observed several discordant findings to those of Ding et al. Expression of this schIL-27 by TAC T cells did not improve *in vitro* anti-tumor cytotoxicity or *in vivo* anti-tumor efficacy. These findings may be due to lower output of schIL-27 generated by the TAC T cells compared to the mIL-27 produced by the transgenic OT-1 cells. This is an inferred hypothesis; in my *in vitro* cultures schIL-27 concentrations decline from ~30-40 ng/mL after activation until they reach an equilibrium of ~5 ng/mL at ~7d post activation. The murine OT-1-IL-27 T cells produced ~10-100 (mean 50) ng/mL by day 5 of culture, which was the latest time point that they evaluated. Furthermore, we were unable to detect schIL-27 in the serum of mice following infusion of IL-27-expressing T cells, although it is possible that the TAC T cells were residing primarily within the tumor and not producing high enough IL-27 levels to be measured in the circulation. In contrast, mIL-27 was readily measurable in the serum of mice treated with murine OT-1-IL-27 T cells where serum concentrations rose steadily to a level of ~10-15 ng/mL by day 20. It is impossible to fairly compare these concentrations given the differences in T cell manufacturing methodology, and the differences in syngeneic and xenograft murine models, but these data do suggest that sustained, high-level IL-27 expression is required to achieve therapeutic benefit.

Although we did not observe any change to anti-tumor efficacy compared to control cells, we did see an intriguing effect on the expression of GM-CSF and TNFα, two cytokines that are critical to the generation of CRS in patients receiving CAR T cell therapy. Two models of CRS without GVHD have been developed that could be employed to determine if the expression differences induced by schIL-27 would manifest as meaningful in controlling engineered T cell-mediated toxicities. The work by Giavridis used immunodeficient beige mice to generate a CRS-like pathology[488]. These mice bear functional monocytes and macrophages which are producers of IL-6 and IL-1, two cytokines thought to underpin CRS toxicities. The Giavridis model requires intraperitoneal injection of $3x10^6$ Raji tumor cells which are grown for 3 weeks to produce a large mass followed by

infusion of a bolus of 30 million CAR T cells, which is 5-10 times larger than the number of CAR T cells typically administered in mouse xenograft models. It is thus difficult to know if this model is truly reflective of CRS as the relative tumor burden, its location in the peritoneum, and T cell dose far exceeds a scaled representation of the human situation. The model developed by Norelli et al. is more complicated but better represents the human system[489]. Using xenotolerant human T cells derived from humanized mice, CAR T cells were generated against two model antigens and adoptive transfer into tumor-bearing humanized mice generated a CRS-like pathology. In this model, the authors implanted $5x10^6$ or $10x10^6$ patient-derived ALL-CM leukemic cell line intravenously to generate low or high tumor burden, respectively. ACT of $2x10^6$ CAR T cells, a more representative dose of CAR T cells, resulted in pathology that correlated with tumor burden. Unlike the previous model, the authors also observed delayed-onset neurotoxicity in this system. Both models implicate the importance of IL-1 and IL-6 in their pathology, which is reflective of CRS in patients.

There are several caveats to consider in the context of forced expression of IL-27 by engineered T cells. Constitutive expression of schIL-27 may result in the generation of a Tr1 regulatory phenotype[490] during manufacturing that may dampen anti-tumor responses. This can be assessed by analyzing surface expression of CD49b and LAG3[491], which I did not do, however given that IL-27-expressing TAC T cells produced tumor regression comparable to control TAC T cells, it seems unlikely that a regulatory phenotype was conferred to the product with constitutive IL-27 expression. A key shortcoming from my work is the inability to confirm IL-27R in the TAC T cells due to the lack of suitable reagents to measure the receptor by flow cytometry. Thus, it is possible that a fraction of schIL-27-expressing TAC T cells are not responding to the schIL-27. Indeed, only 60% - 80% of control TAC T cells demonstrated a response to commercial rhIL-27 as defined by increased levels of pSTAT1 and pSTAT3, which suggests that not all of the control T cells can respond to IL-27. Further, a smaller fraction of the schIL-27-expressing T cells displayed evidence of STAT1/STAT3 phosphorylation, which suggests that the T cells may have adapted to the constitutive schIL-27 expression and have either reduced the expression IL-27R or have modified the downstream signaling.

Given that constitutive expression of schIL-27 may lead to undesirable changes in IL-27R signaling, future work may evaluate inducible expression of schIL-27. An elegant approach to achieve inducible production of cytokine is through an AND-gated synthetic circuit. This strategy was recently used in a proof-of-concept study with inducible IL-2 CAR T cells. The authors in this study gated IL-2 expression under the control of a synthetic receptor targeting a model

antigen, in addition to a tumor targeting synthetic antigen receptor[492]. In doing so, IL-2 production was produced only in the vicinity in tumors bearing the model antigen, resulting in higher expansion of T cells locally, elimination of systemic toxicities, retained autocrine cytokine signaling, and ultimately mediated superior stringent tumor control. One could envision using such a strategy in multiple myeloma where the AND-gate is triggered by a broadly expressed myeloma target, like CD38 or CD138.

Even though my experiments did not reveal a striking benefit to forced expression of schIL-27 by TAC T cells, I believe this approach warrants further study. Syngeneic models may be a better environment to assess the broader impacts of IL-27 on the tumor niche and other immune cells. Further, other engineered T cells, such as CAR T cells, may have greater benefit from the IL-27 signal as a recent publication from Kagoya et al. indicated that enhanced STAT3 signaling can improve the anti-tumor effects of CAR T cell[493].

4.3 Final thoughts and remarks

Engineered T cell therapies are changing the treatment landscape of multiple myeloma, providing optimism for better outcomes in patients with relapsed/refractory disease. Although there are many challenges to overcome, such as the significant adverse effect profiles and the high costs and difficulties in autologous engineered T cell manufacturing, it is clear there is incredible potential. Cilta-cel has achieved deep remissions with impressive durability in many RRMM patients. A 2-year follow up (published June 2022) to the pivotal cilta-cel phase Ib/II CARTITUDE-1 trial has shown median PFS and OS for all-patients has still not been reached[494]. Similarly, a 4-year follow up to the LEGEND-2 trial of cilta-cel found patients who achieved a CR had a median PFS of 28 months and median OS was not yet reached[269]. As efforts move forward there is much more work to be done to understand the biological shortcomings of these current strategies, such as the higher incidence of relapse in RRMM patients who do not achieve a complete response, or those with high-risk cytogenics or high tumor burden.

Impressions on small molecule drugs and cytokine engineering

In this thesis, I investigated next generation engineered T cell strategies for multiple myeloma. My main objective was to augment TAC T cells exogenously by means of the small molecule SMAC mimetic drug LCL161 or constitutive expression of IL-27. The foundational concepts for both these approaches have their respective merits and flaws.

Small molecule drugs are useful and accessible tools that do not have the same intricacies with control that a living drug such as engineered T cells do. When the compound in question has already undergone clinical trials and determined dosing, safety/tolerability, and pharmacokinetics, administration and expected side-effects can be remediated. However, in the case of newer investigational drugs or novel compounds there is considerably more undertaking to move a small molecule compound beyond pre-clinical testing. Such trials would require specific testing with any potential indications and companion engineered T cell product. Furthermore, small molecule discovery is a highly laborious process, and may never yield an acceptably specific and safe compound. Finally, systemic administration of drugs is complicated by distribution to most tissue and organ systems; targeted delivery *in vivo* is not yet clinically viable, though strategies using targeted nanoparticle encapsulation may allow precision delivery in the future[495].

I postulate instead that genetic engineering approaches to utilize signaling molecules or pathways important to T cell biology, such as cytokines or cytokine signaling, is a more permissible avenue with high potential. As an example, the tumor microenvironment contains inhibitory factors and cells that promote tumor progression while suppressing anti-tumor T cell function[496-498]. Chronic antigenic stimulation in these conditions can render T cells exhausted and hypofunctional[499]. Overcoming the inhibitory tumor microenvironment is critical to the success of engineered T cell therapies. Manipulation of microenvironmental cytokines through squelching and/or redirection of negative regulatory signaling is one method to increase the efficacy of anti-tumor T cells. Synthetic dominant-negative receptors can negate inhibitory signaling by binding suppressive factors and terminating the signal. TGFβ is highly expressed in the multiple myeloma microenvironmental niche and contributes to suppression of T cell activation, proliferation, and CTL function[500-502]. BCMA CAR T cells engineered with a dominant negative TGFβRII were resistant to the suppressive effects of chronic TGFβ and retained polyfunctionality[503]. Alternatively, synthetic cytokine receptors can convert the signal of a commonly produced cytokine, such as IL-2 by T cells, to that of another that may be rarer or more difficult to transmit. This was demonstrated with the replacement of the IL-2Rβ intracellular signaling domain with that of the IL-9Rα signaling domain in TCR or CAR T cells. This change resulted in greater anti-tumor efficacy in several animal models of cancer, which was attributed to a robust memory phenotype driven by the IL-9 signal[504].

Thoughts toward the future

Sophisticated genetic manipulation of T cells is only in its infancy, recent strategies in designing genetic circuits to program cellular responses[505], not unlike early development of microprocessors, give us a glimpse of what may be possible in the future as we learn more about the determinants of successful therapy. Researchers are starting to understand what gene signatures and profiles are correlated with superior anti-tumor efficacy; until these foundations are understood, the ceiling is defined by our current knowledgebase. Targeted gene insertion, replacement, activation, or repression with CRISPR systems may allow unprecedented access to the genome. This is a particularly exciting avenue as it allows researchers to bypass natural physiology. However, while genetic engineering opens the doors to some intriguing concepts, cells are still limited by biological processes. Tools such as PROTACs may be viable alternatives to small molecule inhibitors; however, they cannot full recapitulate the complete potential of small molecule drugs. Thus, I envision roles for both small molecule strategies and genetic manipulation to be combined in a rational way based on deep biological interrogation of the features that define successful anti-tumor immunity.

Future strategies need not be limited to αβT cells, or even T cells altogether. Designed cell products comprised of several immune cell subsets could generate a transplantable *in situ* immune response and create a multi-modal anti-tumor effect. Anti-tumor immune populations other than T cells, such as M1-polarized macrophages, could modulate the tumor microenvironment and contribute to generating an endogenous response through antigen-spreading and presentation of neo-epitopes to retain durability in remission. Natural killer cells have potent anti-tumor potential. When cultured *ex vivo* with IL-21, NK cells retain metabolic fitness and resist the suppressive tumor microenvironment to mediate direct cellular cytotoxicity with their complement of cytotoxicity receptors[506]. Utilizing endogenous populations is another means of diversifying the anti-tumor cell repertoire. For example, CAR T cells expressing PD-1 blocking scFvs have been described[507, 508], however T cells could be engineered to secrete full-length antibodies targeting tumor or stromal antigens[509] to control the tumor environment and bring in NK cell and macrophage-mediated ADCC and ADCP, respectively.

Ultimately, cancer treatment will likely always be multi-modal and require combination strategies to achieve the best response, and immunotherapy is another promising tool in this race.

Appendix I – Investigating the necessity of TCR for TAC function using CRISPR/Cas9

Introduction

Gene-editing is a valuable tool for cell therapies. CRISPR can direct sequence-specific genomic modifications to knock-out, knock-in, or otherwise modify genes. Modified CRISPR/Cas9 systems can be even used to inhibit or activate gene expression without DNA sequence modification[510]. Using CRISPR libraries, entire gene-sets can be evaluated for their contributions to T cell function[511]. This strategy has been used to develop new understanding of mediators or gatekeepers of anti-tumor function in engineered T cells[512, 513]. Many clinical trials of gene-edited T cells are underway. Some strategies include knock-out of T cell inhibitory pathways such as PD-1[514, 515], knock-out of the TCR itself to prevent graft-versus-host disease and permit safer use of allogeneic T cells[514, 515], or deletion of genes thought to mediate CRS such as GM-CSF[516].

I sought to establish gene-editing protocols for primary human TAC T cells using CRISPR/Cas9 for use in my research and applied this tool to confirm the requirement of the TCR for TAC receptor function.

Validating Neon electroporation for CRISPR/Cas9 delivery to T cells

The CRISPR system consists of a Cas nuclease enzyme that complexes with a guide RNA that directs the nuclease to a specific genetic sequence. There are many Cas enzymes from different organisms (reviewed in ref[517]), each with their own intricacies and differences. The most predominantly utilized is the *Streptococcus pyogenes* Cas9 enzyme. The functional Cas9 unit is a ternary complex consisting of the Cas9 enzyme bound to a duplex guide RNA (gRNA). This gRNA is comprised of an invariant transactivating RNA (tracrRNA) bound to a CRISPR RNA (crRNA) which is complementary to the chosen target sequence. The tracr:crRNA duplex can be synthesized as a single guide RNA (sgRNA) for easier handling and use.

There are several methods by which to deliver and utilize CRISPR/Cas9 editing in molecular biology, such as lentivirus encoding the Cas9 and gRNA, *in vitro* transcribed Cas9 mRNA and gRNA, or pre-complexed Cas9/gRNA ribonucleoprotein (RNP). Transient expression or exposure to the Cas9 nuclease is preferred because extended presence of Cas9 within a given cell can lead to off-target editing effects[518]. As such, I elected to generate and deliver pre-formed RNP using electroporation due to its high efficiency and lower reagent requirements compared to other methods.

To establish the method for gene editing of primary human T cells, I elected to target the TCR α-chain constant region gene (*TRAC*) as this would allow facile monitoring of the editing process through flow cytometry analysis of αβTCR expression; the protein expressed by the TRAC locus is critically required for

surface expression of the αβTCR and disruption of TRAC leads to loss of expression. To this end, I utilized a triple sgRNA pool targeting TRAC exon 1 developed by Synthego and complexed it with Cas9 enzyme. RNP was delivered by electroporation to T cells using the Neon electroporator from ThermoFisher. The BCMA TAC was utilized to establish the CRISPR/Cas9 editing protocol.

Various electroporation parameters were evaluated without RNP on non-transduced primary human T cells to find conditions that maintained cell viability and division. Six different conditions were selected based on a review of the literature and discussions with collaborators (Table I-1) and tested with $2x10^5$ T cells. Of the tested conditions, #2 (1600 V, 10 ms, 3 pulses), #5 (1400 V, 30 ms, 1 pulse), and #6 (1400 V, 10 ms, 3 pulses) maintained the highest viability (94%, 89%, and 93%) and cell recovery ($3.7x10^5$, $4.2x10^5$, and $4.9x10^5$). Conditions #2 (1600 V, 10 ms, 3 pulses) and 6 (1400 V, 10 ms, 3 pulses), which differed only in voltage, were chosen based on viability. I next employed these electroporation parameters with TRAC RNP into non transduced primary human T cells. Both conditions resulted in high efficiency knockout of the TCR: #2 11% TCR+, #6 22% TCR+ (Figure I-1). Electroporation at 1600 V (condition #2), but not 1400 V (condition #6) maintained viability and cell growth comparable to mock and non-electroporated cells (Figure I-1). Given the high viability and efficiency of gene editing, we elected to continue with condition #2 electroporation parameters: 1600 V, 10 ms pulse width, and 3 pulses.

Condition	Cells edited (#)	Voltage (V)	Pulse width (ms)	Pulses (#)	Live cells (#)	Viability
1	2.00E+05	2100	20	1	7.62E+04	45%
2		1600	10	3	3.69E+05	94%
3		2400	12	2	5.86E+05	83%
4		2200	20	1	2.35E+04	57%
5		1400	30	1	4.16E+05	89%
6		1400	10	3	4.93E+05	93%

Table I-1. Optimization of Neon electroporation conditions with non-transduced human T cells. Primary human T cells were activated with CD3/CD28 Dynabeads and cultured for 4 days prior to Neon electroporation. Washed T cells were electroporated as indicated above. 48 hrs after electroporation viability and total cell number were assessed. n=1, donor L11.

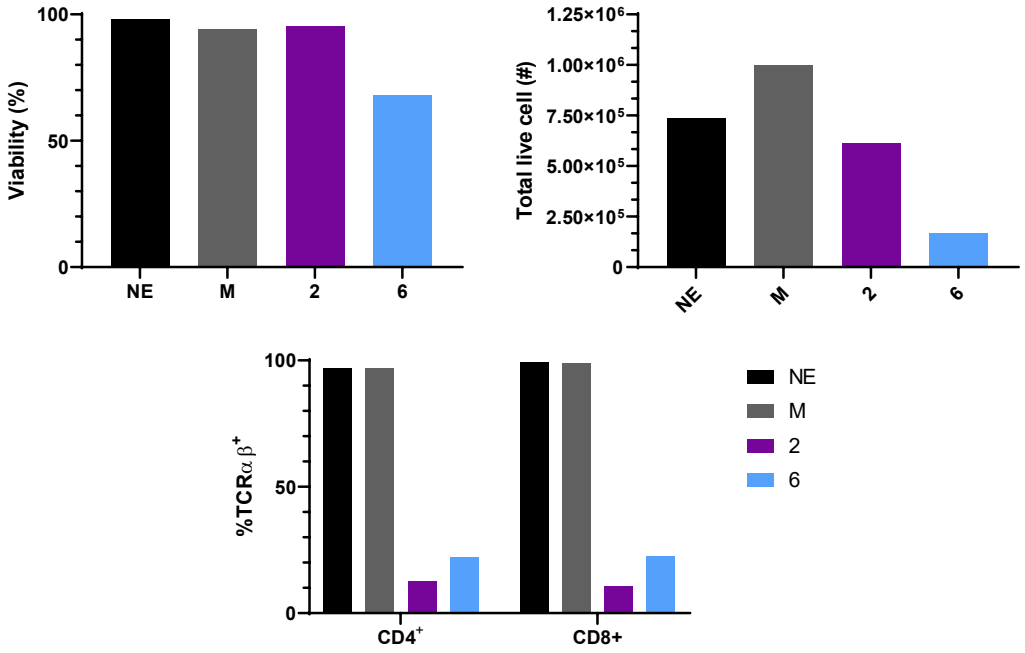


Figure I-1. TRAC multi-guide sgRNA RNPs efficiently knockout TCR expression in primary human T cells. Primary human T cells were activated with CD3/CD28 Dynabeads and cultured for 4 days prior to Neon electroporation. Washed T cells were electroporated as above with 2 µL TRAC multi-guide sgRNA RNPs. 48 hrs after electroporation viability and total cell number were assessed. M = mock; NE = non-electroporated. n=1, donor L10.

I next evaluated the CRISPR-editing protocol with T cells expressing a TAC receptor. TRAC-edited TAC T cells expanded similarly to mock-edited or non-electroporated TAC T cells (Figure I-2). TAC expression was reduced in the TRAC-edited group as seen with a reduction to NGFR+ TAC+ cells with a concurrent increase to NGFR+TAC- cells, indicating that transduced T cells were not lost and rather TAC expression was reduced on some transduced cells. Because the TAC receptor binds to CD3ε, and the CD3 complex is mostly retained within the cell in the absence of binding to the TCR[519], it is likely that TAC receptor bound to CD3ε during protein synthesis and is also within the cell in the TRAC knock-out cells, thereby reducing the overall expression of the TAC receptor.

Optimizing gene-editing by CRISPR/Cas 9 in TAC T cells

In addition to electroporation parameters such as voltage and pulse width, RNP formulation, RNP mass, and the number of cells electroporated may all affect the efficiency of gene editing. Therefore, I revisited and further fine-tuned the protocol with these considerations in mind (Table I-2). I further included previous conditions #5 (1400 V, 30 ms, 1 pulse) and #6 (1400 V, 10 ms, 3 pulses) again.

- To assess the impact of cell number, I assessed 2-fold decrease (condition #A, $1x10^5$ cells) or increase (condition #B, $4x10^5$ cells).
- RNP formulation is a molar ratio of gRNA:Cas9. In condition #C I altered the molar ratio to 1:1 in place of the previous 2.5:1.
- To assess the impact of RNP mass, I doubled the mass from 20 pmol to 40 pmol in condition #D.

Viability post-electroporation was quite variable, but all conditions stabilized with the exception of condition #D (20 pmol RNP) (Figure I-3B). No condition had considerable deviation in fold expansion except for condition #6 which altered electroporation parameters (1400 V 10 ms 3 pulses) (Figure I-3A). Conditions #2 and #B, both of which employed 1600 V, 10 ms, 3 pulse electroporation but differed in the amount of cells electroporated ($2x10^5$ vs $4x10^5$ cells, respectively) achieved the highest editing efficiency.

Because of this high editing, conditions #2 and #B were tested again in 2 separate donors for further examination. Neither donors showed differences in growth or viability between the two conditions (data not shown). Donor L11, but not donor L10, displayed an increase in editing with condition B vs 2 (79% KO vs 61% KO) (Figure I-4A). To assess the frequency of insertion/deletion (indel) events that disrupt the gene, we employed Synthego's ICE tool, which is a genomic DNA analysis tool designed to accommodate edits using multiple gRNAs[417]. Genomic DNA from non-electroporated, mock, and TRAC-edited cells was collected and the region surrounding the gRNA-target sites was amplified by PCR and sequenced by

Sanger sequencing. The indel frequency is reported in Figure I-4B. Both mock samples reported an indel frequency of 0% as expected of non-edited cells. However, L10 condition #2 ($2x10^5$ cells), L10 condition B ($4x10^5$ cells), and L11 condition 2 ($2x10^5$ cells) all showed indel frequencies at least 2-fold less than the knockout rate as determined by flow analysis of TCR expression. It is possible that the ICE analysis is underreporting the editing frequency. An alternative explanation is that the true editing frequency is ~30% but TCR knock-down is higher due to allelic exclusion in T cells where one of the TCRα and β chain alleles is silent. In this way, heterozygous edits could show up phenotypically as higher editing. Nevertheless, engineered human T cells can successfully and efficiently be edited in the TRAC-locus using a multi-gRNA approach and neon electroporation.

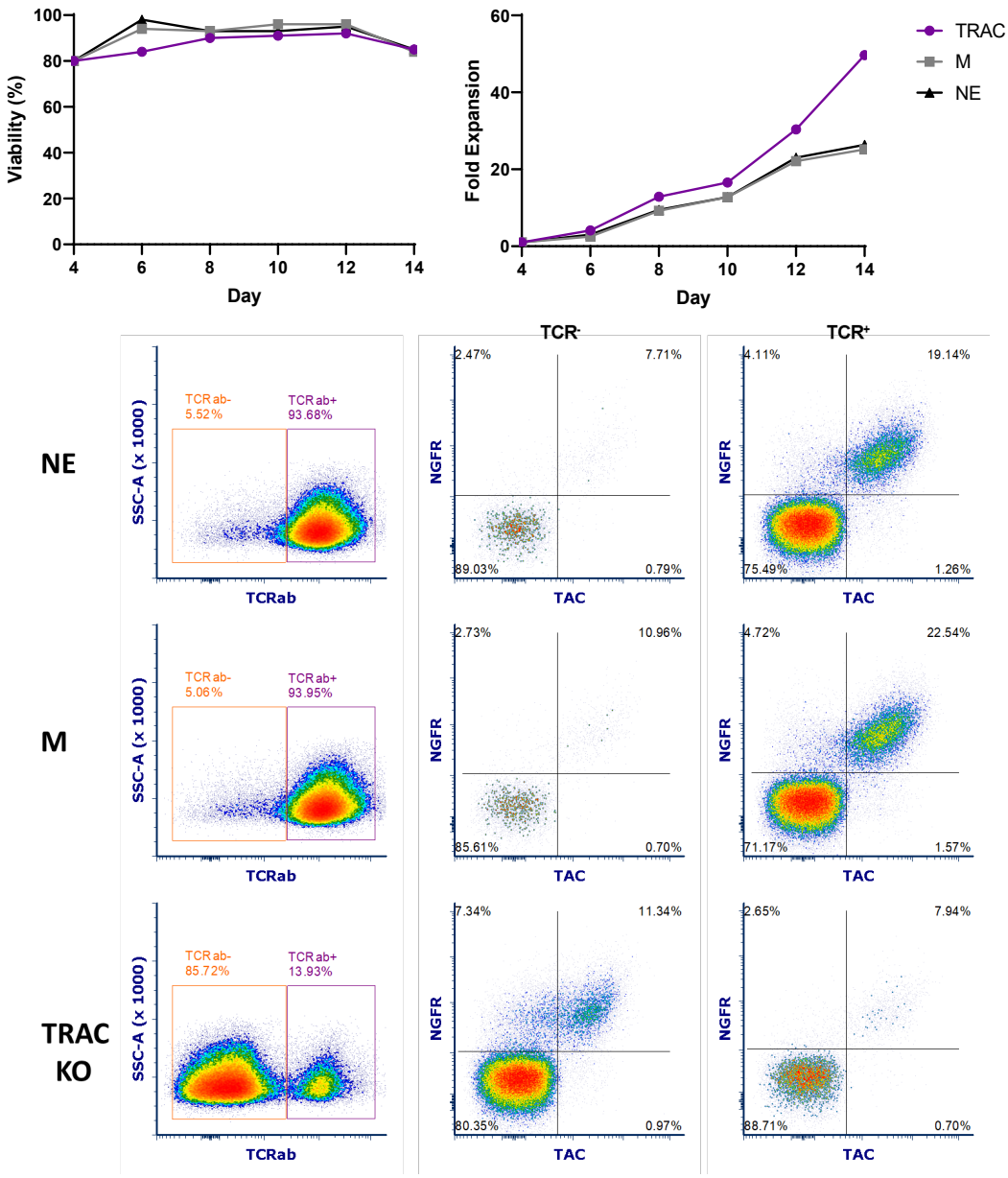


Figure I-2. TRAC editing in TAC T cells does not alter T cell expansion but reduces TAC receptor expression. TAC T cells were generated through standard protocol. T cells were edited on d4 after Dynabead removal, and then cultured until day 14. n=1, donor L9.

Condition	Cells edited (#)	Voltage (V)	Pulse width (ms)	Pulses (#)	gRNA:Cas9	RNP vol.
A	1.00E+05	1600	10	3	2.5:1	2 µL
B	4.00E+05	1600	10	3	2.5:1	2 µL
2	2.00E+05	1600	10	3	2.5:1	2 µL
5	2.00E+05	1400	30	1	2.5:1	2 µL
6	2.00E+05	1400	10	3	2.5:1	2 µL
C	2.00E+05	1600	10	3	1:1	2 µL
D	2.00E+05	1600	10	3	2.5:1	4 µL

Table I-2. Optimization of Neon electroporation conditions with TAC-transduced human T cells. Primary human T cells were activated with CD3/CD28 Dynabeads, transduced to express the TAC receptor, and cultured for 4 days prior to Neon electroporation. Washed T cells were electroporated as indicated above with TRAC-targeting RNP.

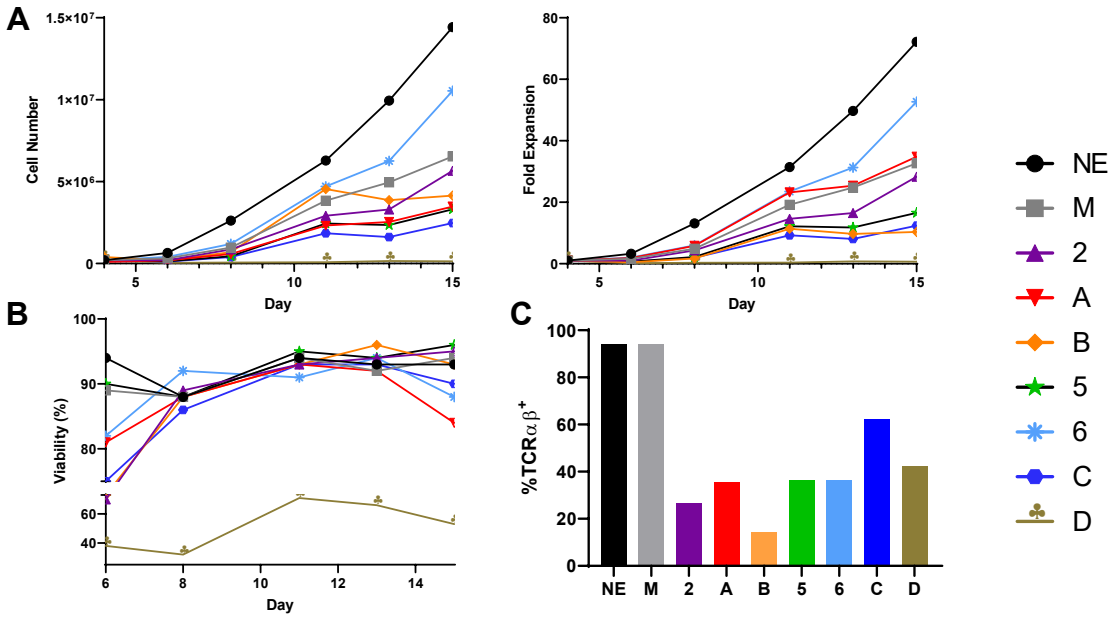


Figure I-3. Optimization of Neon electroporation and Cas9/gRNA RNP parameters. Primary human T cells were activated with CD3/CD28 Dynabeads, transduced to express the TAC receptor, and cultured for 4 days prior to Neon electroporation. Washed T cells were electroporated as indicated above with TRAC-targeting RNP. T cells were cultured for 15 days, and expansion, viability, and TRAC-editing were assessed. n=1, donor L9.

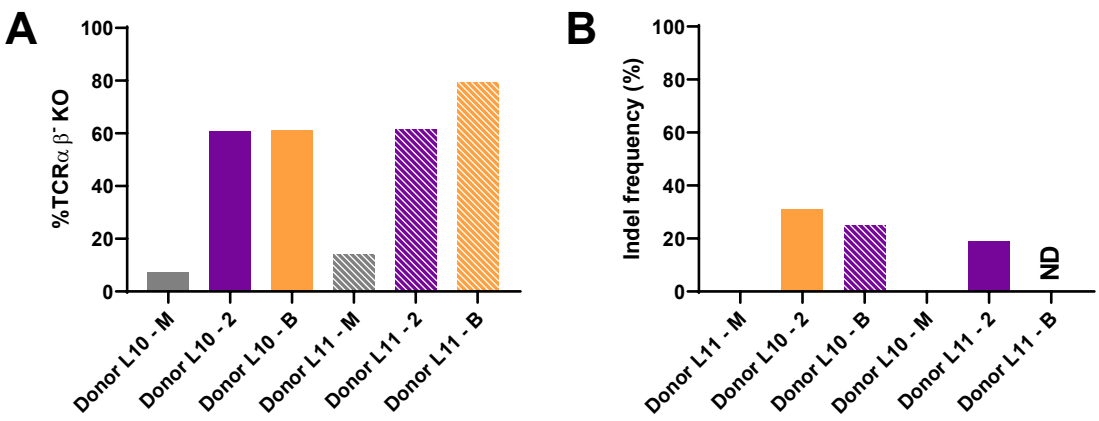


Figure I-4. Editing-efficiency analyzed by ICE does not correspond to phenotypic-expression. Primary human T cells were activated with CD3/CD28 Dynabeads, transduced to express the TAC receptor, and cultured for 4 days prior to Neon electroporation. T cells were edited with condition 2 or B as outlined in Table I-2 with TRAC RNPs. After 8 days of culture, cells were ***A*** phenotyped by flow cytometry for TCRαβ expression, and ***B*** genotyped by sanger sequencing and ICE analysis. n=2 (1 per L10 & L11).

TRAC-edited TAC T cells do not to respond to cognate antigen

I next determined what impacts TRAC-editing would have on engineered T cells. In addition to BCMA-specific TAC T cells, I also utilized a second generation BBζ anti-BCMA CAR. CAR T cells contain the necessary signaling moieties for T cell activation within their receptor, and so should maintain functionality in the absence of TCR. TRAC-edited TAC and CAR T cells were stimulated with 1:1 with $5x10^5$ KMS-11 tumor cells for 4 hrs and the frequency of IL-2 and/or TNFα-producing cells was assessed. This approach also allowed us to definitively answer a longstanding question within the Bramson lab: does the TAC receptor require the TCR for functionality?

Mock electroporation of TAC T cells did not alter the expression of TNFα or IL-2 in comparison to non-electroporated counterparts (data not shown). Both TAC and CAR T cells were successfully edited to remove TCR with 94% and 89% knockout, respectively (Figure I-5A). When TRAC-edited TAC T cells were stimulated the transduced portion of cells (ie. the TAC-engineered T cells) did not respond to the BCMA-antigen as indicated by the almost complete abolition of TNFα and IL-2 production (Figure I-5B). Conversely, CAR T cells maintained cytokine production in the absence of the TCR, albeit at a reduced capacity.

I next substituted the magnetic Dynabead activator to a soluble T cell activator (StemCell Technologies' ImmunoCult Activator) as the soluble activator eliminates the need for bead removal steps that impact T cell viability, which simplifies the manufacturing workflow. I compared two forms of the ImmunoCult Activator (anti-CD3/CD28 and anti-CD3/CD28/CD2) and found T cells activated with ImmunoCult activator had greater editing efficiency than those activated using the magnetic Dynabeads (Figure I-6). Using this new activator, I repeated TRAC KO in TAC and CAR engineered T cells. A 28ζ CAR was included alongside the BBζ CAR to confirm applicability to different CAR platforms. Loss of TCR expression was equivalent amongst all three groups (Figure I-7A). Once again, CAR T cells maintain cytokine production in response to stimulation with KMS-11 tumor cells, regardless of the presence of TCR (Figure I-7B). TCR-edited TAC T cells however were not able to produce TNFα and/or IL-2 in response to stimulation. With these data I concluded that the TAC receptor requires the TCR for cytokine production in response to antigen-positive tumor cells, and likely for other functions.

This pilot work set the stage for the gene editing described in Chapter 2 of this thesis.

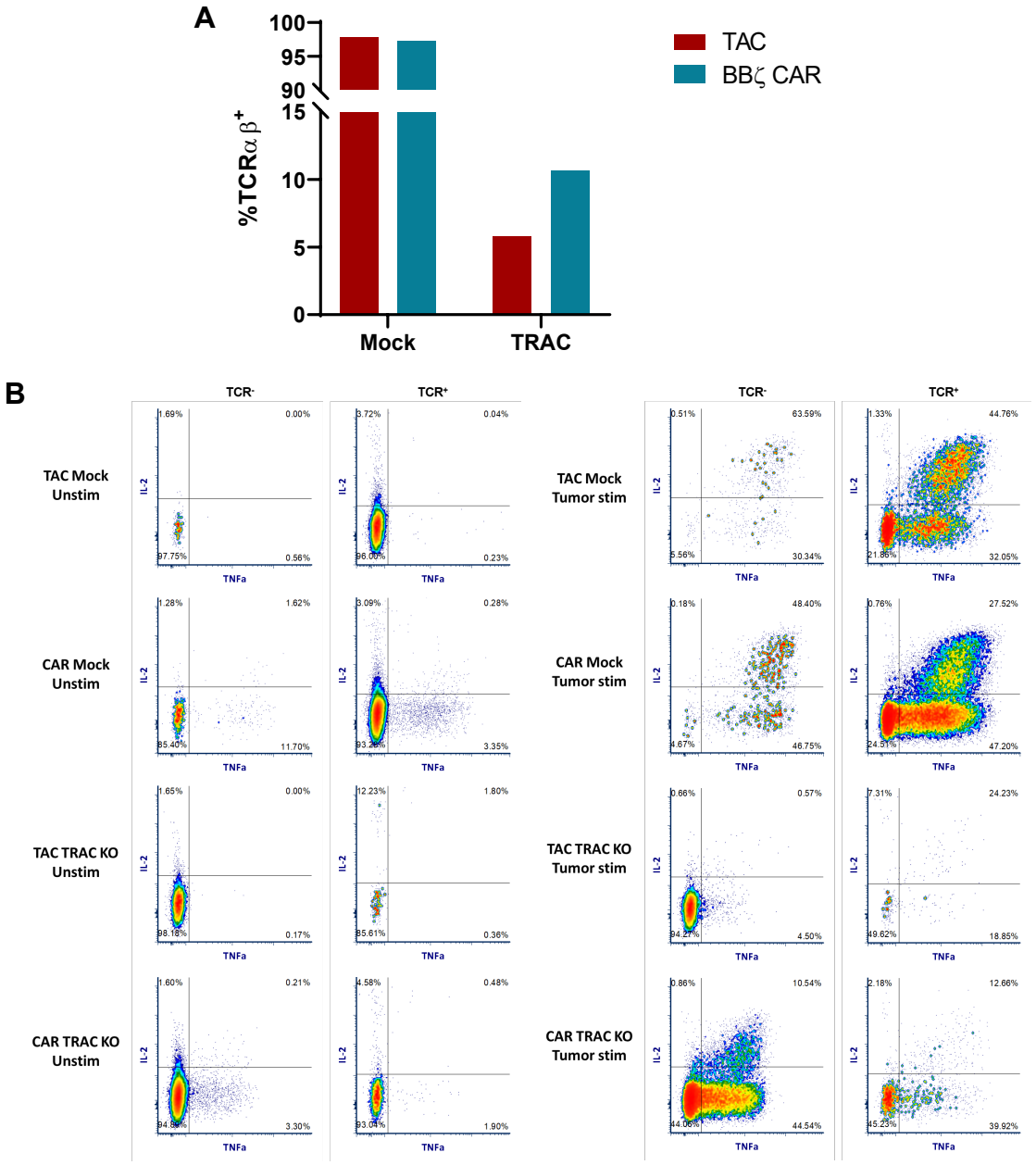


Figure I-5. The TAC receptor requires the TCR for functionality. Primary human T cells were activated with CD3/CD28 Dynabeads, transduced to express the TAC or CAR, and cultured for 4 days prior to Neon electroporation. T cells were edited with TRAC RNPs. After 10 days of culture, cells were ***A*** phenotyped by flow cytometry for TCRαβ expression, and ***B*** stimulated with KMS-11 tumor cells (E:T 1:1 $5x10^5$ tumor cells) for 4 hrs then stained and analyzed by flow cytometry for intracellular cytokines. Cytokine gating: singlets > live > lymphocytes > NGFR+ > TCRαβ+/-. n=1.

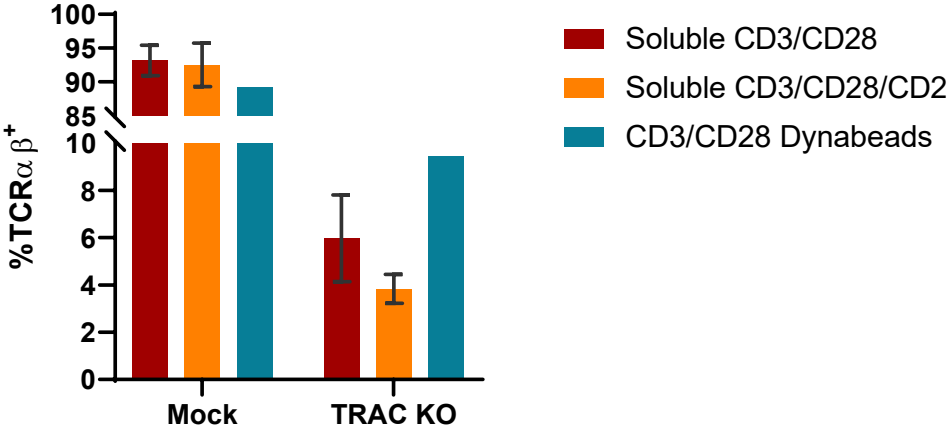


Figure I-6. T cells activated with a soluble activator are amenable to CRISPR/Cas9 editing. Human PBMCs were activated with either soluble activator or the Dynabead magnetic bead activator. PBMCs activated with soluble activators or Dynabeads were edited on day 3 or 4 post-activation, respectively. Cells were then cultured until day 9 post-activation, then stained and assayed for TCR expression by flow cytometry. Soluble activator data is from two independent experiments; Dynabead data is from one experiment.

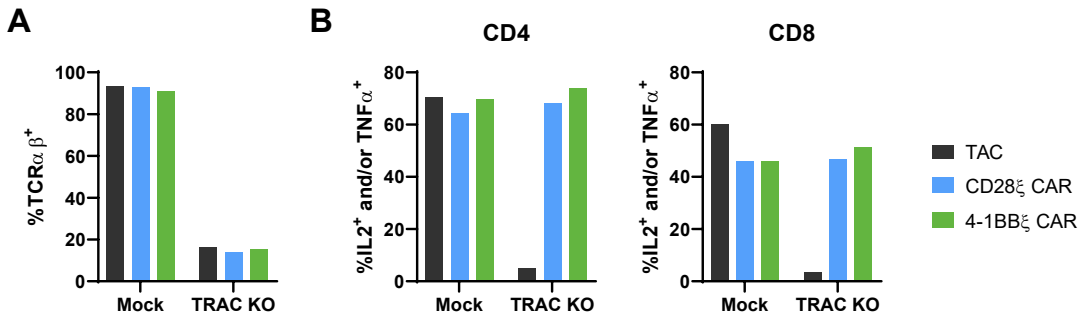


Figure I-7. TAC T cells require TCR for activation against BCMA+ myeloma cells. Human PBMCs were activated with anti-CD3/CD28/CD2 soluble activator and edited on day 3 post-activation. Cells were cultured for a total of 14 days then **A** phenotyped for TCR expression, and **B** stimulated 1:1 with KMS-11 tumor cells for 4 hrs in the presence of brefeldin A. Cells were then permeabilized and stained intracellularly for retained IL2 and TNFα. Data represents a single experiment with 1 donor.

Materials and Methods

The methods and reagents utilized in this appendix were identical to those presented in chapter 2 with the following exceptions and additions:

1. The Cas9 enzyme used in this section was the Synthego S.p. Cas9 2NLS.
2. The sgRNA sequence for targeting the TRAC loci were (5' – 3'):
    a. CUCUCAGCUGGUACACGGCA
    b. GAGAAUCAAAAUCGGUGAAU
    c. ACAAAACUGUGCUAGACAUG
3. KMS-11 multiple myeloma cells were cultured as MM.1S cells previously indicated.

Appendix II – mRNA-engineered TAC T cells

Introduction

Retroviruses, such as pseudotyped lentivirus, are the predominant method to engineer T cells within the field of cancer immunotherapy. Lentiviral engineering yields a product that is stable and can be cultured to desired numbers. In cases where the target ligand for an engineered T cell leads to *on-target, off-tumor* toxicities, the stable expression of a synthetic antigen receptor encoded by a lentivirus will promote the engineered T cells to expand and target healthy tissues resulting in potentially lethal toxicities. Engineering T cells with mRNA presents an alternative method that would provide transient expression of synthetic antigen receptors, which could enable targeting of "imperfect" tumor antigens that are expressed on the tumor and healthy tissues. Expression of the synthetic antigen receptor on mRNA-engineered T cells will only persist for a short period of time before the mRNA is degraded or lost through cell division. Such a transient expression strategy provides an avenue to investigate newer, less understood, or tumor targets with broad expression. For example, CD123 is a potential target in acute myeloid leukemia, however as CD123 can also be expressed on hematopoietic progenitor cells there is considerable risk for myeloablative toxicities[520]. A pre-clinical study by Tasian et al. has shown that 3 infusions of anti-CD123 mRNA-engineered CAR T cells given *in vivo* can be used to eradicate acute myeloid leukemia without producing severe toxicity, which then allows for hematopoietic stem cell transplant as the CAR T cells do not persist[521].

Here, I explored the possibility of using mRNA-engineering to generate TAC T cells.

Design of TAC mRNA DNA template

The TAC mRNA was produced using an *in vitro* transcription system. When designing *in vitro* transcribed (IVT) mRNA, several factors must be considered: **i) the choice of 5' and/or 3' untranslated region (UTR)**, **ii) incorporation of a poly(A) sequence into the DNA template or post-transcriptional enzymatic addition of a poly(A) tail**, and **iii) which 5' cap structure should be used**.

IVT mRNA can be enzymatically capped with a 7-methylguanylate (m^7G) cap structure (known as cap 0). This is the 5' cap that is generated in eukaryotic cells. The cap 0 can be further modified by methylation of ribose-2'-O to generate a cap 1 structure, which can enhance translation in some systems[522, 523]. Alternatively, a cap analog can be used by some *in vitro* transcription systems. Rather than determining this empirically step-by-step, we followed the findings of Zhao et al. who investigated these individual aspects for generating mRNA-engineered CAR T cells[523]. Based on this data, the BCMA-specific TAC was

appended with 2 copies of the human β-globin 3' UTR (exact sequences can be found here[524]), a sequence of 100 adenines (the optimal length of the poly(A) sequence appears to be partially dependent on the individual sequence preceding it[525]), and a linearization restriction site. The final DNA template was generated by GenScript Biotech (note that GenScript was unable to produce 150 A nucleotides as used in the original citation, thus the lower value of 100) (Figure II-1).

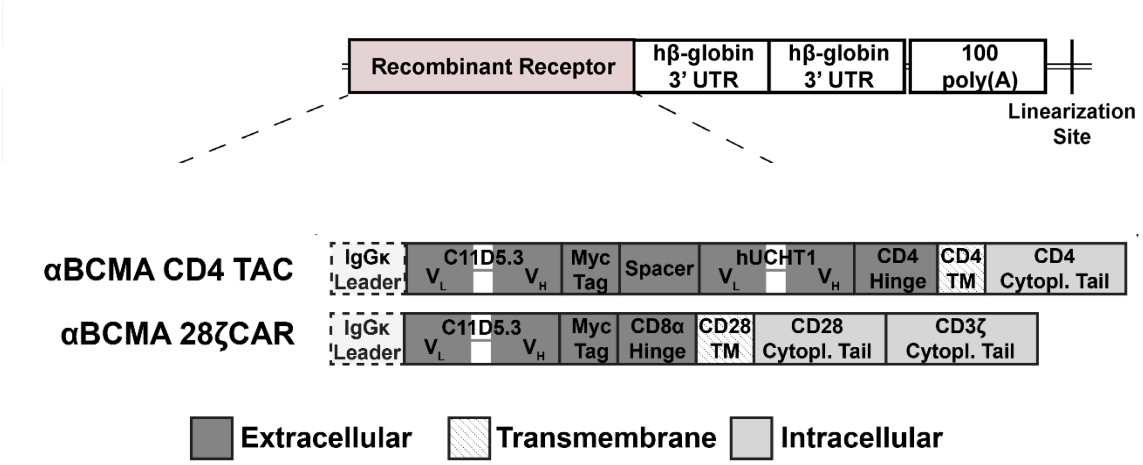


Figure II-1. Schematic diagram indicating the components of the BCMA-specific TAC and CAR template DNA used for IVT mRNA. Molecular schematic outlining the components of the BCMA-specific TAC. Labels indicate the leader sequence, scFv, Myc tag, humanized UCHT1 scFv, CD4 hinge, transmembrane (TM), cytoplasmic tail regions.

Proof of principle with control luciferase mRNA and transfer into T cells

In the first stage of this work, we employed a commercial kit for producing an mRNA encoding firefly luciferase and evaluated different methods (electroporation and nucleofection) to introduce the luciferase mRNA into primary human T cells. A vector encoding luciferase with a 30 poly(A) tail under the T7 promoter was purchased from Promega (cat# L4741) and mRNA was produced used the mMESSAGE mMACHINE T7 kit. Luciferase mRNA was successfully produced as a clean mRNA product (Figure II-2).

To assess mRNA uptake and efficiency, cryopreserved cultured non-transduced T cells were thawed and let to rest for 48 hrs in media containing IL-2 and IL-7. An aliquot of $1x10^6$ T cells were electroporated or nucleofected with 1 μg[526] or 1.6 μg (equivalent to 3 pmol for luciferase) of mRNA, respectively. One day after engineering, T cells were counted and assessed for efficiency of mRNA transfer by staining with an anti-luciferase antibody for flow cytometric analysis. T cells that were electroporated showed greater recovery, viability, and better mRNA transfer efficiency (nucleofection 45.2% vs electroporation 65.9%) when compared to nucleofected T cells (Figure II-3 and Figure II-4).

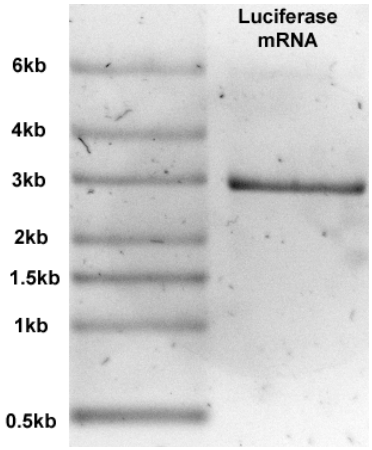


Figure II-2. Bleach gel of luciferase mRNA. Luciferase mRNA was visualized on a 1% agarose:bleach gel after 1 hr of 100 V electrophoresis.

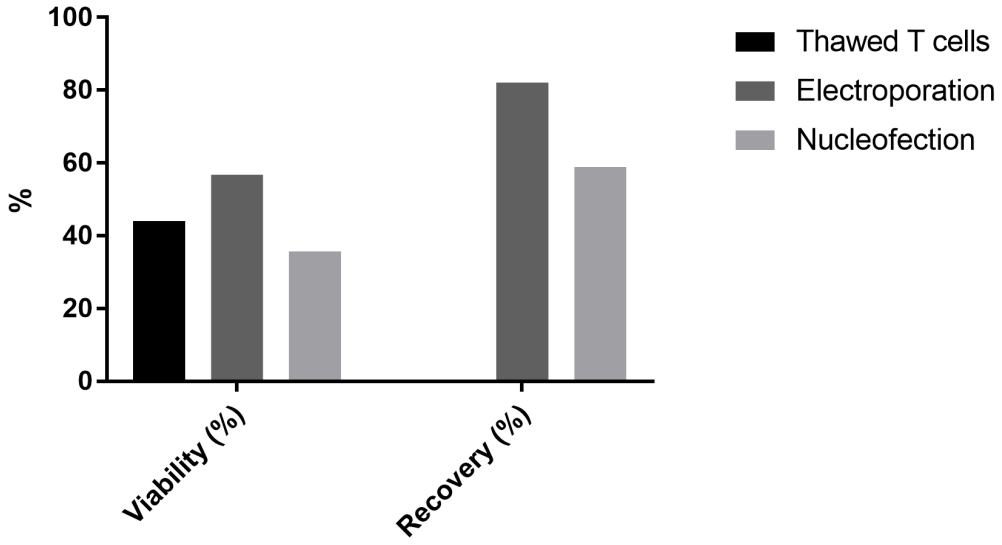


Figure II-3. Viability and recovery of T cells undergoing mRNA electroporation of nucleofection. mRNA-engineered T cells were stained with trypan blue, and viability and cell counts were acquired with a Countess II FL cell counter.

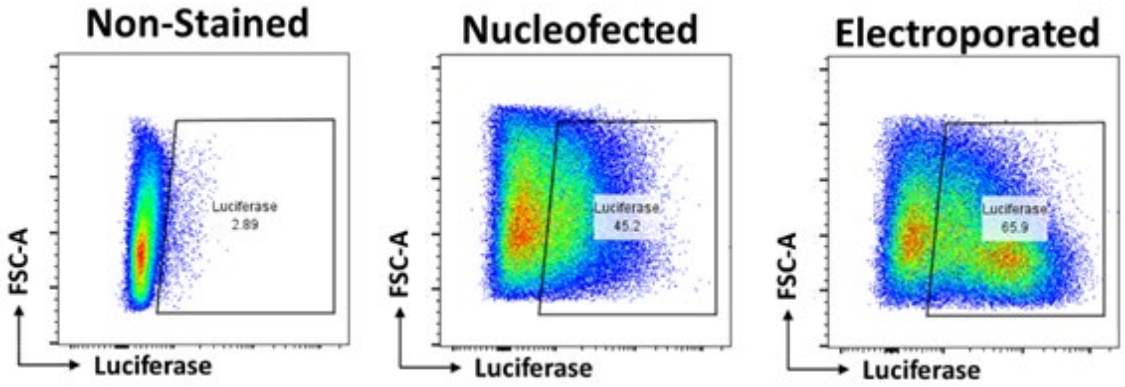


Figure II-4. Analysis of luciferase expression on T cells after mRNA-engineering. $5x10^5$ T cells were first stained with mouse anti-luciferase mAb (Abcam; clone: Luci17), then stained with goat anti-mouse IgG. Cells were then analyzed on a LSRFortessa flow cytometer.

Generation of TAC mRNA and electroporation of T cells

Having determined that electroporation was a more efficient method for delivery mRNA to primary T cells, the next step was to deliver an mRNA encoding the TAC receptor into primary T cells.

The BCMA-TAC template DNA (Figure II-1) was ordered from GenScript and cloned into the pGEM-4Z vector (Promega) within the *EcoRI*/*HindIII* restriction sites. Attempts at generating mRNA using pGEM-4Z BCMA-TAC plasmid (henceforth referred to as pGEM-BCMA-TAC) linearized with SapI and purified using PCR clean up columns (ThermoFisher) failed repeatedly or produced mRNA with multiple products (data not shown). Linearization of pGEM-BCMA-TAC plasmid with *AvrII* and subsequent purification using phenol:chloroform:isoamyl alcohol extraction produced a pure enough template to facilitate efficient mRNA production (Figure II-5). A small amount of larger product was also present, which is likely the DNA template (5079 bp in size). DNase after IVT was used to remove the template DNA prior to mRNA purification, however incomplete digestion likely occurred. Primary human T cells were cultured from cryopreserved PBMCs for a period of 10 days, then $2.5x10^6$ T cells were electroporated with 2.5 µg of mRNA. After 24 hours, control and electroporated T cells were stained for the TAC receptor and evaluated by flow cytometry. T cells electroporated with the BCMA-TAC mRNA showed expression of the TAC receptor (CD4 45.1%; CD8 40.3%)(Figure II-6). Previous studies using mRNA to generate CAR T cells have shown efficiencies in the 90% range[527], suggesting that there was room for improvement.

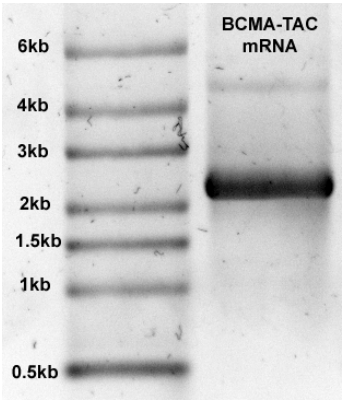


Figure II-5. Bleach gel of BCMA-TAC mRNA. BCMA-TAC mRNA was visualized on a 1% agarose:bleach gel after 1 hr electrophoresis using 100 V.

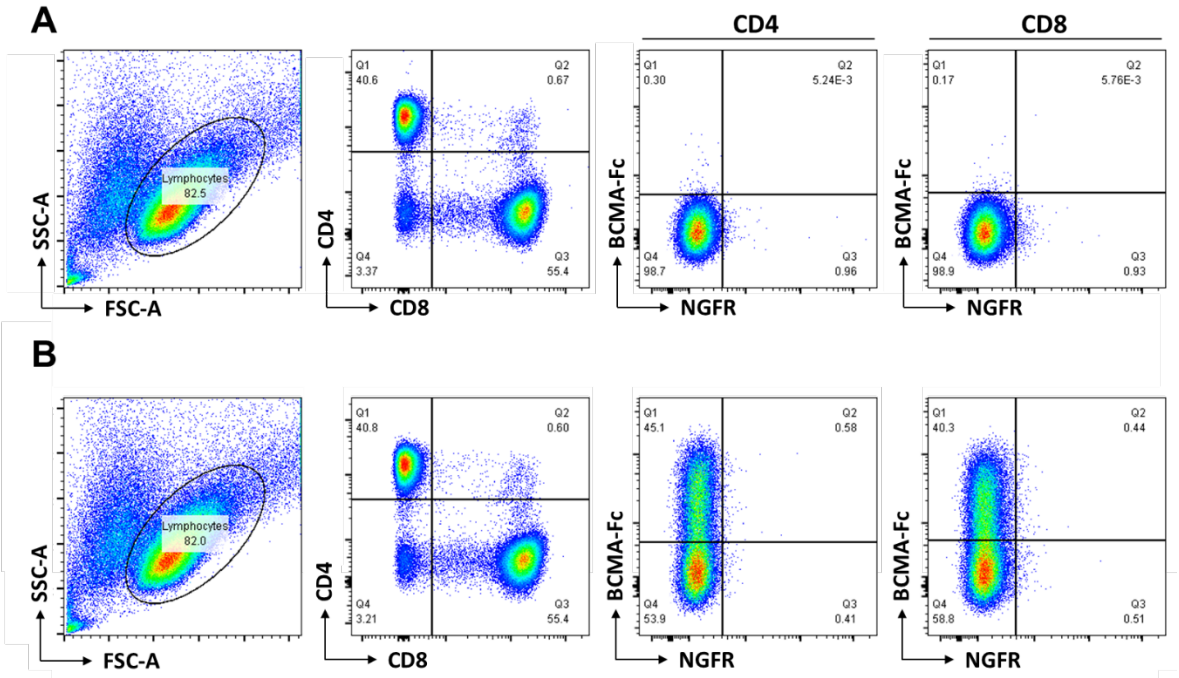


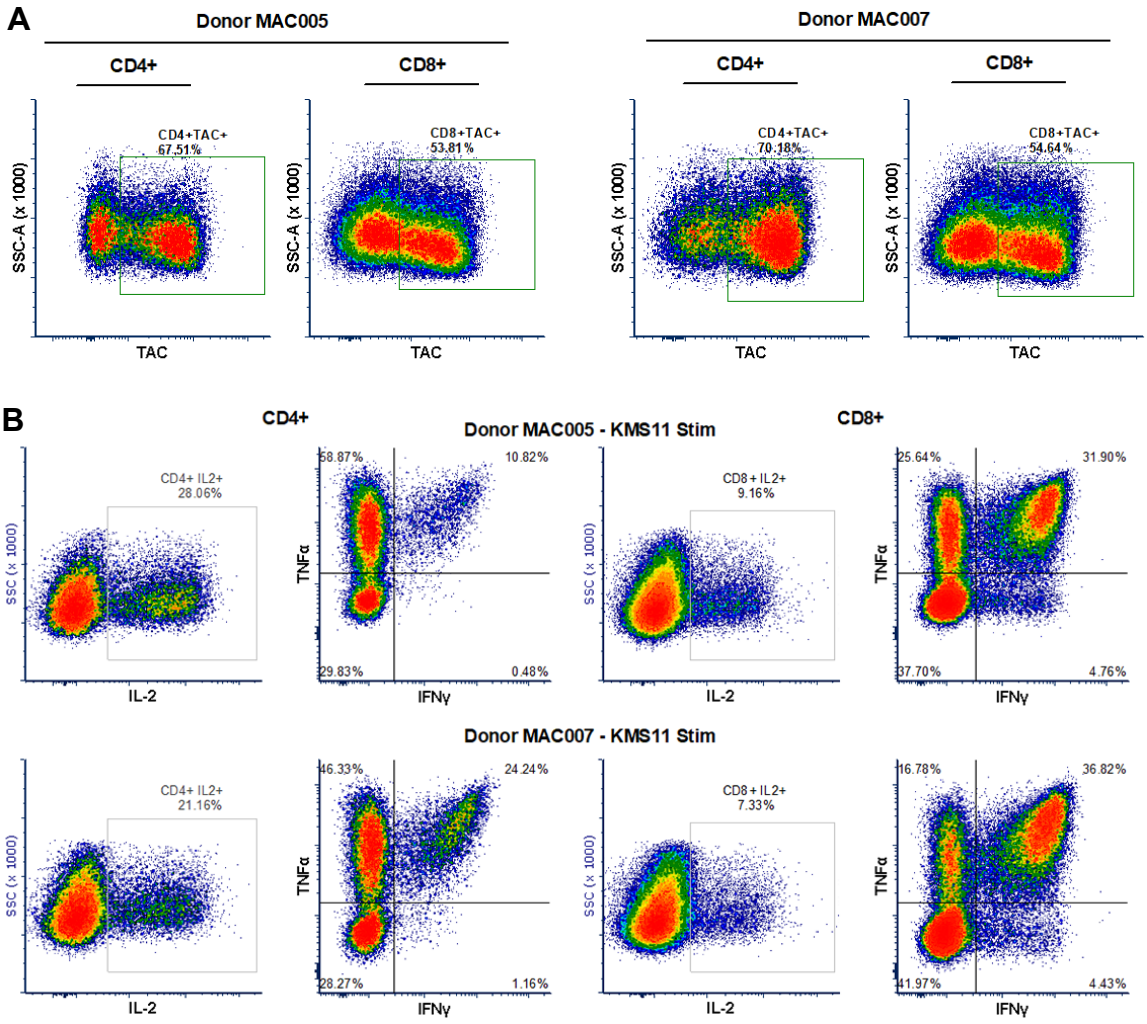
Figure II-6. Analysis of BCMA-TAC expression on T cells after mRNA-engineering. 5x10^5 ***A*** Mock-electroporated or ***B*** BCMA-TAC mRNA-electroporated T cells were first stained with recombinant human BCMA-Fc (R&D Systems), then stained with goat anti-human IgG. Cells were then analyzed on a LSRFortessa flow cytometer.

mRNA TAC T cell effector function and phenotype

These experiments were repeated in two additional donors to evaluate the IVT mRNA TAC. The percent of cells expressing the TAC receptor was higher in this manufacturing round (CD4 68.8%±1.34 SD; CD8 54.2%±0.42 SD), but still lower than the reported efficiencies seen in the literature (Figure II-7A). T cells engineered with the TAC mRNA displayed expected effectors functions in response to BCMA+ KMS-11 multiple myeloma cells (Figure II-7B-D). T cells engineered with mRNA TAC were high frequency producers of TNFα and IFNγ, with >70% of CD4 and >60% of CD8 TAC T cells producing cytokine (Figure II-7B). mRNA TAC T cells were capable of killing >50% of KMS-11 myeloma cells when co-cultured for 24 hrs at an E:T of 1:1 (Figure II-7C). mRNA TAC T cells also underwent 4 rounds of division within 72 hrs when stimulated 2:1 with KMS-11 cells (Figure II-7D).

The *in vitro* transcription kit used thus far uses co-transcriptional capping, which is the incorporation of a 5' cap analog during transcription. Comparative studies by Zhao et al. determined that RNA produced using enzyme-based post-

transcriptional capping and polyadenylation was superior to RNA produced using a cap analog[523]. Therefore, we investigated the enzyme-based mScript T7 kit to generate mRNA with cap1 structure and additional polyadenylation. TAC mRNA generated using the mScript kit contained transcripts of variable length, likely due to variability of the polyadenylation enzyme in processing the primary transcripts (Figure II-8A). The mRNA product resulted in high TAC receptor expression (CD4 89.4%; CD8 72.2%) after electroporation into cultured T cells (Figure II-8B).



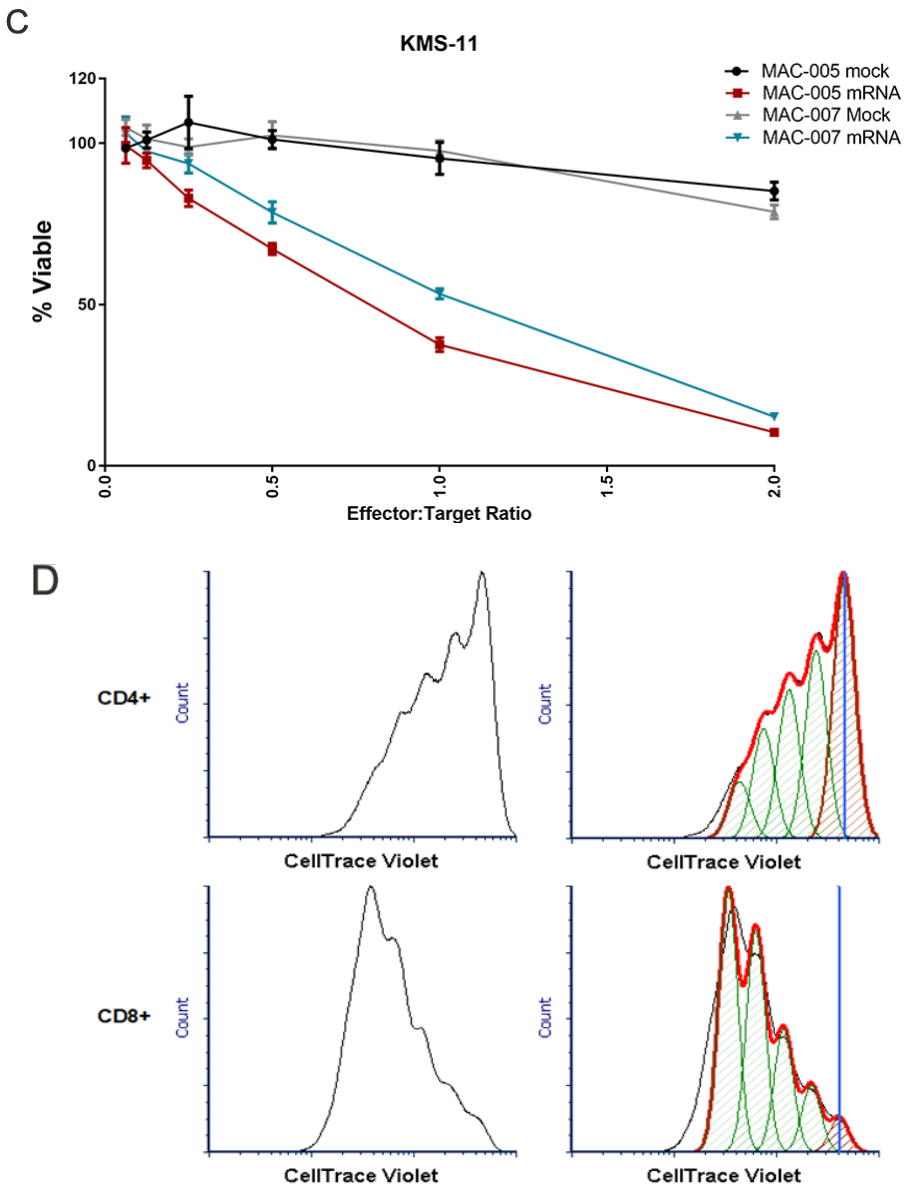


Figure II-7. T cells engineered with the TAC mRNA function as expected. T cells engineered with TAC mRNA were rested 24 hrs after electroporation, then assayed for ***A*** TAC expression by flow cytometry, or co-cultured with BCMA-positive KMS-11 tumor cells for varying lengths of time and assessed for ***B*** production of the cytokines TNFα, IFNγ, and IL2 through flow cytometry, ***C*** ability to kill as measured through loss of luciferase activity, and ***D*** proliferative capacity as measured through CellTrace Violet dye dilution.

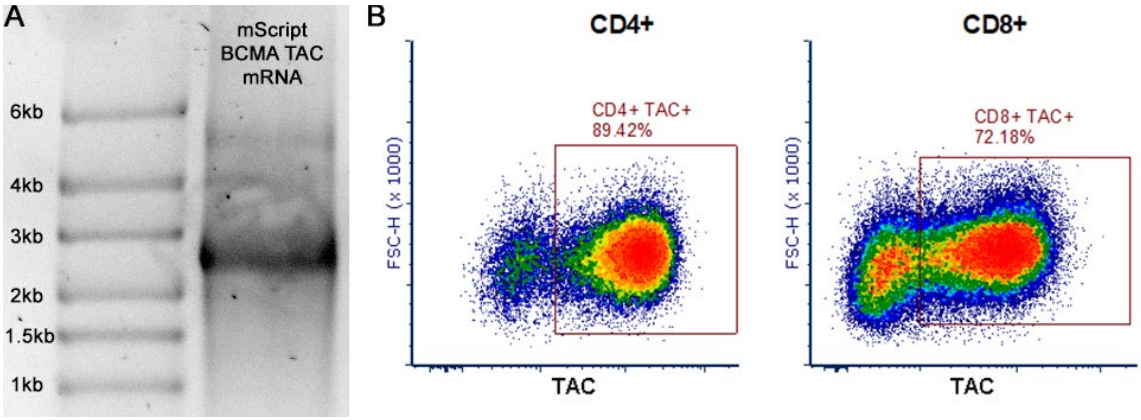


Figure II-8. Electroporated mScript T7 mRNA results in high TAC expression in T cells. *A* mScript TAC mRNA was visualized on a 1% agarose bleach gel *B* Representative phenotype data of T cells engineered with mScript TAC mRNA after resting for 24 hrs post electroporation, similar results generated in multiple independent experiments, n=5.

In vitro *and* In vivo *comparison of mRNA- vs lentivirus-engineering*

We next compared TAC T cells generated with IVT mRNA to TAC T cells produced through lentiviral transduction. To determine if any differences seen are unique to the TAC receptor or are a general property of mRNA vs lentivirus engineering, a second-generation 28ζ anti-BCMA CAR was created in the mRNA-template framework (Figure II-1). Lentivirus-engineered T cells were positively sorted by magnetic bead separation using the tNGFR transduction marker to produce T cell products with similar frequencies of TAC and CAR T cells.

Both CAR and TAC mRNA electroporation displayed high efficiency by 24 hours post-electroporation (TAC 82%; CAR 94%)(Figure II-9). The process of electroporation induced activation of some T cells, likely through an influx of calcium[528], as seen by upregulation of the activation marker CD69 (CD4 mRNA TAC 34.1% vs CD4 LV TAC 5.4%; CD8 mRNA TAC 26.2% vs CD8 LV TAC 1.2%). Interestingly, CD69 upregulation was lower on LV TAC T cells than LV CAR T cells (CD4 LV TAC 5.4% vs CD4 LV CAR 8.8%; CD8 LV TAC 1.2% vs CD8 LV CAR 6.3%)., possibly due to tonic signaling from the CAR (Figure II-10). The most striking observation seen in T cell samples electroporated with the mRNA TAC was the increased percentage of cells producing IFNγ, TNFα, and IL2 relative to T cells engineered with lentivirus (Figure II-11). This phenomenon was not observed with CAR T cells. With regard to proliferation, the mRNA-engineered T cells performed quite poorly relative to T cells engineered with lentivirus. This was true for T cells engineered to express either TAC or CAR and the results were consistent for both stimulations with KMS-11 myeloma cells or antigen-loaded

beads (Figure II-12). Both mRNA TAC and CAR failed to undergo any division with the exception of <2 rounds when stimulated with beads coated with 25 ng BCMA-Fc per 1 million beads. Conversely, LV CAR underwent at least 4 divisions and LV TAC underwent 3-4 divisions with 100 ng BCMA-Fc beads or KMS-11 cells, but not with beads coated with a lower concentration of BCMA-Fc.

To determine how these *in vitro* assays compare with *in vivo* efficacy, IVT mRNA- and lentivirus-engineered TAC T cells were evaluated in a xenograft model of multiple myeloma. Tumor-bearing mice were split into 3 treatment groups: non-transduced T cells, lentivirus-engineered TAC T cells, and mRNA-engineered TAC T cells. While lentivirus-engineered TAC T cells were able to produce regressions in tumor load, the mRNA-loaded TAC T cells provided no benefit relative to the non-engineered control T cells (Figure II-13).

We suspected that the duration of TAC expression may have been too short to support the degree of T cell attack required to mediate regression of established tumors. Indeed, investigation of receptor expression over time shows that the majority of both CAR and TAC expression is gone by 48 hrs post electroporation (Figure II-14). qPCR analysis using TAC-specific primers supported the notion that the mRNA is quickly degraded within the T cells (Figure II-15).

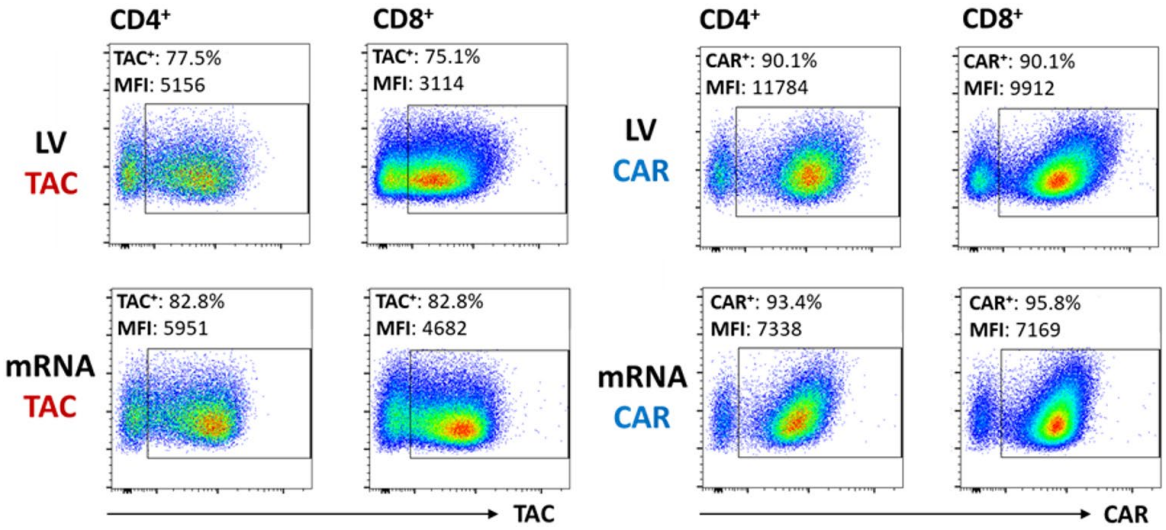


Figure II-9. Sorted lentivirus-engineered T cells have comparable receptor expression to mRNA-engineered T cells. T cells were CAR or TAC lentivirus on day 1, then cultured for 14 days. mRNA T cells were generated by culturing non-transduced T cells for 13 days, then electroporating with mRNA on day 13. Samples were collected on day 14 of culture for phenotyping by flow cytometry. MFI: mean fluorescence intensity.

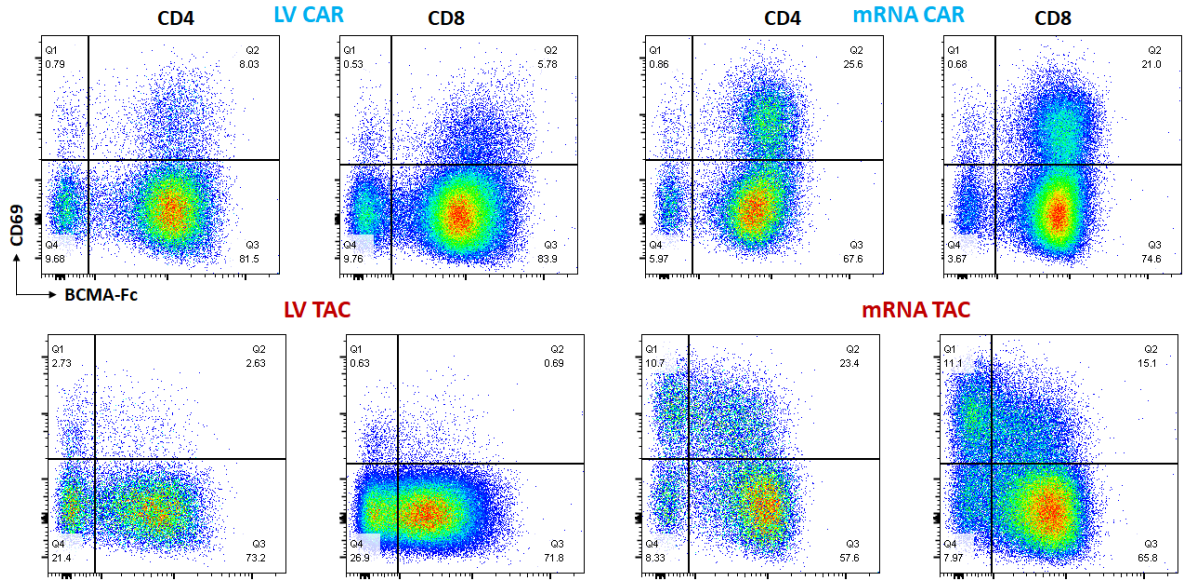


Figure II-10. Electroporation induces expression of CD69 in T cells. T cells were electroporated with either TAC or CAR mRNA and rested for 24 hours before staining and flow cytometry analysis of CD69 and TAC expression.

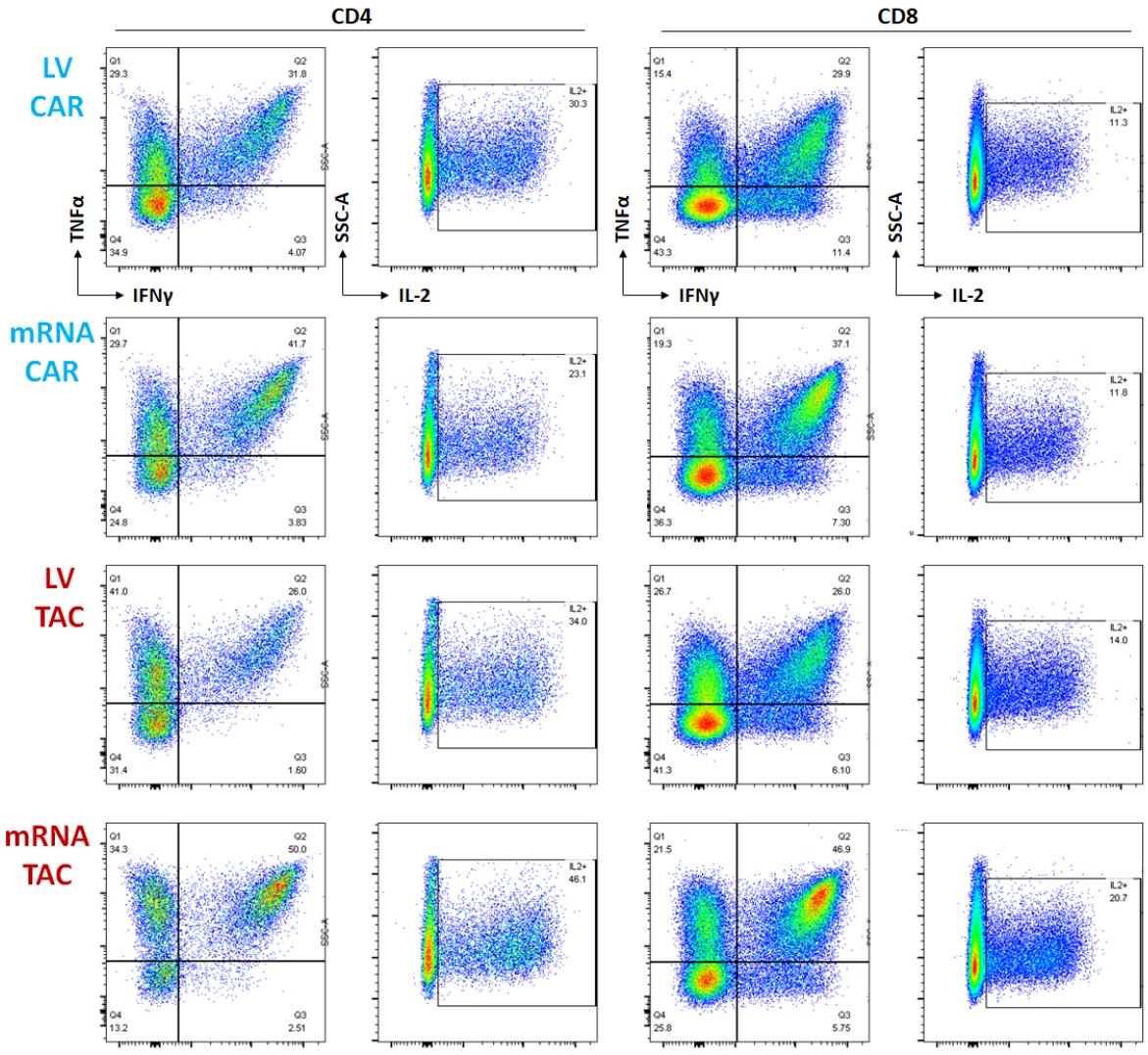


Figure II-11. mRNA TAC T cells robustly produce cytokines. mRNA- or lentivirus-engineered T cells were co-cultured with KMS-11 tumor cells at an effector:target ratio of 2:1 for 4 hrs. Cells were then stained intracellularly for the cytokines IFNγ. TNFα, and IL2.

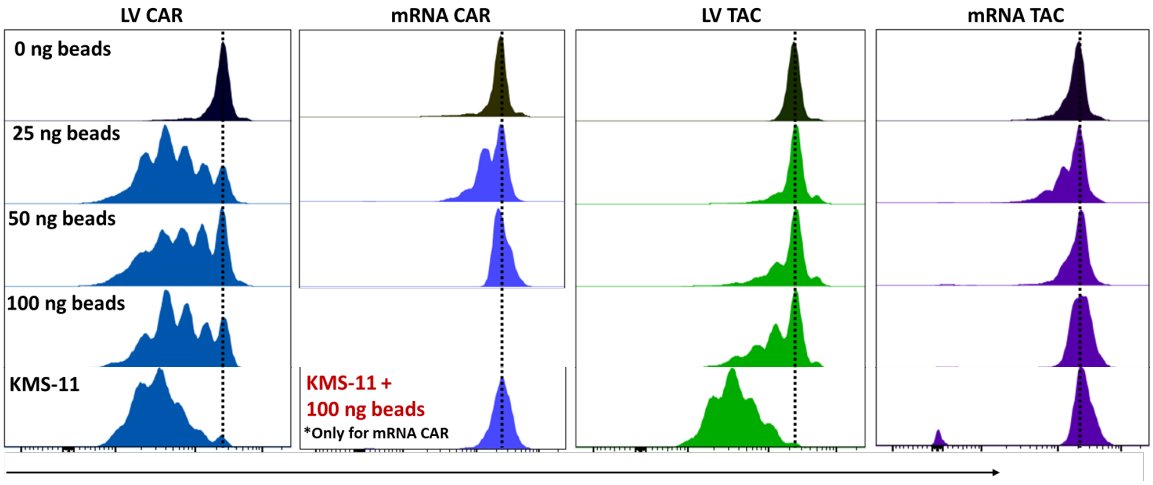


Figure II-12. mRNA engineered cells do no proliferate compared to their lentiviral counterparts. CTV-labeled mRNA- or lentivirus-engineered T cells were co-cultured with KMS-11 tumor cells or antigen-coated beads at an effector:target ratio of 2:1 for 3 days, after which cells were analyzed using a LSR Fortessa flow cytometer.

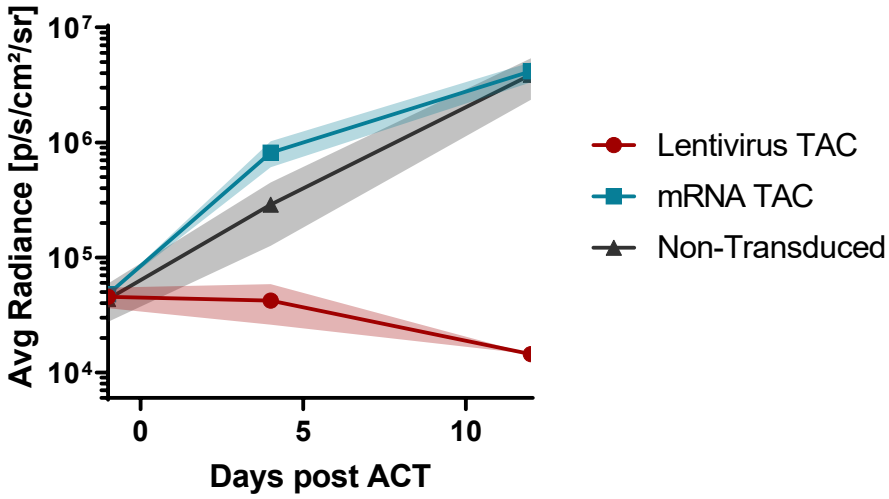


Figure II-13. mRNA TAC T cells fail to induce regression in the KMS-11 multiple myeloma mouse model. Male mice 6-8 weeks old were inoculated with $1x10^6$ KMS-11 tumor cells intravenously. 12-days post implantation, mice were injected intravenously with either: mRNA TAC T cells at $3.5x10^6$ TAC^+ T cells per mouse ($4.2x10^6$ cells total per mouse), lentivirus TAC T cells at $2.2x10^6$ TAC^+ T cells per mouse ($8x10^6$ cells total per mouse), or non-transduced T cells at $8x10^6$ T cells per mouse. n=4 per group.

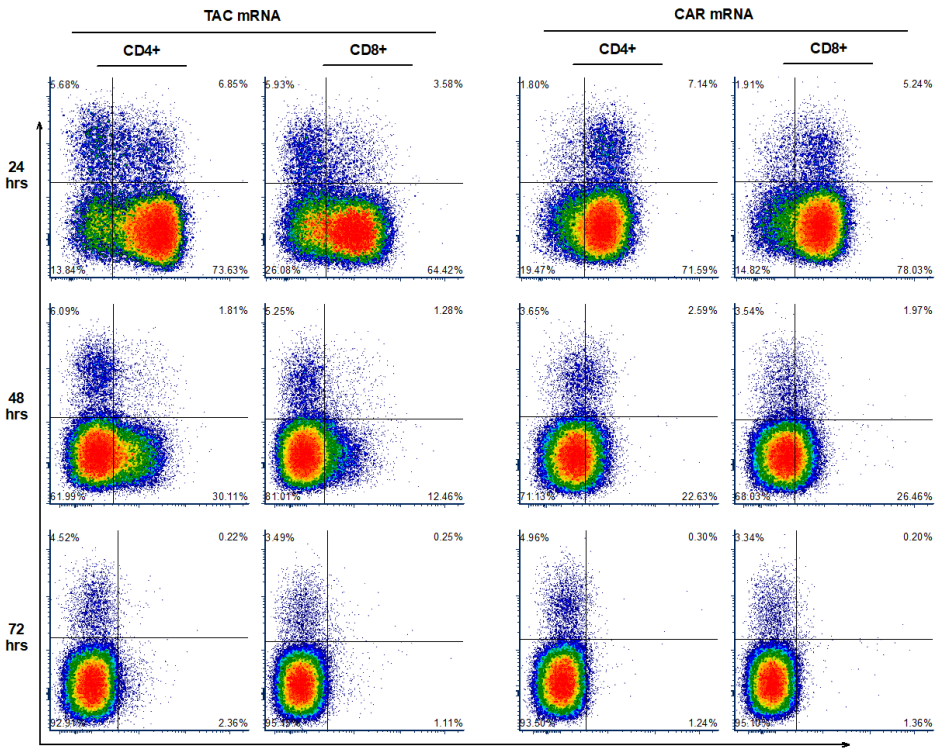


Figure II-14. IVT mRNA-based TAC and CAR receptor expression rapidly declines within 48 hrs of electroporation. T cells were electroporated with either TAC or CAR mRNA as per standard protocol and receptor expression was evaluated at 24, 48, and 72 hrs after electroporation by flow cytometry.

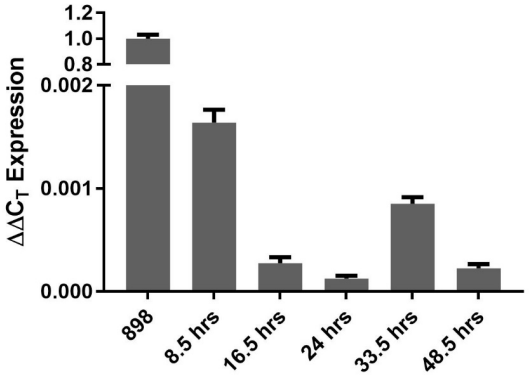


Figure II-15. TAC mRNA is rapidly lost from electroporated cells. T cells were electroporated with TAC mRNA as per standard protocol. Total RNA was taken from T cell samples for cDNA generation and subsequent qPCR analysis with TAC-specific primers.

Discussion

Our data generated suggested there may be unique biology to the T cells engineered with TAC receptor through IVT mRNA, which warrants further investigation. However, stability of the mRNA after electroporation is a large hurdle that will limit the ultimate utility of this approach. As the mRNA cassette design is taken from published work, we believe it is unlikely that the issue is due to the mRNA structure. Experiments investigating an extended 5' capping reaction (up to 2 hrs) or polyadenylation (up to 1 hr) did not improve persistence of the mRNA in the T cells (data not shown). We believe that the short duration of TAC/CAR expression following transfection with IVT mRNA is likely due to mRNA degradation.

A recent study by Foster *et al.* describes several strategies to enhance mRNA stability[529]. The researchers highlight the negative impact of dsRNA contamination within the IVT mRNA product and show improvements to mRNA expression and downstream effector function by dsRNA removal with RNase III. Additionally, detection by innate immune factors can also result in faster mRNA removal. Although this is less of an issue with electroporation as the mRNA bypasses detection by vesicular TLRs, the RNA may still be detected as foreign in the cytoplasm. Inclusion of modified nucleotides such as pseudouridine (Ψ) or 1-methylpseudouridine (m1Ψ) could potentially reduce the probability of this occurring.

Beyond the stability of the mRNA within the cells, mRNA-engineered T cells suffer from their lack of persistence because the mRNA species will be lost over successive rounds of proliferation following T cell expansion. Further, despite high level expression of TAC/CAR, the mRNA-engineered T cells display poor proliferation, which suggests that the engineering process may negatively impact T cell expansion – a key feature of therapeutic T cells. Although multiple infusions may overcome this, it is a brute force solution that provides no advantages over lentiviral-engineered cells. Give that the persistence, survival, and stemness of an anti-tumor T cell product appear to be correlates of clinical efficacy, we do not believe that introducing synthetic antigen receptors using mRNA will ultimately prove to be clinically useful.

Materials and Methods

The methods and reagents utilized in this appendix were identical to those presented in chapter 2 with the following exceptions and additions:

1. T cells were activated using anti-CD3/anti-CD28 Dynabeads and cultured with 660 IU/mL (10 ng/mL) rhIL-2, instead of 1.5 ng/mL, for the duration of the manufacturing period.
2. KMS-11 multiple myeloma cells were cultured as MM.1S were previously described.
3. *In vitro* Luciferase Cytotoxicity Assay
    a. To evaluate cytotoxicity, $5x10^4$ luciferase engineered KMS-11 cells were co-cultured with T cells in a white flat bottom 96-well plate (Corning) at indicated effector:target for 8 hrs at 37°C. After co-culture, 0.15 mg/mL D-Luciferin (Perkin Elmer) was added per well and luminescence was measured using a i3 SpectraMax (Molecular Devices) across all wavelengths. Tumor cell viability was calculated as: ((Emission – Background)/(Tumor cell alone – Background)) * 100%. Each condition was tested in triplicate.
4. *In vitro* Stimulation and Ki67 Assay
    a. Cells were stimulated as in described for cytokine production in *chapter 3* with the following modifications: cells were stimulated for a period of 24 hrs without the use of BD GolgiPlug or 0.02 M EDTA, and the following antibodies were used for staining, anti-CD4-Pacific Blue (BD Pharmingen), anti-CD8-AF700 (OKT8 clone; eBioscience), anti-CD69-BV650 (BD Horizon), and anti-Ki67-FITC.
5. *In vitro Transcription by mMESSAGE mMACHINE T7*
    a. IVT mRNA was generated using the Ambion mMESSAGE mMACHINE T7 Kit (ThermoFisher). As per manufacturer guidelines, all plasmid DNA templates were linearized by restriction digest using enzymes that produce 3' overhangs. The control luciferase vector and BCMA-TAC mRNA vector were linearized using XmnI (NEB) and AvrII (NEB), respectively. Linearized BCMA-TAC template DNA was purified by phenol:chloroform:isoamyl alcohol (PCI) extraction. Briefly, digested DNA was mixed 1:1 with PCI and phase separation was induced by centrifugation. Following centrifugation, the aqueous phase was removed gently and back extracted with water to remove any left-over PCI. The aqueous phase was once again removed, mixed with chloroform:isoamyl alcohol 1:1 and the aqueous phase was removed as above. NH_4OAc was added to the extracted solution to a final concentration of 0.75 M, mixed, and then 100% ice cold ethanol was

added at 2.5x volume. This solution was incubated overnight at -20°C, then DNA was precipitated by repeated 80% ethanol washes.

b. Linearized template DNA was used to generate mRNA following manufacturers instructions. Eluted mRNA was purified using QIAGEN RNeasy Mini purification kit and eluted in nuclease-free water prior to storage at -80°C.

c. Samples were taken from each batch of mRNA produced to be quantified by NanoVue (GE Healthcare) and assessed for quality by inspection on an agarose:bleach gel[530] alongside of a ssRNA ladder (ThermoFisher). Samples were loaded with 2x RNA gel loading buffer (ThermoFisher) on a 1% agarose:bleach gel. After electrophoresis, gels were incubated with 1mg/mL ethidium bromide for 15 minutes, destained in 1x TAE for 15 minutes, and finally visualized using an AlphaImager UV Imager.

6. In vitro *Transcription with the T7 mScript Transcription System*

a. IVT mRNA was generated using the T7 mScript Transcription System (CELLSCRIPT). As per manufacturer guidelines, all plasmid DNA templates were linearized by restriction digest using enzymes that produce 5' overhangs. The BCMA-TAC mRNA vector was linearized using AvrII (NEB). Linearized BCMA-TAC template DNA was purified by phenol:chloroform:isoamyl alcohol (PCI) extraction or column purification using . Briefly, digested DNA was mixed 1:1 with PCI and phase separation was induced by centrifugation. Following centrifugation, the aqueous phase was removed gently and back extracted with water to remove any left-over PCI. The aqueous phase was once again removed, mixed with chloroform:isoamyl alcohol 1:1 and the aqueous phase was removed as above. NHR_4ROAc was added to the extracted solution to a final concentration of 0.75 M, mixed, and then 100% ice cold ethanol was added at 2.5x volume. This solution was incubated overnight at -20°C, then DNA was precipitated by repeated 80% ethanol washes. For column purification, digested template was ran on a 1% agarose TAE gel for 1 hr at 100 V. The band corresponding to the linear product was gel extracted using the QIAquick Gel Extraction kit (QIAGEN) and eluted in nuclease-free water (Ambion).

b. Linearized template DNA was used to generate mRNA following manufacturers instructions. Eluted mRNA was purified using QIAGEN RNeasy Mini purification kit and eluted in nuclease-free water prior to storage at -80°C.

c. Samples were taken from each batch of mRNA produced to be quantified by NanoDrop OneC (ThermoFisher) and assessed for

quality by inspection on an agarose:bleach gel[530] alongside of a ssRNA ladder (ThermoFisher). Samples were loaded with 2x RNA gel loading buffer (ThermoFisher) on a 1% agarose:bleach gel. After electrophoresis, gels were incubated with 1mg/mL ethidium bromide or 1x GelRed Nucleic Acid Stain (Biotium) for 15 minutes, destained in water for 15 minutes, and finally visualized using an AlphaImager UV Imager.

7. *mRNA Electroporation*
    a. T cell electroporation was accomplished using a BTX ECM830 Electro Square Ware Porator[526]. T cells to be electroporated were washed 3 times in warm Opti-MEM (ThermoFisher), then resuspended at $1x10^8$ cells/mL in Opti-MEM. $1x10^7$ T cells/100 µL of Opti-MEM were mixed with 10 µg mRNA/$1x10^7$ T cells, which was then transferred to a BTX Electroporation Cuvette Plus (2 mm) and immediately electroporated with a single pulse of 500 V with a pulse length of 1 ms. Electroporated cells were immediately transferred to an appropriately sized vessel containing T cell media with IL-2 and IL-7 that has been pre-equilibrated within the incubator.
8. *mRNA Nucleofection*
    a. T cell nucleofection was accomplished using a Amaxa Nucleofector 2b system (LONZA) following the Amaxa Human T cell Nucleofector Kit (LONZA) protocol. Briefly, $1x10^6$ T cells were centrifuged at 200 RCF for 10 minutes and resuspended in 100 µL nuclefector solution. T cells were then mixed with 3 pmol of RNA (~1.6 µg in the case of luciferase mRNA) and nucleofected using program T-023 on the machine. T cells were then transferred as above.
9. *qPCR Analysis of Gene Expression*
    a. RNA samples were extracted from T cells using the QIAGEN RNeasy Plus Mini kit as per manufacturers guidelines. Genomic DNA was removed using the included G-eliminator columns. Samples were eluted in nuclease-free water and stored at -20°C until used to generate cDNA. cDNA was produced using the SuperScript IV First-Strand Synthesis (ThermoFisher) kit as per manufacturers protocol using oligo(dT)R_{20}R primers and 500 ng total RNA. cDNA was diluted 10x with nuclease-free water, and 5 µL was used per well for qPCR analysis. qPCR was accomplished using either the TaqMan Fast Advanced Master Mix (ThermoFisher), Luna Universal Probe Master Mix (NEB), or PerfeCta SYBR Green SuperMix, ROX (Quantabio), as per manufacturer guidelines on a Applied Biosystems StepOnePlus RT-PCR system. CBLB was probed for using either the Hs00909783_m1 or

Hs00180288_m1 TaqMan Gene Expression Assay using the FAM dye. TAC was probed using custom primers previously validated in our lab (Vivian Lau, 500 nM, unpublished data), with the SYBR Green. B2M (Hs00187842_m1) and RPL13A (Hs04194366_g1) TaqMan Gene Expression Assays were used as endogenous controls. Data was analyzed following the GE Healthcare $\Delta\Delta CR_TR$ analysis method[531].

10. Adoptive Transfer into the KMS-11 Xenograft Model of Multiple Myeloma

a. All experiments were approved by the McMaster Animal Research Ethics Board. 7-8-week-old male NOD.Cg-Rag1P^{tm1Mom}PIl2rgP^{tm1Wjl}P/SzJ (NRG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (Stock #007799), or bred in-house. Male mice (6-12-weeks-old) were injected with 1x10^6 KMS-11 effLuc cells intravenously to establish disease. One dose of thawed, cryopreserved engineered T cells was administered 12-days post-tumor inoculation. Tumor burden was monitored through bioluminescent imaging. Briefly, 10 µL/g of a 15 mg/mL D-Luciferin solution (Perkin Elmer) was injected intraperitoneally 14 minutes prior to dorsal and ventral imaging using an IVIS Spectrum (Perkin Elmer). Images were analyzed using Living Image Software v4.2 for MacOSX (Perkin Elmer), and dorsal and ventral radiance was summed.

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