

**CHARACTERIZATION OF ENGINEERED T CELLS  
FOLLOWING ANTIGEN STIMULATION**

**CHARACTERIZING THE RESPONSE OF TAC- AND CAR-  
ENGINEERED T CELLS FOLLOWING ANTIGENIC  
STIMULATION**

By VIVIAN WING CHONG LAU, B.SC.

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## LAY ABSTRACT

Cytotoxic T cells are also known as “resident killer” cells of the immune system, as they can seek and eliminate diseased or infected tissue, including cancer cells. However, cancer cells can evade elimination by T cells over time. Genetic engineering of T cells allows us to re-arm T cells against cancer cells. T cells isolated from a patient are genetically modified to recognize cancer cells specifically. So far, these modified T cells have been successful against several leukemias. However, the side effects of this treatment can be substantial and life-threatening, due to the massive reaction of the T cells against the cancer cells following infusion. We explore the biology of two different types of engineered T cells to better understand the interaction between T cell and tumour cell. Our results aim towards mitigating the side effects of T cell treatment, while investigating how we can improve its effectiveness for the future.

## ABSTRACT

T lymphocytes engineered with chimeric antigen receptors (CARs) have shown remarkable success in the treatment of leukemias. Conventional CARs seek to recapitulate TCR and costimulatory signals through fusion of T cell signaling elements into a single receptor. The robust anti-tumor activity of CAR T cells is often accompanied by debilitating toxicities due to excessive T cell activation and cytokine production following infusion. Our lab has generated a novel chimeric receptor termed T cell antigen coupler (TAC), which is designed to engage native T cell signaling domains for cellular activation. In a murine xenograft model, we previously found that TAC T cells mediated rapid tumour regression in the absence of toxicities. Comparatively, CAR T cells elicited significant lethal toxicities to the mice due to reactivity against an unspecific antigen that resulted in excessive proliferation and cytokine production *in vivo*. Here, we report that TAC and CAR T cells have fundamentally different biology, both at rest, and during activation. TAC T cells were more sensitive to the context of stimulation compared to CAR T cells. Whereas TAC T cells can discriminate between antigen bound to a bead, or antigen present on a cell, CAR T cells do not make the same distinction and responds equally well to both. Compared to several different CAR constructs, TAC T cells are less prone to tonic signaling and T cell differentiation in the absence of antigen. These findings support that TAC T cells may pose a safety benefit as a cancer immunotherapy, due to its distinct biology from CAR T cells that enables them to require more stringent contexts for activation.

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## LIST OF ALL ABBREVIATIONS AND SYMBOLS

### Abbreviations:

ACT - adoptive cell transfer  
ADCC - antibody-dependent cell-mediated cytotoxicity  
Akt - protein kinase B  
ANOVA - analysis of variance  
AP-1 - activator protein 1  
APC - antigen-presenting cell  
BCMA - B cell maturation antigen  
BiTE - bispecific T cell engager  
CAIX - carbonic anhydrase IX  
CAR - chimeric antigen receptor  
CD - cluster of differentiation  
CFSE - carboxyfluorescein succinimidyl ester  
CO<sub>2</sub> - carbon dioxide  
CRS - cytokine release syndrome  
CTLA4 - cytotoxic T lymphocyte-associated protein 4  
CTV - CellTrace Violet  
DARPin - designed ankyrin repeat protein  
DC - dendritic cell  
DMEM - Dulbecco's Modified Eagle Medium  
DNA - deoxyribonucleic acid  
EC<sub>50</sub> - concentration of a drug that gives half-maximal response  
E:T - effector:target  
EF-1 $\alpha$  - elongation factor-1 alpha  
FBS - fetal bovine serum  
Fc - fragment crystallizable region  
Fc $\epsilon$ RI - high-affinity IgE receptor  
GM-CSF - granulocyte-macrophage colony-stimulating factor  
HEK - human embryonic kidney  
HER2 - human epidermal growth factor receptor 2  
HLA - human leukocyte antigen  
huUCHT1 - humanized UCHT1  
IFN - interferon  
IgE - immunoglobulin E  
IgG - immunoglobulin G  
IL - interleukin  
ITAM - immunoreceptor tyrosine-based activation motif  
IU - international units  
JAK-STAT - Janus kinase/signal transducers and activators of transcription  
LAG-3 - lymphocyte-activating gene 3  
MAPK - mitogen-activated protein kinase  
mCMV - minimal cytomegalovirus  
MFI - mean/fluorescence intensity  
MHC - major histocompatibility complex

MOI - multiplicity of infection  
mRNA - messenger RNA  
mTOR - mammalian target of rapamycin  
NCI - National Cancer Institute  
NFAT - nuclear factor of activated T-cells  
NGFR - nerve growth factor receptor  
NK cell - natural killer cell  
NRG - NOD.Cg-Rag1<sup>tm1Mom</sup>Il2rg<sup>tm1Wjl</sup>/SzJ  
N.S. - not significant  
PBMC - peripheral blood mononuclear cell  
PBS - phosphate buffered saline  
PD-1 - programmed cell death protein 1  
PD-L1 - programmed death ligand 1  
PI3K - phosphoinositide 3-kinase  
PSMA - prostate-specific membrane antigen  
qPCR - quantitative polymerase chain reaction  
rh - recombinant human  
RNA - ribonucleic acid  
RT - room temperature  
scFv - single chain variable fragment  
SPICE - simplified presentation of incredibly complex evaluations  
TAC - T cell antigen coupler  
TCR - T cell receptor  
TIL - tumor infiltrating lymphocyte  
TIM-3 - T-cell immunoglobulin and mucin-domain containing-3  
TNF - tumor necrosis factor  
TNFRSF - tumor necrosis factor receptor superfamily  
tNGFR - truncated nerve growth factor receptor  
ZAP-70 - zeta-chain-associated protein kinase 70

Symbols:

$\alpha$  alpha  
 $\beta$  beta  
 $\delta$  delta  
 $\gamma$  gamma  
 $\zeta$  zeta  
 $\mu$  micro  
°C degrees Celsius  
cGy centigray  
 $\kappa$  kappa  
% percent

## DECLARATION OF ACADEMIC ACHIEVEMENT

I, Vivian W.C. Lau, declare that I have independently authored and assembled the contents of this thesis, with editorial assistance from Professor Bramson. Listed below are the contributions from colleagues in the providing of materials and/or methods used in this thesis.

Christopher W. Helsen – conceived, cloned, and constructed the T cell antigen coupler (TAC) receptor, and HER2-TAC constructs

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## **1.0 Introduction**

### **1.1 The early history of cancer and the immune system**

The concept of immunosurveillance was first proposed in 1909 by German physician scientist, Paul Ehrlich, as the process by which ‘aberrant’ cells such as cancer cells remain latent in healthy individuals due to mechanisms that suppress their growth.<sup>1</sup> In essence, immunosurveillance in the context of cancer suggests that although tumour cells naturally develop and accumulate over time, their deviation from healthy cells enables them to evoke an immunological response that results in their eradication, thereby preventing further uncontrolled growth.<sup>2</sup> Forty years later, speculations surrounding the concept were supported by several important observations. In 1943, Ludwik Gross observed that mice inoculated intradermally with sarcomas were able to resist engraftment and subsequent reinoculation, despite the tumours originating from genetically identical hosts.<sup>3</sup> Further studies showed that chemically-induced tumours in mice can induce a “state of immunity”, such that subsequent attempts to engraft a secondary tumour of the same type resulted in tumour rejection.<sup>4,5</sup> An intriguing comment came from F. MacFarlane Burnet’s review on curative cancer treatments in 1957, who suggested:

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“A slightly more hopeful approach, which, however, is so dependent on the body’s own resources that it has never been seriously propounded, is the immunological one.”

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The acknowledgement that cancer could be considered a disease of the immune system foreshadows the modern paradigm that a patient’s own immune system can be used for cancer treatment. Ultimately, these observations support the foundations of modern cancer immunotherapy which seeks to enhance the immune system to be able to eradicate tumour cells and maintain a state of immunity against neoplasias.

### **1.2 Modern cancer immunotherapy targets cancers by enhancing immune function**

According to the theory of cancer immunosurveillance, clinical manifestation of cancer occurs when the immune system has failed to eliminate and suppress neoplastic cells over time.<sup>6</sup> Tumours can develop despite the presence of a functional immune system, through immunosuppressive strategies that allow cancer cells to evade immune recognition. Traditional cancer therapies involve surgical resection of the tumour, chemotherapy to reduce tumour growth, and radiation therapy to eradicate the tumour cells. In comparison to traditional therapies, cancer immunotherapies employ a more targeted approach by recognizing components of the tumour itself, or promoting immune cells to facilitate an anti-tumour response.<sup>7</sup> There are several major classes of cancer immunotherapies currently employed alone, or in combination with other immunotherapies or traditional therapies. These can be broadly categorized into cell- and non-cell-based strategies, with examples and proposed mechanisms of action summarized in Table 1.

**Table 1.** Examples of cancer immunotherapy agents, with indications and proposed mechanisms of action.

Type of Agent	Examples of Agents (Trade names)	Indication(s)	Proposed Mechanism of Action	Reference
Cytokines	IL-2 (Aldesleukin)	Melanoma, renal cell cancer	Broad immunostimulation	8
	Type I interferons (Intron-A, Sylatron)	Leukemias, melanoma	Broad immunostimulation	9
Checkpoint inhibitor monoclonal antibodies	Anti-CTLA-4 (Ipilimumab)	Melanoma	Blocks inhibition of lymphocyte activation	10
	Anti-PD-1 (Nivolumab)	Melanoma		11
	Anti-PD-L1 (atezolizumab)	Bladder cancer		12
Tumour antigen-specific monoclonal antibodies	Anti-CD20 (rituximab)	Leukemias	Antibody-dependent cellular cytotoxicity (ADCC) by NK cells	13
	Anti-HER2 (trastuzumab)	Breast cancer	Inhibition of tumour cell proliferation; ADCC	14
Bispecific antibodies and T cell engagers	CD3-CD19 bi-specific T cell engagers (blinatumomab)	Leukemias	Recruitment of T cells to specific antigen-expressing tumour cells	15
Peptide-based vaccines	Oncophage (Vitespen)	Melanoma	Tumour peptides are presented on MHC-Class I molecules for lymphocyte activation	16
Dendritic cell vaccines	Sipuleucel-T (Provenge)	Prostate cancer	Priming of lymphocytes with DCs loaded with prostatic acid phosphatase and GM-CSF	17
Oncolytic viruses	T-vec	Melanoma	Lysis of infected tumour cells; broad immunostimulation	18
Adoptive cell therapy	Tumour-infiltrating lymphocytes	Melanoma	Lysis of tumour cells by antigen-specific T cells	19,20
	TCR-engineered T cells	Melanoma		21
	Chimeric antigen receptor T cells (Kymriah, Yescarta)	Leukemias		22,23

Cell-based therapies overcome several limitations of other biological therapies, such as monoclonal antibodies. Cells are living components that can adapt and respond to their environment, which can make them more amenable to the heterogeneous nature of tumours. As an example, the longevity of T cells and their intrinsic capability to differentiate into memory populations make them highly useful in suppressing tumour growth over the long term. However, live cell therapies are disadvantaged by their degree of personalization as they typically employ the patient's own cells. This significantly increases the cost of the therapy, and makes their manufacturing, preparation, and dosing difficult to manage. Nevertheless, cell-based immunotherapies remain a promising form of cancer therapy as demonstrated by their breakthrough success when used for adoptive cell transfer.

### **1.3 The T cell receptor is responsible for antigen recognition by T cells**

T lymphocytes are one of two primary white blood cell-types of adaptive immunity, which can mount specific immune responses. Over the past 50 years, the development of microscopic techniques, advancement in gene manipulation, and the use of transgenic animal models have led to significant improvements in our understanding of T cell activation. *In vitro* culturing of engineered T cells was made possible by several discoveries spanning the 1970s and 1980s. Examples of these landmark discoveries include the identification of IL-2 as a primary T cell growth factor<sup>24</sup>, discovery of T cell receptor complex components<sup>25-28</sup>, and uncovering the critical role of costimulation during T cell activation.<sup>29,30</sup> These concepts will be described in greater detail in subsequent sections.

T cells recognize and respond to antigens, which are defined as substances that can elicit an antibody response from the host immune system.<sup>31</sup> Antigen-presenting cells (APCs), such as dendritic cells, specialize in processing whole antigens such as viral proteins into peptides, which are presented on major histocompatibility complex (MHC) molecules. Recognition of peptides in the context of MHC molecules by T cells is dependent on the T cell receptor (TCR), a transmembrane protein consisting of a heterodimeric  $\alpha\beta$  or  $\gamma\delta$  subunit.<sup>32</sup> A majority of circulating T cells carry  $\alpha\beta$  TCRs; each  $\alpha$  and  $\beta$  subunit contains highly polymorphic variable domains which grants significant diversity in the peptide-antigens recognized by a given T cell repertoire.<sup>31</sup> While the TCR does not possess signaling domains of its own, it is non-covalently associated with the CD3 complex, which is composed of three dimers, CD3 $\epsilon$ -CD3 $\delta$ , CD3 $\epsilon$ -CD3 $\gamma$ , and CD3 $\zeta$ -CD3 $\zeta$ .<sup>33,34,35</sup> Signal transduction through the TCR relies on the CD3 complex, which contains intracellular immunoreceptor tyrosine-based activation motifs (ITAMs).<sup>26,36,37</sup> ITAMs are conserved amino acid sequences containing tyrosine residues, which are phosphorylated by tyrosine kinases to serve as the first step in signal transduction following ligand engagement.<sup>38</sup> The TCR-CD3 complex contains a total of 10 ITAMs; one on each of CD3 $\delta$ ,  $\epsilon$ , and  $\gamma$  intracellular domains, and six are located within the intracellular tails of the CD3 $\zeta$  homodimer.<sup>39</sup> Early experiments that showed that ITAM phosphorylation leads to downstream T cell activation, measured by activation marker CD69 upregulation, or cytokine production.<sup>26</sup> Subsequent studies identified tyrosine kinases Lck and zeta-associated protein-70 (ZAP-70) as key mediators for initiating signal transduction through ITAM phosphorylation.<sup>36,40</sup>

#### 1.4 T cell activation initiates a cascade of biophysical and biochemical downstream effects

The process of TCR-CD3-MHC ligation and is known as TCR triggering, and results in several biochemical and biophysical changes that ultimately result in T cell proliferation, cytokine production, and/or effector function, depending on the extent of cellular activation.<sup>41</sup> Several models for T cell triggering exist, each describing the biophysical events that result in complete signal transduction. There is currently no unifying theory that consolidates all the theories for TCR triggering. This is further complicated by variability in the types of T cells used in each study, methods used to determine kinetics measurements, and whether coreceptors, costimulatory receptors, and adhesion molecules are also contributing to the quality of the interaction.<sup>42</sup>

One theory suggests that when a T cell recognizes an antigen, an immunological synapse forms which segregates signaling components on the plasma membrane.<sup>43</sup> This *kinetic-segregation* model proposes that phosphatases, such as CD45, are excluded from the region where TCR:peptide-MHC are bound, which allows for sustained ITAM phosphorylation by tyrosine kinases in close proximity through formation of microclusters.<sup>44,45</sup> Another model, the *kinetic-proofreading* model, suggests that productive binding events only occur if TCR are bound long enough for a series of phosphorylation steps to occur. Intermediate steps between TCR engagement allows for error correction, where fast off-rates from weak interactions can allow the T cell to reverse biochemical modifications.<sup>46</sup> Compared to strong agonists that fully phosphorylate all tyrosine residues of the CD3 $\zeta$  homodimer, stimulation by weak TCR agonists or antagonists only resulted in partial  $\zeta$ -chain phosphorylation.<sup>47,48</sup> However, later experiments attested that T cell activation is not necessarily an “all or nothing” response. These studies showed that signals originating from weak TCR agonists, as determined by their rapid  $k_{\text{off}}$  measurements, can accumulate over time and produce T cell effector functions such as CD69 upregulation or cytokine production. However, these responses were significantly delayed compared to signals triggered by strong TCR agonists.<sup>49</sup> Furthermore, coreceptors CD4 and CD8 can stabilize weak TCR:peptide-MHC interactions by binding to non-variant regions of MHC class II and class I, respectively.<sup>50</sup> One primary function of coreceptors is to recruit kinases such as Lck, which phosphorylate  $\zeta$ -chains of the TCR and ZAP-70.<sup>51,52</sup> CD4 and CD8 bind to MHCs with fast kinetics. Several studies have shown that only CD8 is important for low affinity TCR:peptide interactions, and CD4 has little to no impact on TCR triggering.<sup>53,54</sup> This suggests that the primary function of coreceptors is to deliver Lck and facilitate the early steps in signal transduction.<sup>55</sup>

A third model with significant experimental evidence describes serial engagement of multiple TCR molecules by a single peptide-MHC during TCR triggering.<sup>56</sup> On a macro scale of TCR triggering, individual TCR and ligand interactions may last a few seconds, whereas interactions between a single T cell and target cell, such as an APC, takes place over several hours. At low ligand densities of <100 per APC, each peptide-MHC molecule can trigger up to 200 separate TCRs, which amplifies the signal, extends the duration of signaling, and allows for accumulation of signaling intermediates that lead to complete T cell activation.<sup>57</sup> Several studies have correlated serial triggering with sustained signaling, and showed its role in promoting

downstream T cell cycle progression, and differentiation.<sup>58–60</sup> Consequently, TCR downmodulation following antigenic stimulation has also been used as a marker for sustained signaling or productive binding.<sup>56,61,62</sup> From a functional perspective, downmodulation acts as a negative feedback mechanism that limits responsiveness to further T cell signaling, thereby preventing overstimulation and prohibiting excessive inflammatory responses.<sup>63–66</sup> Interestingly, ligand-independent mechanisms have also been shown modulate TCR expression; as an example, engagement of the programmed cell death-1 (PD-1) pathway was demonstrated to induce ubiquitin ligase expression and subsequent targeting of TCR for degradation.<sup>67</sup>

### **1.5 Costimulation, in addition to TCR triggering, is required for T cell activation**

Early reports of costimulation in T cells highlighted the role of CD28 in augmenting cytokine production in human T cells following stimulation through the TCR-CD3 complex.<sup>29</sup> CD28 can enhance TCR triggering by lowering the threshold of TCR occupancy required, and acts as an amplifier of early TCR signaling.<sup>68</sup> In the context of infection, upregulation of costimulatory molecules by APCs can provide appropriate signal amplification that leads to T cell activation. Conversely, antigen recognition by the TCR in the absence of costimulation during initial antigen encounter will result in TCR hyporesponsiveness, or T cell anergy.<sup>69,70</sup> This is the basis for the “two-signal” concept of T cell activation mentioned earlier, which dictates that T cells will optimally activate only when signal 1 (antigen recognition by the TCR) and signal 2 (costimulatory signals) are presented concurrently.<sup>71,72</sup> Functionally, CD28 signaling activates the PI3K pathway, which leads to Akt and mTOR signaling. Synergistic with TCR triggering, CD28 activation optimally induces IL-2 production through Akt signaling<sup>73</sup>, which increases glycolytic metabolism in T cells to support biosynthesis.<sup>74</sup> Although CD28 may be the most well-studied costimulatory receptor in T cells, its function, overlaps with other costimulatory receptors belonging to the TNF receptor superfamily (TNFRSF). TNFRSF receptors include, but are not limited to, 4-1BB (or CD137), CD27, and OX40.<sup>72</sup> Together, costimulation from the TNFRSF receptors perform a variety of functions, and share commonalities in their ability to upregulate anti-apoptotic genes such as Bcl-2 and Bcl-xL which can render activated T cells more resistant to cell death than non-activated counterparts.<sup>75,76</sup>

A hallmark of adaptive immunity conferred by T cells is their ability to differentiate into long-lived memory populations.<sup>77,78</sup> Transformation of T cells into long lived effector cells requires CD28<sup>79,80</sup>, or CD27, 4-1BB, and OX40, collectively.<sup>81,82,83</sup> Memory T cells can be broadly classified into two subgroups; central memory T cells (T<sub>CM</sub>), and effector memory T cells (T<sub>EM</sub>).<sup>79</sup> T<sub>CM</sub> cells are considered superior for tissue homing functions compared to T<sub>EM</sub> as they express receptors important for extravasation, such as CD62L and CCR7.<sup>84</sup> Conversely, T<sub>EM</sub> cells are better at patrolling the periphery and exerting immediate cytotoxic functions upon antigen encounter. T<sub>CM</sub> also have greater proliferative capacity, and have demonstrated superior anti-tumour immunity compared to T<sub>EM</sub>.<sup>85,86</sup> Based on these findings, some groups select for specific memory T cell subsets prior to ACT, either by immunoseparation using magnetic beads<sup>87–89</sup>, or cytokine cocktails that preserve memory T cell phenotypes during *ex vivo* expansion.<sup>90</sup>

## 1.6 Expression of coinhibitory receptors negatively regulates T cell activation

Immune responses are tightly regulated via costimulated and coinhibitory pathways. Coinhibition is the opposing function of costimulation, whereby coinhibitory receptors are expressed following T cell activation to prevent overactivation and excessive inflammation.<sup>72</sup> Coinhibitory pathways are also essential for preventing potentially dangerous reactivity against self-antigens, which can result in autoimmune disease if dysregulated.<sup>91</sup> Coinhibitory receptors serve as direct negative feedback regulators, and multiple mechanisms exist to shut down T cell activation.<sup>92</sup> The most well-studied coinhibitory receptor is cytotoxic T lymphocyte-associated protein 4 (CTLA4), which peaks in expression 24–48 hours following T cell activation.<sup>93</sup> CTLA4 binds to equivalent ligands as CD28 with 10–20-fold higher affinity, thereby terminating CD28 costimulation through competition.<sup>92</sup> Similarly, programmed cell death protein 1 (PD-1) is another well-characterized coinhibitory receptor whose interaction with its ligand PD-L1 results in recruitment of phosphatases, and termination of CD28 signaling through dephosphorylation of tyrosines required for signal transduction.<sup>94</sup> Although expression of coinhibitory receptors are normally transient following T cell stimulation, chronically stimulated T cells express multiple coinhibitory receptors at persistent levels over time.<sup>95</sup> In the context of cancer, tumour-reactive T cells are constantly exposed to high antigen load from tumour cells, leading to ‘exhaustion’ and eventual loss of anti-tumour immunity.<sup>96</sup> T cell experience progressive loss of effector functions en route to exhaustion. Loss of cytokine production (e.g. IFN $\gamma$ , TNF $\alpha$ , and IL-2), cytolytic capacity, and proliferation is followed by apoptosis of dysfunctional T cells.<sup>97</sup>

The tumour microenvironment is capable of suppressing T cell cytotoxicity through upregulation of ligands that activate T cell coinhibitory pathways.<sup>98</sup> Chronically stimulated T cells in models of murine viral infection can be rescued from exhaustion through blocking of coinhibitory pathways using antibodies.<sup>99</sup> Following these observations, patients treated with monoclonal antibodies targeting the PD-1/PD-L1 pathway have shown objective response rates above 70% in some cancers including melanoma, and Hodgkin’s disease.<sup>100</sup>

As will be discussed shortly, the use of T cells for cancer immunotherapy face similar challenges in balancing stimulation and inhibition within the complex tumour microenvironment. The focus on modern T cell therapies have focused on strategies that activate T cells specifically against tumour cells, and complementary agents that can increase their potency.

## 1.7 Harnessing the power of anti-tumour T cells using adoptive cell transfer

A leading form of cell-based immunotherapy is adoptive cell transfer (ACT) of T lymphocytes, in which tumour-specific T cells are infused into a patient to mediate an anti-tumour effect.<sup>101</sup> T cells are particularly attractive for use as an immunotherapeutic due to their abundance in peripheral blood, and their natural ability to exert direct cytolytic activity against tumour cells.<sup>6</sup>

In the first ACT study of patients with metastatic cancers, T cells were isolated from peripheral blood and expanded *ex vivo* with interleukin-2 (IL-2), a growth factor for T cells. Out of 25 patients, almost half showed marked tumour regression, and one melanoma patient

experienced complete remission of all metastatic lesions.<sup>102</sup> These results led to subsequent trials wherein T cells are isolated directly from metastatic melanoma sites, expanded *ex vivo* with IL-2, and reinfused into the patient.<sup>103</sup> These tumour-specific T cells, termed tumour-infiltrating lymphocytes (TILs), were combined with a pre-infusion lymphodepleting regimens to improve persistence and expansion of TILs following reinfusion. TILs continue to show promising results for metastatic melanoma. Recent clinical trials show that a majority of patients experience a partial response to treatment at a minimum, and a subset of patients show complete remission.<sup>19</sup> However, efficacy of TILs remains restricted to melanoma, due to difficulties in isolation of TILs from other types of solid tumours. Despite this limitation, TILs attest to the potential of harnessing T cells for their natural anti-tumour capacity.

### **1.8 Non-specific T cells in peripheral blood can be rendered tumour-specific**

The development of recombinant viruses for gene transfer gave rise to the opportunity to genetically modify human T cells.<sup>104–106</sup> Genetically engineered T cells could be expanded into large quantities *ex vivo* while retaining transgene expression in all daughter populations, prior to reinfusion into the patient. T cells engineered to express a high affinity TCR against a specific peptide grants T cells the ability to recognize rare, tumour-associated antigens.<sup>107</sup> This strategy has experienced success against metastatic melanoma<sup>108,109</sup>, and multiple myeloma<sup>21</sup>, where the infused T cells were well-tolerated and induced tumour regressions in several patients.

Due to the highly specific nature of the interaction between TCR and MHC molecules, however, TIL therapy and TCR-engineered T cells are restricted by their dependence on expression of specific human MHC molecules (i.e. human leukocyte antigen (HLA)) haplotypes, limiting their application to a subset of patients. Furthermore, many cancers downregulate HLA in response to selective pressure by immune cells that recognize transformed cells through antigens presented on HLA molecules.<sup>110</sup>

In the late 1980's, Eshhar and colleagues described the first chimeric receptor that provided T cells with antibody-like specificity against a chosen antigen.<sup>111</sup> This “chimeric T cell receptor”, or “T-body”, was designed to overcome the issues of HLA restriction, described in the previous paragraph, by redirecting T cell activation towards surface-expressed antigen in an HLA-independent manner. T-bodies employed a binding domain which was a derivative of a monoclonal antibody known as a single-chain antibodies (scFvs). Single-chain antibodies are fusion proteins consisting of variable regions of the heavy and light chains of immunoglobulins, linked by a short peptide.<sup>112</sup> More importantly, scFvs retained equivalent antigen specificity compared to the whole immunoglobulin<sup>113</sup>, and could even be engineered to increase their stability and affinity.<sup>114,115</sup> T-bodies were designed to mimic T cell receptor (TCR) signaling by consolidating a T cell's antigen-recognition complex into a single synthetic protein capable of eliciting T cell activation. T-bodies composed of an scFv fused to a  $\gamma$  or  $\zeta$  chain from the TCR-CD3 complex were able to activate T cell hybridomas through antigen-dependent stimulation, leading to IL-2 production and lysis of target cells.<sup>116</sup> Over the years, T-bodies continued to evolve as our knowledge of T cell signaling improved. T-bodies are now known as chimeric

antigen receptors (CARs) and CAR-engineered T cells are the most successful form of adoptive cell therapy commercially available.<sup>117</sup>

### **1.9 First-generation CAR designs are based on the earliest TCR activating signal**

As described above, the signal transduction moieties incorporated into CARs were derived from our understanding of fundamental T cell receptor signaling. CARs incorporating only one activating signal, such as a CD3 $\zeta$  intracellular domain, are now referred to as first-generation receptors. Early experiments comparing the intracellular domain of the Fc receptor for IgE (Fc $\epsilon$ RI)- $\gamma$  to the CD3 $\zeta$  cytosolic tail of the TCR complex showed enhanced *in vivo* tumour control by CD3 $\zeta$  variants.<sup>118</sup> These results supported its adoption as the dominant signaling domain used in future CAR constructs.

Structurally, CARs exist as homodimers on the surface of T cells, but also form heterodimers with endogenous CD3 $\zeta$  chains from the TCR.<sup>119</sup> In a mouse T cell line that lacks endogenous expression of CD3 $\zeta$ , CAR expression can rescue expression of TCR-CD3 complexes, indicating that CARs interact directly with the complex, and may be providing a source of CD3 $\zeta$  that restores complex stability. In addition to physical interaction between CARs and endogenous TCR-CD3 complexes, CARs can activate T cells both directly (i.e. independently of the TCR) and indirectly by utilizing components of the TCR.<sup>120</sup> Interestingly, progressive truncation of the CD3 $\zeta$  tail in CARs revealed that stimulation through the CAR was largely unaffected by the third ITAM residue.<sup>120</sup> However, loss of the first or second ITAM resulted in reduced responsiveness to antigen stimulation.

Despite early experiments demonstrating that CARs bearing a simple CD3 $\zeta$  signaling domain were capable of tumour lysis *in vitro*<sup>116</sup>, such CAR designs failed to promote T cell proliferation or IL-2 production.<sup>121</sup> In a phase I trial involving two refractory follicular lymphoma patients, first-generation CAR T cells could not be detected beyond 7 days following infusion.<sup>122</sup> Patients observed neither toxicities nor clinical responses, indicating that these CAR T cells were not capable of persisting long enough for anti-tumour activity. Trials targeting different antigens in ovarian cancer<sup>123</sup>, neuroblastoma<sup>87</sup>, and renal cell carcinoma<sup>124</sup>, observed similar deficiencies in persistence of the first-generation CAR T cells.

### **1.10 Second-generation CARs show enhanced T cell activation and persistence**

The lack of persistence of first-generation CAR T cells prompted further consideration of the CAR design. As the understanding of costimulation grew during the 1990's, it became clear that signaling via the TCR alone would render a T cell non-function (i.e. anergic) whereas concomitant signaling via the TCR and the CD28 costimulatory receptor would lead to robust T cell proliferation and functionality. This knowledge led to the discovery that addition of a costimulatory domain to first-generation CAR T cells was crucial for *in vivo* persistence and anti-tumour efficacy in both mouse models, and human clinical trials.<sup>125,126</sup> It remains uncertain which costimulatory domain provides optimal survival signals. The two dominant costimulatory domains used in second-generation CARs are derived from CD28, or 4-1BB (a.k.a. CD137),

which activate distinct signaling pathways in T cells.<sup>127</sup> Clinical data shows that 4-1BB CARs have longer T cell persistence in patients, detectable in peripheral blood up to 6 months following infusion, whereas CD28 CARs typically lasted 8 weeks or less.<sup>128</sup> This can be explained by the distinct signaling pathways activated by CD28 and 4-1BB. CD28 signals trigger IL-2 production, which is an important cytokine for T cell progression through the cell cycle, followed by upregulation of anti-apoptotic genes such as Bcl-X<sub>L</sub>.<sup>129</sup> Comparatively, 4-1BB belongs to the tumour necrosis factor receptor superfamily and has been shown to promote long-term survival and persistence of T cells *in vivo*.<sup>130</sup> For CAR T cells, 4-1BB signals enable enhanced survival compared to CD28, with an increase in the frequency of central memory T cells, which are in a less differentiated state than the effector T cell populations found in CD28 CARs. Although it may seem that 4-1BB CARs are outperforming CD28 CARs currently in CD19-positive cancers, it remains plausible that CD28 CARs may confer greater efficacy against other tumour types. In solid tumours where metabolic requirements are constrained by the tumour's nutrient uptake<sup>131</sup>, it is possible that CD28 signaling can sensitize T cells towards lower glucose concentrations<sup>132</sup>, thereby improving anti-tumour efficacy against hard-to-treat tumours.

Careful consideration is required to select the costimulatory domain to achieve optimal functionality, as some domains can reduce efficacy<sup>133</sup>, or induce of tonic signaling in the absence of antigen.<sup>134</sup> In the context of CAR T cells, extracellular ligand-binding differs significantly from TCR-based stimulation because the single chain antibodies typically used for antigen recognition is at least 2-3 orders of magnitude higher in affinity.<sup>135-137</sup> Furthermore, it is not known whether CARs utilize all or some of the kinases involved in TCR signaling, since CARs have ITAM signals and costimulation built into a single receptor. Recent studies have shown that CARs are capable of kinase recruitment and phosphatase exclusion, similar to TCR-based signaling.<sup>45</sup> While second-generation CARs led to improvements in anti-tumour efficacy, robust CAR activation and rapid tumour clearance also resulted in significant onset of toxicities in many patients. Potential unpredicted off-tumour toxicities associated with the use novel scFvs add to the toxic profile of these promising new therapeutics.

### **1.11 Toxicities observed in patients treated with CAR T cells**

The onset of severe toxicities can hinder anti-tumour efficacy and cause severe systemic damage despite on-target effects.<sup>138,139,140</sup> As mentioned previously, the use of scFvs and other antigen binding domains circumvents MHC expression on target cells, and theoretically allows for targeting of any antigen of choice. However, the antigen must be surface expressed and ideally at high enough levels for differentiation between healthy and cancerous cells. On-target, off-tumor toxicity is of significant concern to solid tumors where targets that are exclusively expressed in the tumor are rare. Early clinical trials using CAR T cells targeting carbonic anhydrase IX (CAIX) for treatment of renal cell carcinomas saw liver toxicities in patients following infusion, due to CAIX expression on bile duct epithelial cells.<sup>124,141</sup> Similarly, infusion of HER2 CAR T cells caused serious lethal on-target toxicities in one patient, due to reactivity of CAR T cells against HER2 expressed by lung epithelial cells.<sup>142</sup> To mitigate off-tumor toxicities, several groups have engineered split-signal CARs that utilize Boolean gating to

control activation based on two tumour-associated signals, rather than the tumour antigen alone. One example of this was demonstrated by Kloss *et al.*, who expressed two receptors on the surface of primary T cells; the first receptor consisted of a single chain antibody recognizing CD19, fused to CD3 $\zeta$ , while the second receptor consisted of a second single chain antibody recognizing prostate-specific membrane antigen (PSMA), fused to a 4-1BB intracellular domain.<sup>143</sup> Because CD19 is expressed by both healthy and malignant B cells, and PSMA is only expressed by malignant cells, this strategy ensured that only malignant cells expressing both antigens would activate both signal 1 and signal 2 in CARs. This idea could in theory be applied to any set of antigens, where healthy tissues only express the targeted antigen at low levels, and rarer tumour-specific antigens are restricted to tumour cells.

Another well-documented adverse event following CAR T cell infusion is immune activation leading to cytokine release syndrome (CRS).<sup>144</sup> CRS is a group of symptoms caused by the infused CAR T cells, as well as bystander immune cells such as macrophages, which results in massive production of pro-inflammatory cytokines. This systemic inflammatory response can be life-threatening, and manifests in up to 70-100% of patients treated with CAR T cells against CD19.<sup>145</sup> Onset and severity of CRS is correlated with initial tumour burden and disease progression.<sup>146</sup> Although management of toxicities to counteract CRS is possible, many treatments can diminish CAR T cell activity and negatively impact anti-tumour efficacy.<sup>147</sup>

One strategy to control toxicities is the inclusion of “suicide genes” such as inducible caspase-9 that are co-expressed with CARs following T cell engineering, such that caspase-dependent apoptosis can be triggered in CAR T cells following specific administration of a single drug.<sup>143,148</sup> Suicide genes allow for CAR activity to be actively modulated following administration to mitigate excessive systemic toxicities. Furthermore, genetically modified T cells can be removed from a patient following treatment to prevent the risk of developing leukemia.

### **1.12 Tonic signaling in CAR T cells hinders anti-tumour efficacy**

A major pitfall of incorporating fixed signaling domains in CAR T cells is the manifestation of tonic signaling, which primarily occurs due to spontaneous aggregation of the extracellular single chains used for antigen recognition.<sup>134,149</sup> In CAR constructs where tonic signaling is prominent, upregulation of coinhibitory receptors such as PD-1 and lymphocyte-activating gene 3 (LAG-3) was observed.<sup>134</sup> Expression of coinhibitory receptors, also referred to as “checkpoint receptors”, severely limits T cell activation and renders them dysfunctional due to chronic stimulation through the T cell receptor.<sup>150</sup>

T cell dysfunction resulting from tonic signaling is independent of antigen stimulation. The use of constitutive strong promoter can result in high surface expression of the CAR, which exacerbates tonic signaling.<sup>149</sup> Several groups have created inducible CARs, where receptor expression can be controlled by inducible or conditional promoters. One example of this strategy was successfully demonstrated by two groups, who created independent Tet-ON and Tet-OFF expression systems for CAR induction. In the Tet-OFF system, removal of doxycycline from the culture media enabled CAR expression in up to 90% of T cells by 96 hours.<sup>151</sup> In the Tet-ON

system, T cells were expanded *ex vivo* in the absence of doxycycline and CAR expression, and adoptively transferred into tumour-bearing mice with and without doxycycline treatment.<sup>152</sup> Mice receiving both CAR T cells and systemic doxycycline showed significant reduction of leukemic tumour burden, and prolonged survival compared to constitutively-expressed CARs. These studies show that not only are inducible CARs feasible as part of the manufacturing process, but they also pose a therapeutic improvement by mitigating tonic signaling inherent in many CAR constructs.

### 1.13 Direct comparisons between CAR- and TCR-based stimulation

Comparing T cell stimulation following CAR and TCR ligation is challenging due to the dissimilarity in binding interactions between the receptors and their targets (cell surface antigens and MHC/peptide complexes, respectively). Although it is possible to generate a single chain antibody that binds to a specific peptide-MHC, it is unlikely to generate a combination that has the same affinity and functional avidity compared to its interaction with native TCR complexes.<sup>153,154</sup> Harris *et al.* derived a TCR  $\alpha$  and  $\beta$  heterodimer linked to CAR intracellular domains that exhibited similar binding affinity as the native  $\alpha\beta$  dimer recognizing the same peptide-MHC.<sup>155,156</sup> Interestingly, CAR constructs exhibited a 10-100-fold reduction in their sensitivity to peptide-MHC complexes when directly compared to TCR counterparts, despite being expressed at a higher surface density on T cells. For all effector cytokines tested, including IFN $\gamma$ , IL-2, and TNF $\alpha$ , the EC<sub>50</sub> (i.e. concentration of peptide required for half maximal cytokine response) was two orders of magnitude lower for TCRs than CARs. As mentioned previously, it is possible that the structure of TCR is highly conserved for optimal ligation with peptide-MHC molecules, whereas linkage of TCR $\alpha\beta$  domains onto CAR signaling scaffolds does not support synapse formation. However, it is worthwhile to note that although CARs are less sensitive to stimulation in these constructs, they can produce higher concentrations of cytokines when stimulated with high levels of peptide.

A separate report compared signaling of CARs and TCRs by transducing the same T cell with a TCR specific for SIINFEKL peptide, and a CAR specific for HER2.<sup>157</sup> Authors showed that stimulation through the TCR led to assembly of specific adhesion molecule “rings”, whereas localization of the same molecules were indiscriminately distributed when stimulated through the CAR. Furthermore, proximal and distal phosphorylation of kinases was strong and more rapid following CAR stimulation than through the TCR. This ultimately led to faster recruitment of cytotoxic granules and faster immunological synapse resolution following ligation with a target cell.

These studies indicate that CARs likely do not follow similar mechanisms of T cell activation compared to conventional TCR triggering. Because we cannot yet predict the type of signal that will activate a CAR T cell, this makes toxicities in patients hard to anticipate, and effector functions unpredictable due to variation in tumour burden and antigen availability between patients.

## 1.14 The TAC receptor as an alternative to CARs

Our lab has generated a novel chimeric receptor termed T cell antigen coupler (TAC), which possesses distinct structure and biology compared to CAR T cells. The TAC receptor is designed to activate a T cell through simultaneous recruitment of the tumour target and TCR signaling complex using two independent binding domains. The receptor is composed of three components: (1) an antigen-binding domain, (2) an anti-CD3 $\epsilon$  scFv for TCR recruitment, and (3) a coreceptor domain (hinge, transmembrane, and cytosolic regions) (**Figure 1**). We believe that the TAC receptor operates through native TCR signaling machinery via binding of CD3 $\epsilon$ , and subsequently utilizes kinases such as Lck for signal transduction following antigen recognition.

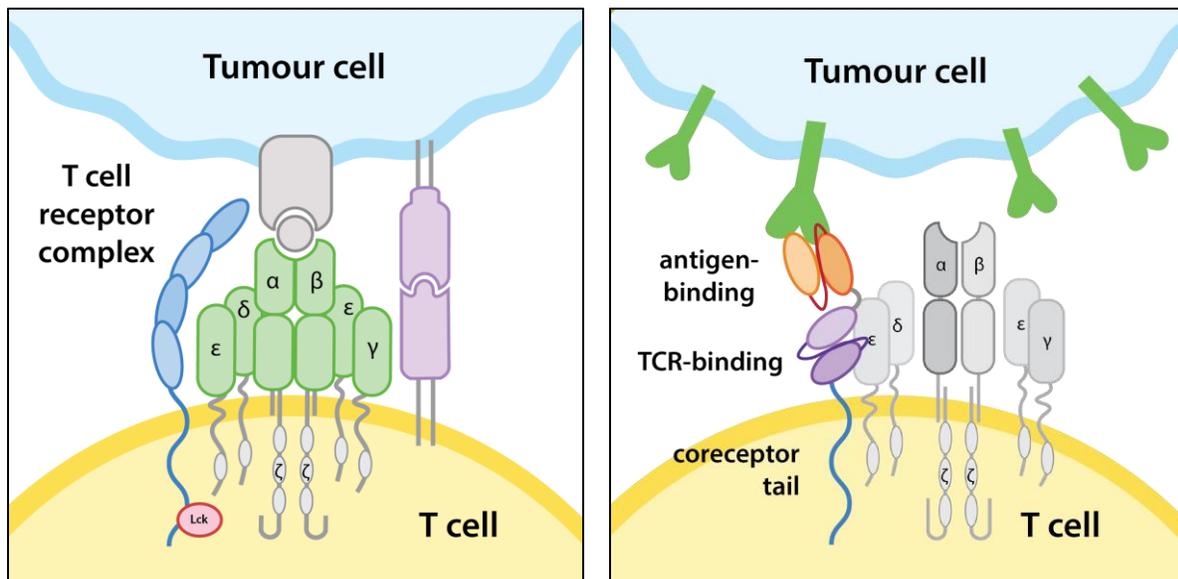
We have recently reported on the rationale for TAC receptor design, and experimental evidence that TAC T cells possess distinct biology and functional efficacy compared to CAR T cells engineered with equivalent antigen binding domains.<sup>158</sup> Although TACs are designed to bind CD3 $\epsilon$  of the TCR complex, this proposed interaction does not appear to trigger autoactivation of T cells with surface TAC expression. Consistent with literature, however, CAR T cells carrying the same binding domain exhibits significant levels of tonic activation that results in checkpoint receptor upregulation and increased T cell differentiation in the absence of antigen-dependent stimulation.

Most striking was the contrast of TAC and CAR T cell efficacy in a murine xenograft model of ovarian carcinoma. While TAC T cells mediated potent and rapid tumour regression, CAR T cells elicited significant and lethal toxicities in all mice, regardless of tumour burden. This off-tumour, off-target response against an unknown antigen was not observed in any dose of TAC T cells tested. Although the effects of this model are attributable to the specific antigen-binding domain used, the question remains why TAC T cells did not cause equivalent toxicities, assuming the same binding domain on TAC or CAR T cells could recognize the same off-target antigen. Based on several clinical reports concerning the severity of toxicities elicited by CAR T cell infusion in human patients<sup>144,147,159</sup>, it would be highly beneficial to employ an engineered T cell approach that offers lower incidence of side effects during treatment.

The data reported in this thesis show that TAC and CAR T cells are fundamentally different, both in the absence of stimulation, and in response to stimulation. Differences during basal states are largely caused by tonic activation of CAR T cells, which was significantly less prominent in TAC T cells. Phenotypic and transcriptional analyses show that TAC T cells were more similar to control T cells expressing only the transduction marker, whereas CAR T cells showed extensive evidence of tonic activation. The more interesting discovery, however, was that TAC T cells were more sensitive to the context of stimulation compared to CAR T cells, as we explored different sources of antigen in the form of bead-bound targets, or cell-based stimulation. CAR T cells were agnostic to the source of antigen and could respond equally well when stimulation with bead-bound antigen or tumour cells. Conversely, TAC T cells only reached optimal activation equivalent to CAR T cells when stimulated with tumour cells, but not bead-bound antigen. This important functional dichotomy between TAC and CAR T cells and potential mechanisms are further explored. The lack of response by TAC T cells towards either

low amounts of antigen, or antigen in the absence of other stimulatory molecules, may pose to be an advantage over CAR T cells in clinical settings.

As noted by Eshhar *et al.* in 1992, therapeutic anti-tumour antibodies were limited to hematological malignancies at the time. We have made significant progress in the development of cell-based therapies to continue to improve their potency against complex tumour microenvironments, while minimizing potential dangerous side effects. Our goal to understand the biological and biochemical mechanisms that underlie engineered T cell therapies can aid to improve the chances of efficacy against solid tumours, and ideally uncouple toxicity from efficacy.



**Figure 1.** Comparison of antigen engagement between conventional TCR-MHC versus chimeric receptor T cell antigen coupler (TAC). Left: In conventional TCR-based stimulation, the TCR-CD3 complex (green) engages with peptide in the context of HLA molecules (grey) for activation of signal 1. Coreceptors (blue) deliver tyrosine kinase Lck for ITAM phosphorylation. Costimulatory receptors (purple) are present during initial antigen encounter to deliver signal 2. Right: In TAC-based stimulation, the antigen-binding domain (orange) specifically binds to tumour-associated antigens on tumour cells. A secondary binding domain (purple) engages with the  $\epsilon$  domain of the TCR-CD3 complex to co-opt the primary signaling machinery of T cells.

## **2.0 Materials and Methods**

**CAR and TAC vector generation.** CAR and TAC receptor transgenes and vectors were designed as previously described.<sup>158</sup> Briefly, the TAC sequence is comprised of an antigen-binding domain linked to a CD3 $\epsilon$ -targeting scFv, followed by the hinge, transmembrane, and cytoplasmic domains of CD4. The CD3 $\epsilon$ -binding domain uses a humanized version of the UCHT1 scFv (huUCHT1). Anti-HER2-TAC uses a HER2-specific H10-2-G3 designed ankyrin repeat protein (DARPin)<sup>160</sup> downstream of an Ig $\kappa$  leader sequence. The HER2-DARPin was cloned into the pUC57 plasmid containing the huUCHT1, CD4 hinge, transmembrane, and cytoplasmic domains, followed by subcloning of the entire HER2-TAC sequence into the pCCL lentiviral transfer vector (kindly obtained from Dr. Megan Levings, University of British Columbia, Vancouver, BC). Anti-HER2-CD28 $\zeta$  CAR consists of the same H10-2-G3 DARPin, and was obtained as previously described.<sup>160</sup> Anti-HER2-4-1BB $\zeta$  CAR consists of the H10-2-G3 DARPin, CD8 $\alpha$  hinge and transmembrane domains, 4-1BB cytoplasmic tail, and CD3 $\zeta$  cytoplasmic tail portions from an anti-CD19 CAR, described in<sup>161</sup>. Anti-BCMA CAR and TAC receptors, the anti-BCMA scFv (C11D5.3) sequence was obtained from patent US20150051266A1<sup>162</sup> and synthesized by Genscript. The scFv was subcloned into existing pCCL TAC and CD28 $\zeta$ -CAR lentiviral backbones using *AscI* and *BamHI* cut sites, replacing the existing scFv with C11D5.3. The pCCL vector contains a bi-directional promoter for the chimeric receptor under EF-1 $\alpha$  promoter, and truncated nerve growth factor receptor (tNGFR) under minimal CMV promoter as a transduction control. For constructs containing puromycin resistance in the transfer vector, receptor transgenes were subcloned using *AscI* and *NheI* cut sites from the pCCL-tNGFR vector, to a pCCL vector with a puromycin-N-acetyltransferase gene replacing tNGFR.

**Lentivirus generation.** Third-generation, self-inactivating and non-replicative lentivirus was produced by transfection of  $12 \times 10^6$  HEK293T cells cultured on 15 cm diameter tissue culture-treated dishes (Nunc) in DMEM supplemented with 10% fetal bovine serum, 1X L-glutamine, 1X HEPES, and 1X penicillin-streptomycin (Gibco). Packaging plasmids pRSV-Rev (6.25  $\mu$ g), pMD2.G (9  $\mu$ g), pMDLg-pRRE (12.5  $\mu$ g), and the pCCL transfer plasmid (32  $\mu$ g) encoding the CAR or TAC transgene were combined with Lipofectamine 2000 (Thermo Fisher Scientific; Cat#11668-019) in Opti-MEM (Gibco; Cat#31985-070) according to manufacturer's guidelines. Ten to twelve hours after transfection, media was replaced with fresh media supplemented with sodium butyrate (Sigma-Aldrich; Cat#B5887) at a final concentration of 1 mM. Media containing lentivirus particles were collected 36-48 hours later and concentrated via ultracentrifugation or Amicon filter concentration (EMD Millipore; Cat#UFC910024). Viral titer was determined by serial dilution of concentrated virus on HEK293T cells. Transduction of tNGFR<sup>+</sup> HEK293T cells was determined by flow cytometry for calculation of titer in TU/mL.

**Lentiviral transduction and culturing of human T cells.** Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors or commercial leukapheresis products (STEMCELL) and isolated by Ficoll-Paque gradient (GE Healthcare) prior to cryopreservation.

T cells from bulk PBMCs were activated with anti-CD3/anti-CD28 Dynabeads (Gibco; Cat#11161D) at a 0.8:1 bead ratio for 18-24 hrs, then transduced with lentivirus encoding for CAR or TAC constructs at a multiplicity of infection (MOI) of 2, 4, or 10, depending on the construct. Control T cells are transduced with lentivirus encoding only for tNGFR at an MOI of 2. T cells were scaled into culture vessels at an approximate concentration of  $1 \times 10^6$  cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum, 1X L-glutamine, 1X sodium pyruvate, 1X HEPES, 1X non-essential amino acids, 1X penicillin-streptomycin, 50  $\mu$ M  $\beta$ -mercaptoethanol. T cell media was further supplemented with 660 IU/mL recombinant human (rh) IL-2 (Peprotech; Cat#200-02), and 10ng/mL rhIL-7 (Peprotech; Cat#200-07) for checkpoint receptor and memory phenotypic staining. For functional assays including T cell proliferation assays, T cell media was supplemented with 100 IU/mL rhIL-2 and 10ng/mL rhIL-7. Engineered T cells transduced with puromycin-acetyltransferase-containing pCCL lentiviruses were transduced as previously described. Forty-eight hours after transduction, media was replaced with fresh media containing 100 IU/mL rhIL-2, 10ng/mL rhIL-7, and 0.625  $\mu$ g/mL puromycin (Invivogen; Cat#ant-pr-1). All T cell cultures were monitored daily and fed according to cell counts every 1-3 days for a period of up to 16 days.

**Cell lines.** Adherent HER2<sup>+</sup> cell lines SKOV-3 and A549, and HER2<sup>-</sup> LOX-IMVI tumour cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, 1X L-glutamine, 1X HEPES, and 1X penicillin-streptomycin (cell lines were obtained from the NCI-60 human tumour cell lines panel, a kind gift from Dr. Karen Mossman, McMaster University, Hamilton, ON). Suspension BCMA<sup>+</sup> cell lines KMS-11 and MM.1s were cultured in RPMI 1640, supplemented with 1X L-glutamine, 1X sodium pyruvate, 1X HEPES, 1X non-essential amino acids, and 1X penicillin-streptomycin (cell lines were a kind gift from Dr. Kelvin Lee from Roswell Park Comprehensive Cancer Center, Buffalo, New York). BCMA<sup>-</sup> cell line K562 were a kind gift from Dr. Jana Burkhardt (Fraunhofer IZI, Leipzig, Germany). CD64/4-1BBL-engineered K562 cells (referred to as K64) were a kind gift from Dr. Carl June (University of Pennsylvania, Philadelphia, PA, USA). All cell lines were cultured under ambient atmosphere adjusted to 5% CO<sub>2</sub>, 37°C, and routinely tested for mycoplasma using Plasmotest mycoplasma detection kit (Invivogen; Cat#rep-pt1).

**Surface detection of receptors by flow cytometry.** Engineered T cells were analyzed for T cell subsets and receptor expression by staining with recombinant Fc proteins directly for receptor specificity. Standard phenotyping of transduced T cells used BCMA-Fc or HER2-Fc proteins for labeling of chimeric receptors, followed by addition of conjugated secondary antibodies against human IgG Fc, CD4, CD8, and NGFR. Detection of PD-1, LAG-3, TIM-3, and memory markers CD45RA, CCR7, CD62L, CD28, CD27 and CD127 was completed using conjugated secondary antibodies. Flow cytometry was conducted on BD LSRFortessa or BD LSRII and analyzed by FlowJo vX software (Treestar). SPICE analysis and visualizations were generated using SPICE v6.0 (NIH).<sup>163</sup>

**Purification of engineered populations.** For assays where flow-sorted T cells were used, T cells were surface labelled with conjugated secondary antibody against NGFR as described above, for separation of receptor-positive T cells on day 7-14 of culture, depending on experimental requirements. T cells were sorted on Beckman Coulter MoFlo XDP Cell Sorter, followed by replenishment of fresh media and expansion of purified populations to day 13-16 as needed. Magnetic separation using EasySep Human CD271 (STEMCELL; Cat#18659) kit was also used for positive-selection of tNGFR<sup>+</sup> T cells on day 7, or 13 of culture, according to manufacturer's guidelines.

**Analysis of T cell proliferation by flow cytometry.** Engineered T cells were labelled with CellTrace Violet or CellTrace CFSE (Invitrogen; Cat#C34557 and Cat#C34553) on day 13-16 of culture. Following stimulation under different conditions, T cell subsets were stained with LIVE/DEAD NearIR Viability Dye (Invitrogen; Cat#L10119) diluted in PBS for 20 minutes at room temperature (RT), followed by surface antibodies against CD4, CD8 and NGFR for 30 minutes at RT. For bead-based stimulations, Protein G polystyrene beads (Spherotech, Cat#PGP-60-5) were incubated with BCMA-Fc or CD86-Fc (R&D Systems; Cat#7625-B2), at a concentration of 5-10x10<sup>6</sup> beads per mL in 0.1% BSA in PBS overnight with physical agitation on a tube rotator at 4°C. For cell-based stimulations, target cells were adjusted to 1x10<sup>6</sup> cells per mL in fresh media, and gamma irradiated at a total dosage of 10,000 cGy (Gammacell 1000). T cells were co-incubated with target beads or cells for 4 days and replenished with 1 volume of fresh media two days following initial stimulation. Flow cytometry was conducted on BD LSRFortessa or BD LSR II and analyzed by FlowJo vX software.

**Surface and intracellular staining for activation and proliferation markers.** For analysis of activation markers, transduced T cells were co-incubated with target beads or cells for the time indicated, followed by labelling of conjugated secondary antibodies against human CD69, CD4, CD8, and NGFR as described previously. Following labelling of surface markers, T cells were permeabilized using BD Cytfix/Cytoperm (BD Bioscience; Cat#554714) for 20 minutes at RT, then stained for intracellular markers Nur77 and Ki-67 for 30 minutes at RT. Flow cytometry was conducted on BD flow cytometers and data was analyzed as previously described.

**Activation-induced cell death assays.** Engineered T cells were co-incubated with BCMA-Fc-coated polystyrene beads as previously described. At each time point, samples were collected into 5mL polystyrene tubes and washed 1X with sterile-filtered binding buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl<sub>2</sub>) at room temperature. T cells were labelled with Annexin V-BV421 (BD Horizon; Cat#563973) and 7-amino-actinomycin-D (BD Pharmingen; Cat#559925) diluted 1:10 into binding buffer, for 15 minutes at room temperature. Cells were washed again in binding buffer, prior to immediate analysis on BD flow cytometers. Data were analyzed as previously described.

**RNA isolation and next-generation sequencing.** Flow-sorted T cells from day 7 were sorted again on day 14 to isolate CD4<sup>+</sup> and CD8<sup>+</sup> populations. Purified populations were stimulated

with Protein G polystyrene beads coated with 50ng of BCMA-Fc per  $1 \times 10^6$  beads, at a T cell:bead ratio of 2:1. Following stimulation period, T cells were collected, and RNA was isolated using RNeasy Plus Mini Kit (QIAGEN; Cat#74134) following manufacturer's guidelines. Illumina sequencing was performed by the Farncombe Metagenomics Facility (McMaster University). RNA integrity was first verified using the Agilent BioAnalyzer, followed by mRNA enrichment and library prep using the NEBNext Ultra II Directional RNA Library Prep Kit along with the NEBNext Poly(A) mRNA Magnetic Isolation Module. Libraries were subject to further BioAnalyzer QC and quantified by qPCR, then pooled in equimolar amounts. Sequencing was performed with the HiSeq Rapid v2 chemistry using onboard cluster generation (2 lanes) and a 1x51 bp read length configuration.

**RNAseq data preprocessing, normalization and statistical analysis.** The mapping of the processed reads was performed by using Tophat<sup>164</sup> and HISAT<sup>165</sup> with hg38 (UCSC) reference genome; reads were counted by using HTSeq<sup>166</sup>. Genes showing less than 10 on average across all samples were removed, resulting in 10,077 genes for the dataset used to generate Figure 5, 11,422 genes for Figures 6, 13 and Table 2, and 11,336 genes for Figures 18, 19 and Table 3. The remaining values were normalized with TMM normalization method<sup>167</sup> and then transformed with voom transformation<sup>168</sup>. Heat maps for transcription factors were generated for CD4+ and CD8+ T cell datasets independently, using the online tool Heatmapper (<http://heatmapper.ca/expression/>). Limma package<sup>169</sup> was used to examine differential expression between the groups of interest, by pairing samples based on the Donors. Obtained p-values were corrected with BH correction for multiple testing<sup>170</sup>, and corrected values <0.05 were considered to be significant. Reactome FI plugin<sup>171</sup> in Cytoscape environment<sup>172</sup> was used to build Protein-Protein Interaction (PPI) Networks and then to examine Pathway enrichment and Gene Ontology (GO) – Biological processes. Additional Gene Ontology analysis, not based on PPI networks, was performed with BINGO<sup>173</sup> plugin in Cytoscape environment. For Figure 5, hierarchical clustering was performed by using a built-in hclust function using all available genes. Volcano plots were built using Mathematica 10 software. Heatmaps were created in MatLab R2015b software.

**Isolation of cells from murine lung tissue.** The McMaster Animal Research Ethics Board approved all murine experiments. Twelve to sixteen-week-old NOD.Cg-Rag1<sup>tm1Mom</sup>Il2rg<sup>tm1Wjl</sup>/SzJ (NRG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (Stock #007799), or bred in-house. Mice were fully anesthetized with isoflurane and dissected to reveal heart and chest cavity. A 25-gauge needle attached to a syringe filled with cold, sterile PBS was used to perfuse the lungs through the left ventricle. All lobes of the lung were removed following perfusion with ~25 mL of PBS and preserved in RPMI media on ice. Type IV collagenase (Worthington Biochemical; cat#: CLS-4) was reconstituted in RPMI at 1 mg/mL, and 0.2  $\mu$ m sterile-filtered. Lung tissues were minced into 1mm pieces using surgical scissors and transferred into the collagenase mixture. DNase I (Roche; cat#: 10104159001) was added to the mixture at a final concentration of 0.4 mg/mL. Tissues were digested for 1 hour

with continuous rotation on a tube rotisserie at 37°C. The digestion mixture was filtered through 70µm cell strainers and total cell numbers and viability was quantified via trypan blue exclusion.

**Antibodies and recombinant proteins.**

Recombinant proteins used: rhErbB2/Fc Chimera (R&D Systems; cat#: 1129-ER); rhBCMA/Fc Chimera (R&D Systems; cat#: 192-BC); rhB7-2/Fc (R&D Systems; cat#: 7625-B2)

Flow cytometry antibodies used: CD4-AF700 (eBioscience; cat#: 56-0048-82); CD4-Pacific Blue (BD Pharmingen; cat#: 558116); CD4-APC-H7 (BD Pharmingen; cat#: 560158); CD8-AF700 (eBioscience; cat#: 56-0086-82); CD8-PerCP-Cy5.5 (eBioscience; cat#: 45-0088-42); LNGFR-BV421 (BD Pharmingen; cat#: 562582); LNGFR-VioBright FITC (Miltenyi Biotec; cat#: 130-104-893); Human IgG (Fcγ)-PE (Jackson ImmunoResearch; cat#: 109-115-098); IFNγ-APC (BD Pharmingen; cat#: 554702); IL-2-PE (BD Pharmingen; cat#: 554566); TNFα-FITC (BD Pharmingen; cat#: 554512); CD69-BV650 (BD Horizon; cat#: 563835); CD27-APC-H7 (BD Pharmingen; cat#: 560222); CD28-PE (BD Pharmingen; cat#: 555729); CD45RA-ECD (Beckman Coulter; cat#: IM2711U); CD62L-APC (BD Pharmingen; cat#: 559772); CCR7-PE-Cy7 (BD Pharmingen; cat#: 557648); PD-1-BV421 (BD Horizon; cat#: 562516); TIM-3-PE-CF594 (BD Pharmingen; cat#: 565560); TIM-3-BV785 (Biolegend; cat#: 345031); LAG-3-AF647 (BD Pharmingen; cat#: 565716); Nur77-PE (eBioscience; cat#: 12-5965-82); Ki-67-FITC (eBioscience; cat#: 11-5699-82); CD86-BV605 (Biolegend; cat#:305430); CD80-PE-Cy5 (BD Pharmingen; cat#: 559370).

### **3.0 Results**

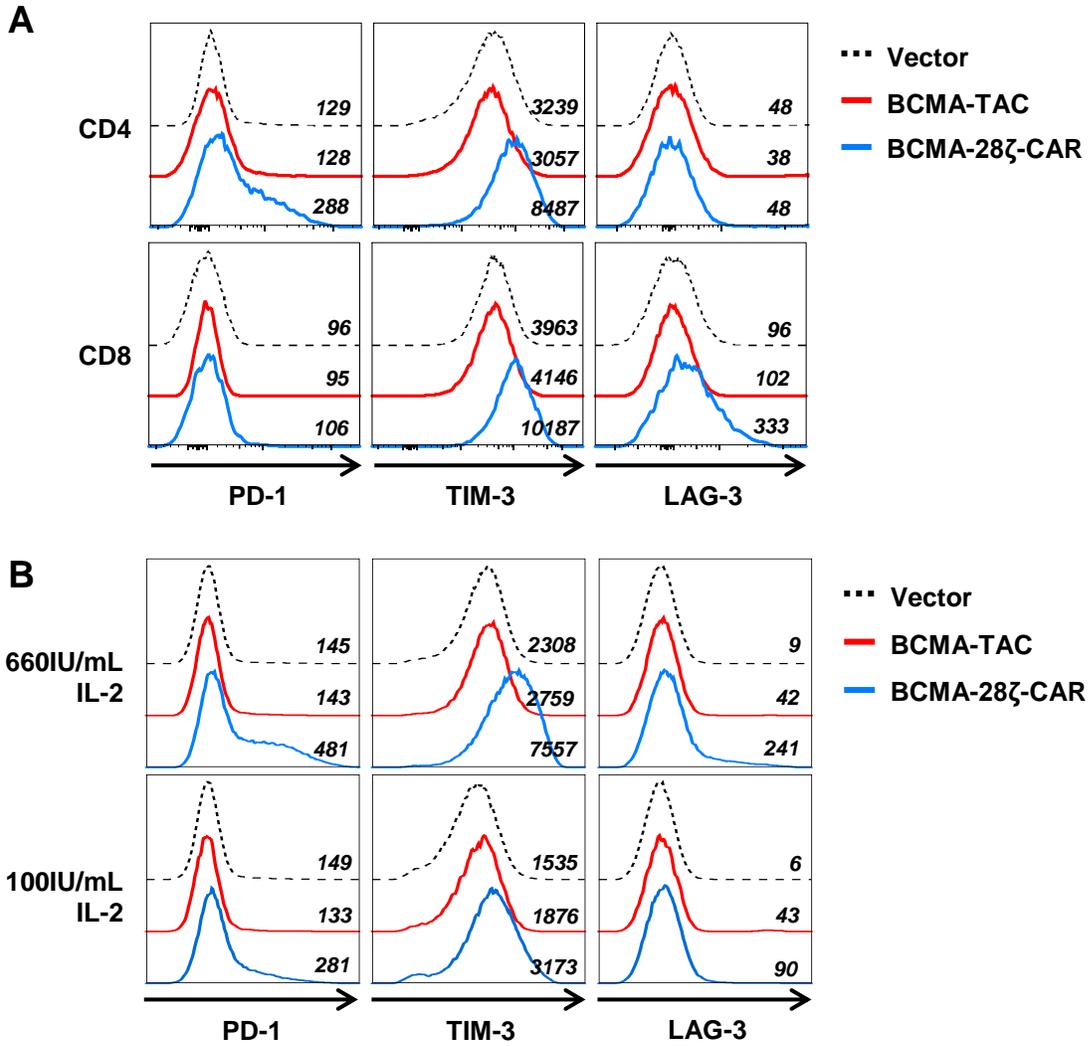
#### **3.1 Fundamental differences between TAC and CAR T cells during non-activated and activated states**

##### **3.1.1 TAC T cells lack tonic signaling and retain a less differentiated phenotype and reduced checkpoint receptor expression relative to CAR T cells**

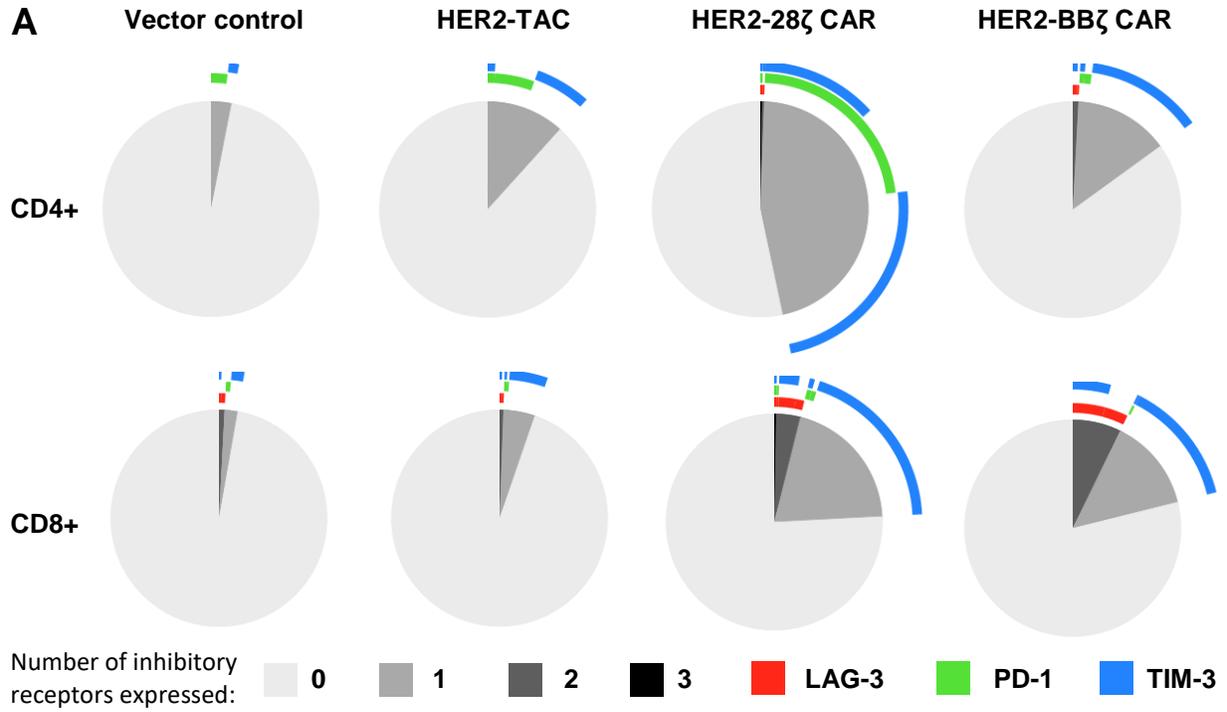
One major hindrance of second-generation CARs is the manifestation of tonic signaling, which primarily occurs due to spontaneous receptor aggregation that leads to antigen-independent activation.<sup>134</sup> Consistent with reports in literature describing tonic signaling in CAR T cells<sup>134,149</sup>, the second-generation CARs used in our experiments exhibited similar signs of T cell activation in the absence of stimulation (**Figure 2A**). Anti-B cell maturation antigen (BCMA) CAR T cells with a CD28 costimulatory domain fused to a CD3 $\zeta$  signaling domain (hereafter referred to as BCMA-28 $\zeta$ -CAR) expressed significantly higher levels of PD-1, TIM-3, and LAG-3 compared to TAC T cells (BCMA-TAC), or vector control. Conversely, TAC T cells show much lower levels of checkpoint receptor expression, despite its proposed interaction with the T cell receptor complex. Checkpoint receptor expression was influenced by the amount of IL-2 present during the manufacturing process, as high IL-2 concentrations drove higher expression of all three markers in CAR T cells; however, even at high IL-2 concentrations, TAC T cells did not significantly upregulate checkpoint receptors relative controls (**Figure 2B**).

To demonstrate that tonic signaling is intrinsic to CARs, but not TAC receptors, we examined T cells engineered with CARs directed against a different antigen (HER2) using a different binding domain (designed ankyrin repeat protein; DARPIn). We employed SPICE analysis to visualize the proportions of T cells expressing one or more checkpoint receptor (**Figure 3**). As observed with BCMA-28 $\zeta$ -CAR T cells, HER2-28 $\zeta$ -CAR T cells significantly upregulated PD-1 and TIM-3. HER2-BB $\zeta$ -CAR T cells, which contain a 4-1BB costimulatory domain instead of CD28, showed less tonic signaling in CD4<sup>+</sup> T cells, comparatively.

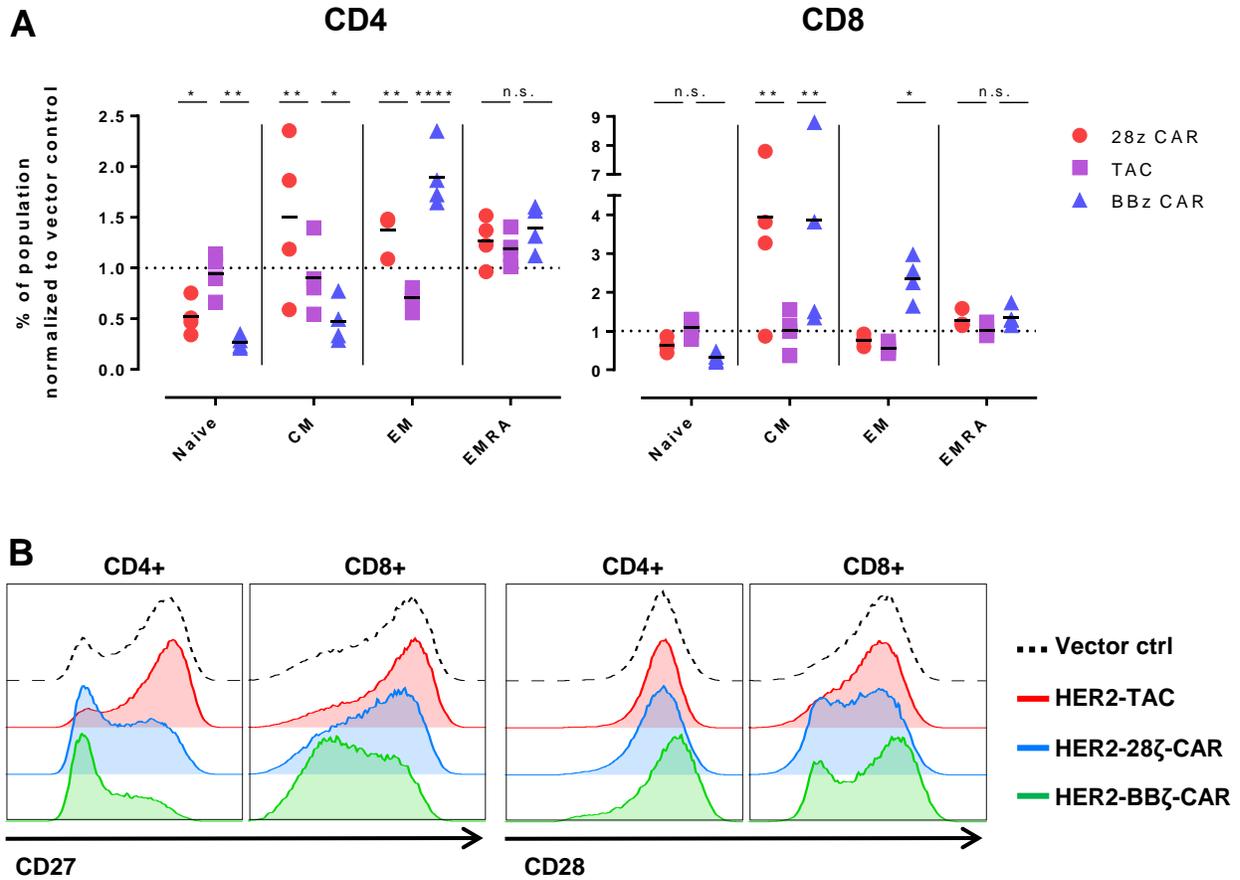
Tonic signaling also accelerated differentiation of the engineered T cells in culture, as determined by CD45RA and CCR7 staining by flow cytometry (**Figure 4A**). Despite lower checkpoint receptor expression, BB $\zeta$ -CAR-T cells biased towards loss of naïve T cells and increased in effector memory T cells. Interestingly, it appeared that HER2-BB $\zeta$ -CARs deliver unique tonic signals compared to HER2-28 $\zeta$ -CARs, as CD27 and CD28 costimulatory receptors were differentially downregulated in T cells engineered with either construct (**Figure 4B**). HER2-BB $\zeta$ -CARs expressed higher levels of CD28 and lower levels of CD27 compared to HER2-28 $\zeta$ -CARs, which showed the opposite. This proposes that tonic signals are derived in part from both the costimulatory domains and  $\zeta$ -chains built into the CAR framework.



**Figure 2.** Tonic signaling in second-generation CD28 $\zeta$ -CAR T cells results in upregulation of checkpoint receptors and increased T cell differentiation. (A) Surface expression of PD-1, TIM-3, and LAG-3 of BCMA-CAR- or TAC-engineered T cells was detected by immunostaining and flow cytometry. Numbers indicate median fluorescence intensity. (B) Expression of checkpoint receptors of CD4<sup>+</sup> BCMA-CAR and TAC T cells cultured in high IL-2 conditions (660 IU/mL) versus low IL-2 conditions (100 IU/mL) during the duration of a 14-day culture period. Data are representative of three donors from three independent experiments in (A), and a single donor from one experiment in (B).



**Figure 3.** Second-generation CAR T cells show upregulation of several checkpoint receptors. SPICE analysis of HER2-engineered T cells was performed to visualize proportions of T cell expressing checkpoint receptors. Pie slices represent % of total T cells expression 0-3 checkpoint receptors, and pie arcs indicate proportion of total population expressing each marker. Data are representative of three donors in three independent experiments.

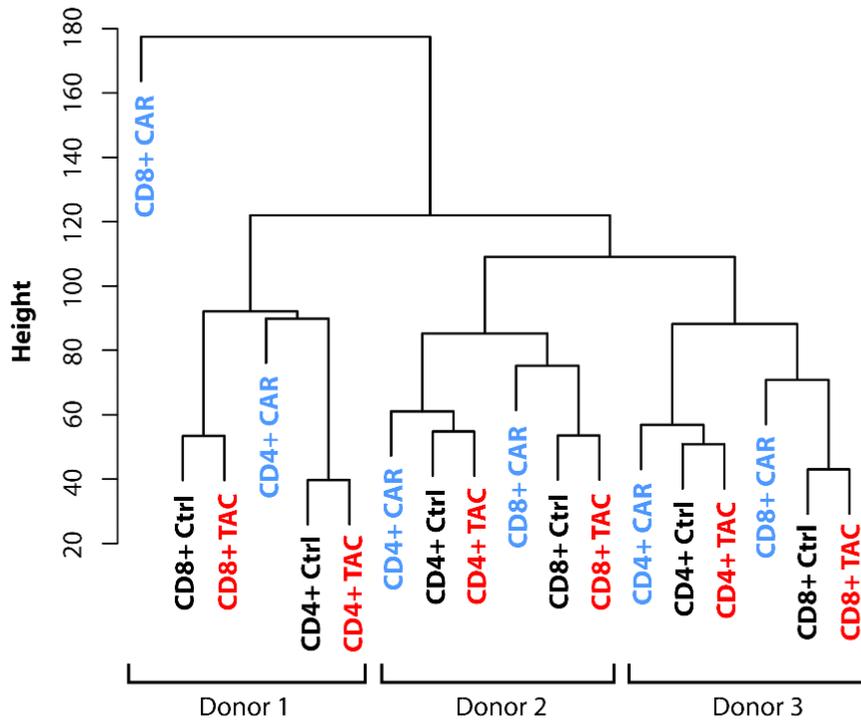


**Figure 4.** Tonic signaling in second-generation CAR T cells leads to T cell differentiation and increased loss of costimulatory receptor expression. (A) Relative proportions of memory T cell populations normalized to vector control, as determined by CD45RA and CCR7 staining by flow cytometry. Population frequencies were determined by Boolean gating on FSC/CD45RA<sup>+</sup> or FSC/CCR7<sup>+</sup> using FlowJo vX software. T cell subsets are defined as naïve (CD45RA<sup>+</sup>, CCR7<sup>+</sup>), central memory (CM) (CD45RA<sup>-</sup>, CCR7<sup>+</sup>), effector memory (EM) (CD45RA<sup>-</sup>, CCR7<sup>-</sup>), and terminal effectors (EMRA) (CD45RA<sup>+</sup>, CCR7<sup>-</sup>). Lines represent the mean of 4 donors. Multiple t-test is used to determine significance. Data are representative of three donors in three independent experiments. (B) Histograms of CD27 and CD28 expression in HER2-TAC- and CAR-T cells from one donor, representative of 4 donors. Numbers indicate median fluorescence intensity. All populations are gated on CD4<sup>+</sup>NGFR<sup>+</sup>, or CD8<sup>+</sup>NGFR<sup>+</sup>.

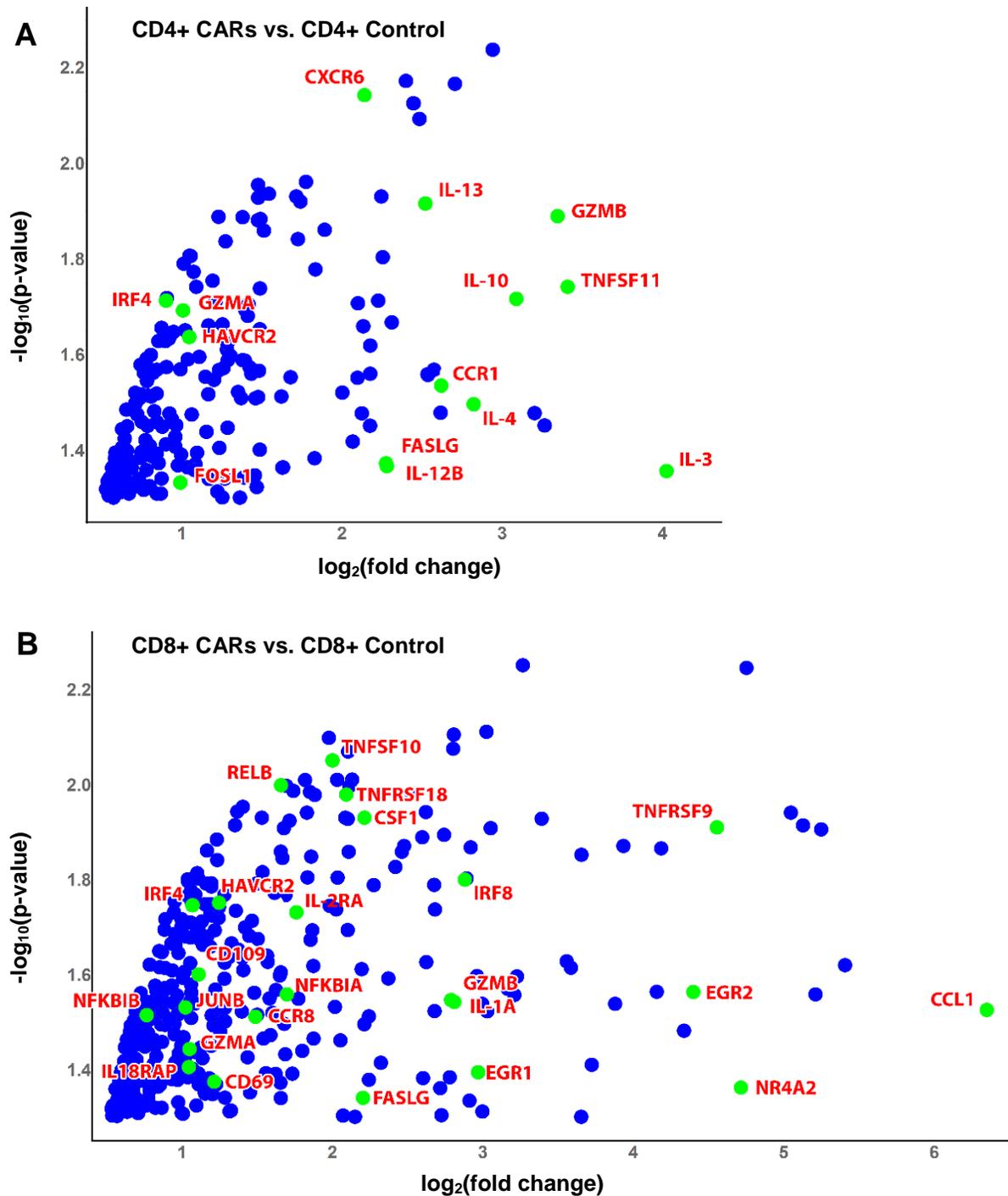
### 3.1.2 Transcriptome analysis indicates significant tonic activation of CAR T cells in the absence of stimulation

To examine transcriptional differences between TAC and CAR T cells in their basal states, BCMA-TAC and BCMA-28 $\zeta$ -CAR T cells were flow-sorted for receptor-positivity and separated by CD4 or CD8 expression. Hierarchical clustering indicated greatest similarity between control and BCMA-TAC T cells, whereas BCMA-28 $\zeta$ -CAR T cells showed greatest dissimilarity, for each subpopulation and donor (**Figure 5**). The extent of tonic signaling in the absence of stimulation present in CAR T cells led to upregulation of >200 genes in CD4+ T cells, and >382 genes in CD8+ T cells, compared to control T cells (**Figure 6A & 6B**). Among the transcripts detected are several markers of activation, such as TNFRSF9 (a.k.a. 4-1BB), and CD69. In comparison, fewer than 10 genes were differentially expressed between TAC T cells and control at baseline (data not shown).

Pathway analysis and GO analysis of protein-protein interaction networks was performed to identify potential pathways upregulated in CAR T cells relative to TAC T cells (**Table 2**). Interestingly, tonic signaling in CAR T cells appear to activate both TCR and CD28 costimulatory pathways, indicating that both the CD3 $\zeta$  and CD28 domains of the intracellular tail contribute to tonic activation. As expected, CAR T cells showed diverse and constitutive cytokine-dependent signaling, as well as transcriptional activation of multiple pathways downstream of TCR activation.



**Figure 5.** Unsupervised hierarchical clustering of non-stimulated CAR and TAC T cells shows greater similarity between TAC and control T cells. RNAseq was performed on engineered T cells sorted into CD4+ and CD8+ populations, and distance between sample groups was determined using average linkage.



**Figure 6.** Transcription profiling of BCMA-28 $\zeta$  CAR T cells using RNAseq shows significant gene upregulation in the absence of stimulation. One-sided volcano plots were generated for visualization of genes upregulated by (A) CD4+ and (B) CD8+ non-stimulated BCMA-CAR T cells compared to control. Each blue dot represents a single gene, plotted by fold change and corresponding p-values. Green dots represent manually selected effector genes of interest. Data was combined from three individual donors as independent biological replicates.

**Table 2.** Pathway enrichment analysis for gene signatures upregulated by BCMA-28 $\zeta$ -CAR T cells relative to BCMA-TAC T cells. Pathway enrichment was performed on the list of genes with greater than 1.5-fold-change between CAR and TAC samples. P-value of indicated pathways are < 0.05.

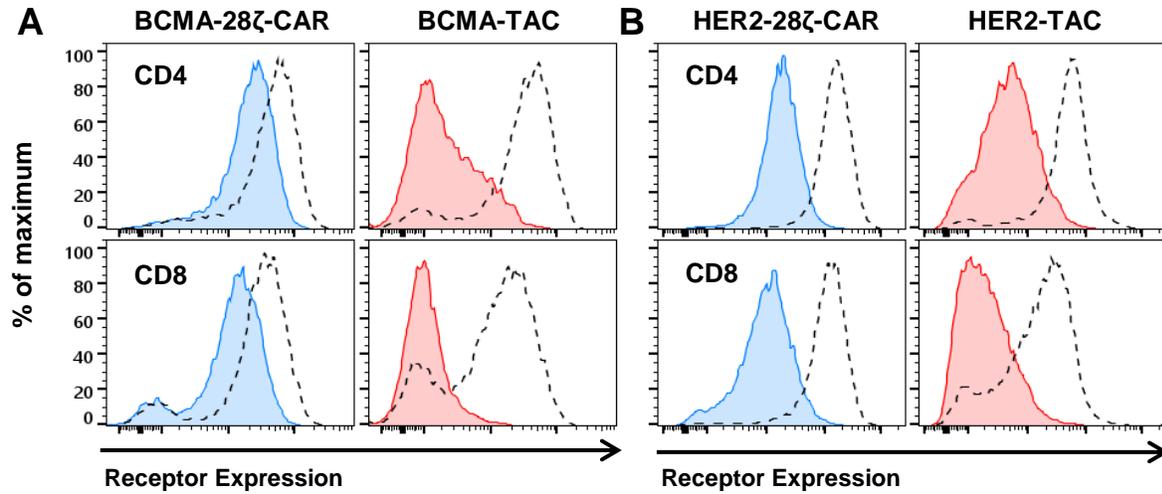
	<b>Upregulated in CD4+ CAR T cells vs. CD4+ TAC T cells</b>	<b>Upregulated in CD8+ CAR T cells vs. CD8+ TAC T cells</b>
<b>TCR signaling and costimulation</b>	T cell activation	
	T cell receptor signaling pathway	T cell receptor signaling pathway
	PI3K-Akt signaling pathway	PI3K-Akt signaling pathway
	Costimulation by the CD28 family	
<b>Transcription factors</b>	NF-kappa B signaling pathway	NF-kappa B signaling pathway
	Calcineurin-regulated NFAT- dependent transcription in lymphocytes	Calcineurin-regulated NFAT- dependent transcription in lymphocytes
	AP-1 transcription factor network	AP-1 transcription factor network
<b>Cytokine Signaling Pathways</b>	Cytokine-cytokine receptor interaction	Cytokine-cytokine receptor interaction
	Chemokine signaling pathway	Chemokine signaling pathway
	JAK-STAT signaling pathway	JAK-STAT signaling pathway
	TNF signaling pathway	TNF signaling pathway
	Inflammation mediated by chemokine and cytokine signaling pathway	
		MAPK signaling pathway
<b>Other pathways</b>	Cell cycle	
	Cell adhesion molecules (CAMs)	Cell adhesion molecules (CAMs)
	Apoptosis	Apoptosis
		Ligand-dependent caspase activation

### 3.1.3 Engineered receptors are downmodulated following antigen-dependent stimulation

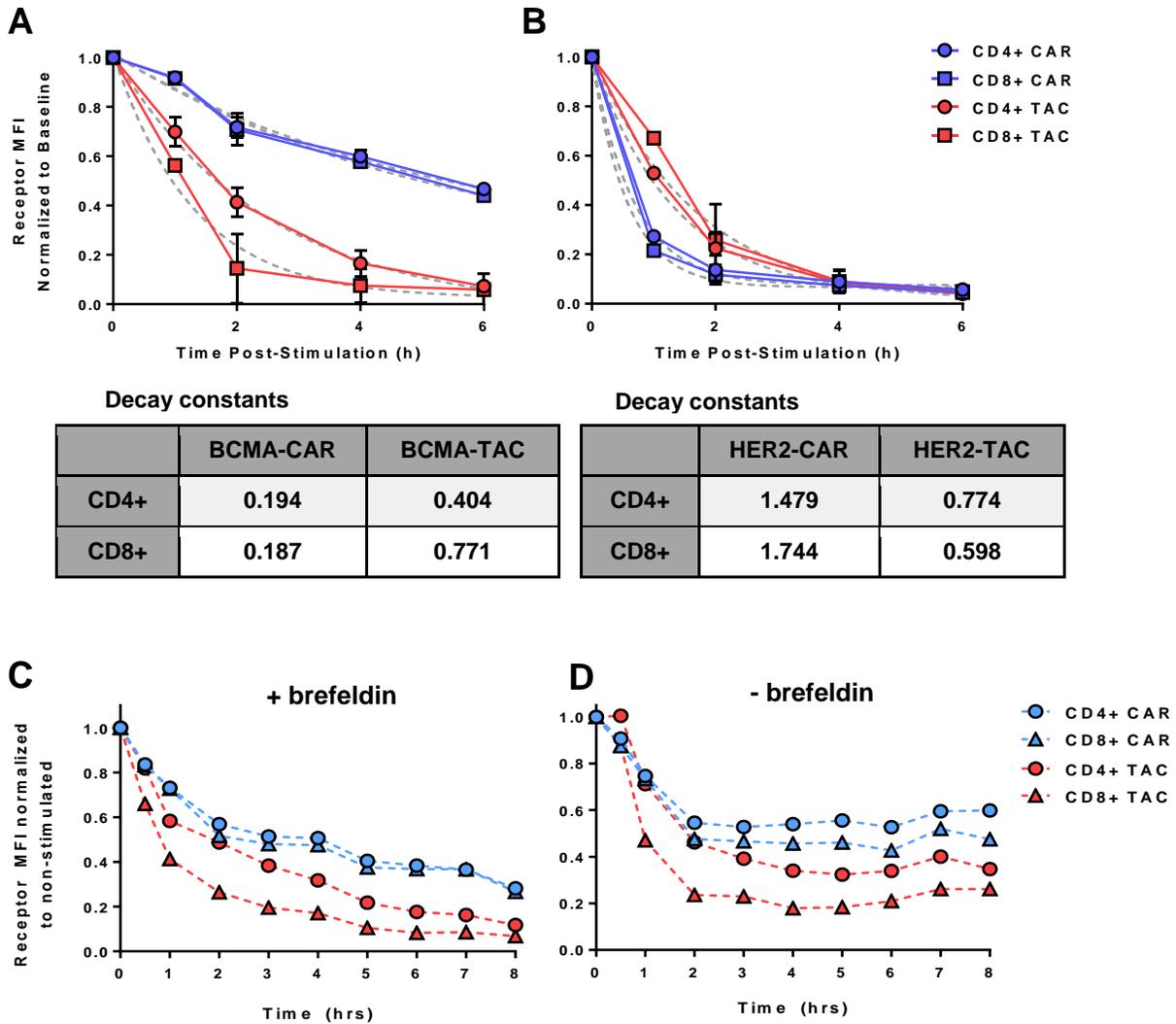
As previously discussed, TCR triggering results in downmodulation of the TCR-CD3 complex, which has been described as a negative feedback mechanism that limits excessive T cell effector functions.<sup>66</sup> To observe whether CAR or TAC receptors downmodulate following stimulation, CAR and TAC T cells were stimulated with plate-bound Fc-conjugated proteins over a specific timeframe, in the presence of protein transport inhibitor brefeldin A. BCMA-TAC receptors were rapidly downmodulated upon stimulation, whereas BCMA-28 $\zeta$ -CARs remained relatively highly expressed (**Figure 7A**). Interestingly, the entire shift of the curve was indicative that all T cells gated as transduction marker tNGFR+ were responsive to stimulation. If only a proportion of T cells were triggered, we would more likely observe bimodal histogram distributions. In comparison, downmodulation of HER2-28 $\zeta$ -CAR T cells was more pronounced than BCMA-28 $\zeta$ -CARs (**Figure 7B**), indicating that the extent of downmodulation was dependent on the target antigen.

Downmodulation of the BCMA-TAC receptor followed a biphasic response, with highest rate of internalization during the first two hours of stimulation, whereas BCMA-28 $\zeta$ -CAR internalization was constant (**Figure 8A**). Anti-HER2 receptors from both CAR and TAC receptor scaffolds were equally downmodulated compared to BCMA receptors, and at similar kinetics (**Figure 8B**).

To further determine whether the chimeric receptors could be recycled back to the surface following receptor ligation, HER2-28 $\zeta$ -CAR and TAC T cells were stimulated with plate-bound proteins in the presence or absence of brefeldin A. In the presence of brefeldin A (**Figure 8C**), BCMA-TAC T cells expressed significantly lower receptor levels than BCMA-28 $\zeta$ -CAR T cells at 6 hours post-stimulation, estimated from the MFI values of non-stimulated T cells. In the absence of brefeldin A (**Figure 8D**), surface expression of both CAR and TAC receptors reached a steady state after approximately 2 hours, indicative of saturated receptor triggering. This indicated that a specific density of antigen likely triggers a finite number of receptors within the first two hours. After this period, the downmodulation, either at a basal level, or due to continuous triggering, of receptors was equivalent to the rate at which receptors were recycled, or newly synthesized.



**Figure 7.** Downmodulation of CAR and TAC receptors following stimulation with plate-bound recombinant Fc-conjugated proteins. Histograms for surface expression of anti-BCMA-engineered T cell receptors (A) and anti-HER2-engineered T cell receptors (B) following stimulation for 4 hours (solid lines), or non-stimulated receptor levels (dotted lines). Data are representative of 3 donors in from 3 independent experiments, and 2 donors from 2 independent experiment for A and B, respectively.



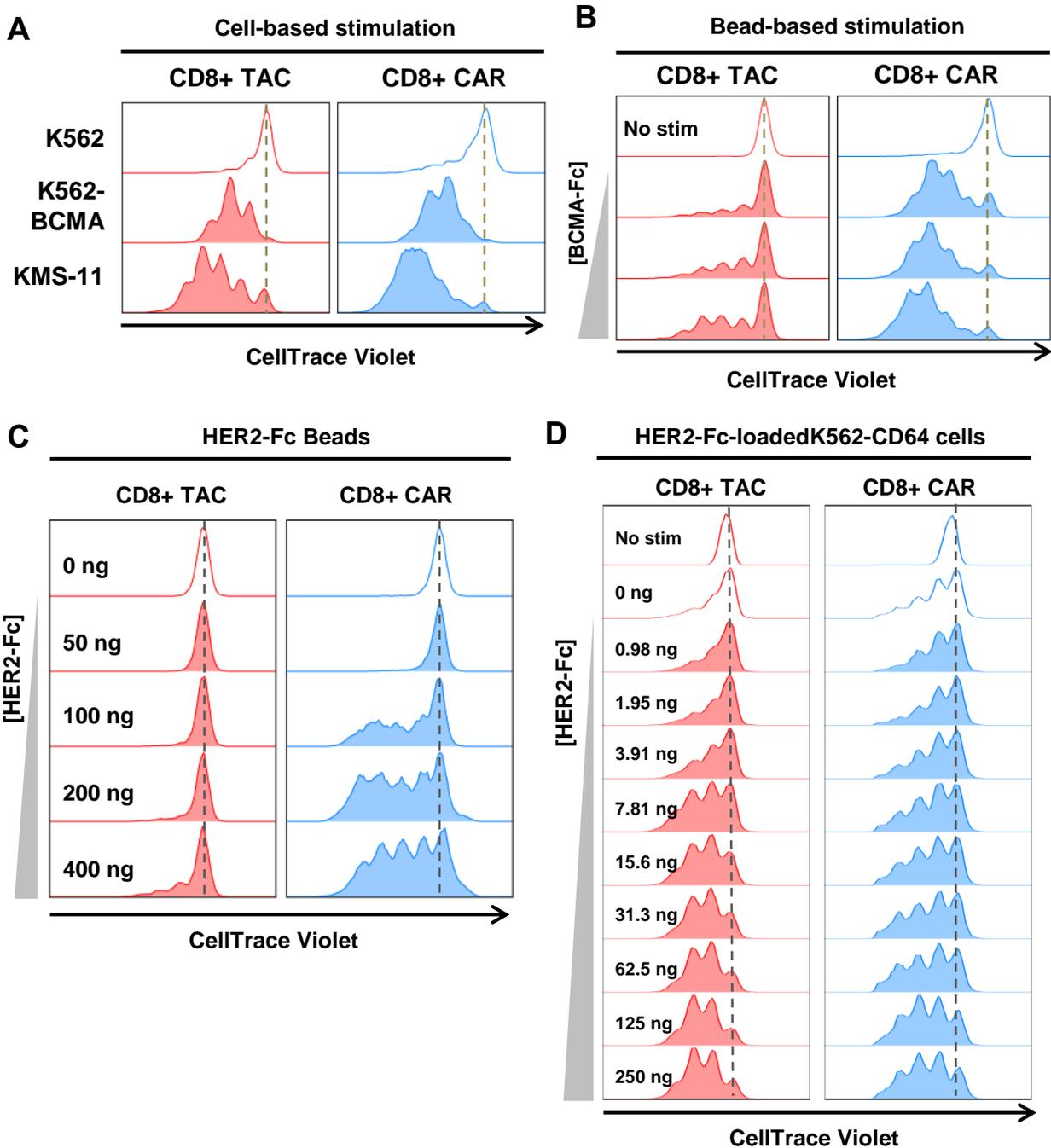
**Figure 8.** CAR and TAC receptors downmodulate following stimulation with Fc-conjugated proteins over time. Median fluorescence intensity (MFI) values of BCMA-Fc-stimulated (A), and HER2-Fc-stimulated (B) T cells are normalized to non-stimulated MFI. Dotted lines represent curve fitting for exponential decay, with decay constant  $k$  calculated from the function  $Y=Y_0 \cdot e^{-kx}$ , where  $Y_0$  is initial quantity, and  $k$  is rate constant. Data are representative of 2 donors from 2 independent experiments. Timecourse analysis of receptor expression following stimulation of BCMA-CAR and TAC T cells, with (C) or without (D) initial addition of brefeldin. MFI values are normalized to non-stimulated MFI of each T cell type, respectively. Data is representative of one experiment. All populations were gated on CD4+NGFR+ or CD8+NGFR+.

### **3.2 Optimal TAC T cell activation is contextually more stringent than CAR T cells**

#### **3.2.1 TAC T cells can discriminate between cell- and bead-based stimulation**

The magnitude, efficiency, and overall response following T cell stimulation can be measured using several parameters, including cytokine production, and proliferation. In solid cancers, presence of proliferating T cells within the tumour are highly associated with improved outcomes, including overall survival and absence of metastatic relapse.<sup>174,175</sup> To compare the proliferative capacity of CAR and TAC T cells against tumour cells expressing the target antigen, T cells were labelled with CellTrace Violet (CTV), a cell permeable fluorescent dye that binds covalently to amine residues, and analyzed by flow cytometry. Response to cell-based stimulation, either by BCMA-positive myeloma cell line KMS-11, or K562 cells engineered to express BCMA, was roughly equivalent between TAC and CAR T cells in all donors tested (**Figure 9A**).

It is well-established that the T cell response to stimulation varies with antigen density. To control the amount of antigen used for stimulation, protein G polystyrene beads (minimum 6.0  $\mu\text{m}$  diameter) were coated with increasing quantities of Fc-conjugated recombinant antigens. Unexpectedly, BCMA-Fc immobilized on beads led to robust CAR, but not TAC, T cell proliferation (**Figure 9B**). At all antigen levels tested and with both anti-BCMA and anti-HER2 TAC T cells, the proliferative response against beads was markedly lower compared to CARs (**Figure 9C**). Conversely, HER2-Fc loaded on K562 cells engineered to express high affinity Fc $\gamma$ RI showed that TAC T cells were much more responsive to cell-based stimulation (**Figure 9D**). Beyond 15.6 ng of HER2-Fc per million K562-CD64 cells, TAC T cell proliferation was more robust compared to CAR T cells, whereas this was not observed for concentrations of up to 400 ng HER2-Fc per million beads.

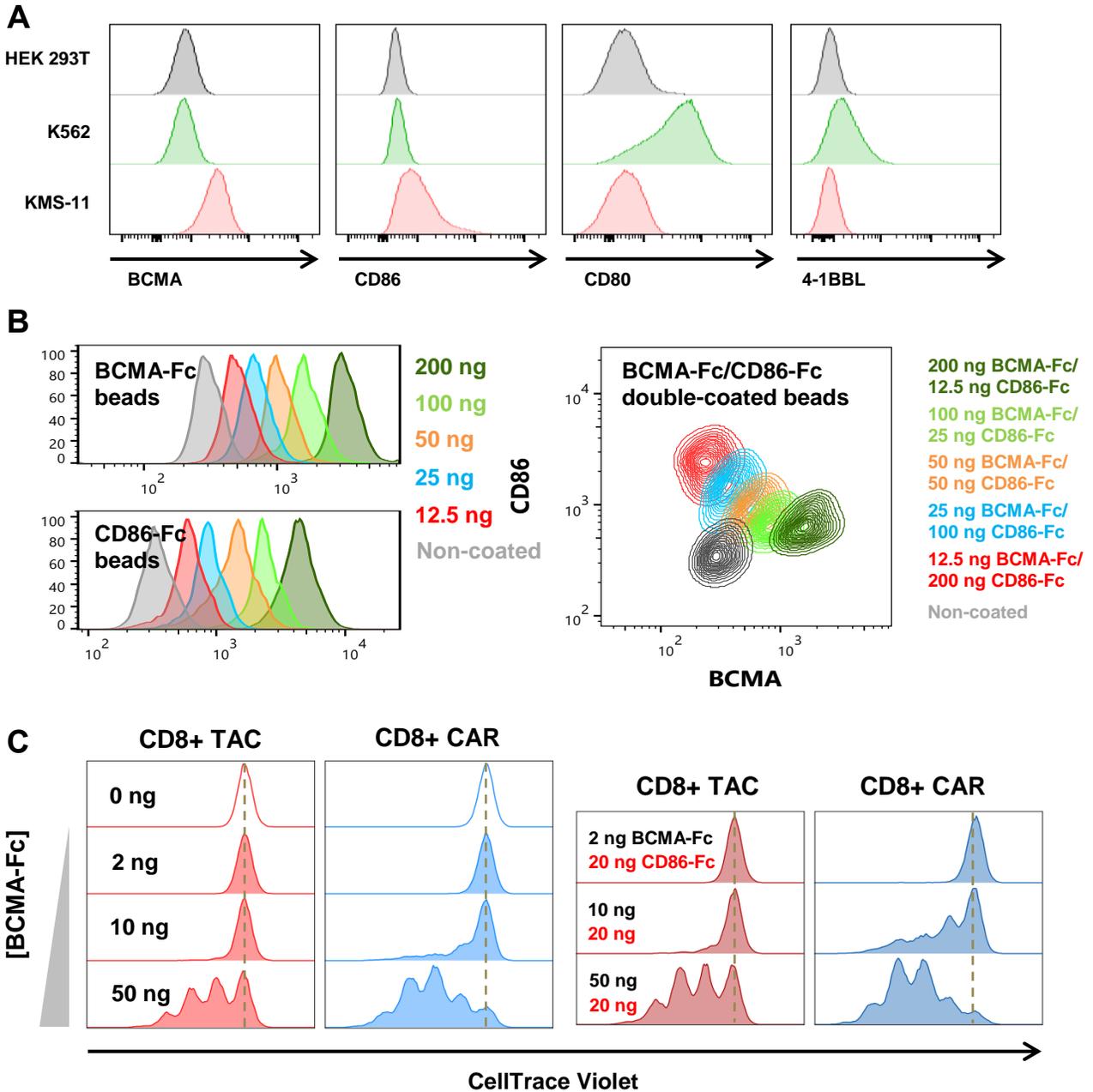


**Figure 9.** Proliferation of CD8+ TAC and CD8 $\zeta$ -CAR T cells stimulated by antigen-positive cells or beads coated with recombinant-Fc-conjugated proteins. T cells were co-cultured with target cells or beads at an effector:target ratio of 2:1 on day 13, 14, or 15 of culture, and analyzed 4 days post-stimulation. Histograms show serial dilution of CellTrace Violet in cell populations that have undergone division. Dotted lines represent fluorescence intensity of parent peak, or undivided, live T cells. (A) BCMA-TAC and BCMA-28 $\zeta$ -CAR T cells stimulated with BCMA-expressing K562 cells, or endogenous BCMA-expressing KMS-11 cells. (B) Differential proliferation of TAC and CAR T cells stimulation with BCMA-Fc-coated polystyrene beads. HER2-TAC and HER2-28 $\zeta$ -CAR T cells stimulated with (C) HER2-Fc-coated beads, or (D) HER2-Fc-loaded K562-CD64 cells. Numbers indicate quantity of protein per million beads or cells. Dotted lines indicate peak fluorescence intensity of non-stimulated, parent population. All plots are gated for CD4+NGFR+, or CD8+NGFR+ expression, and live cells. Data are representative of three donors in three independent experiments.

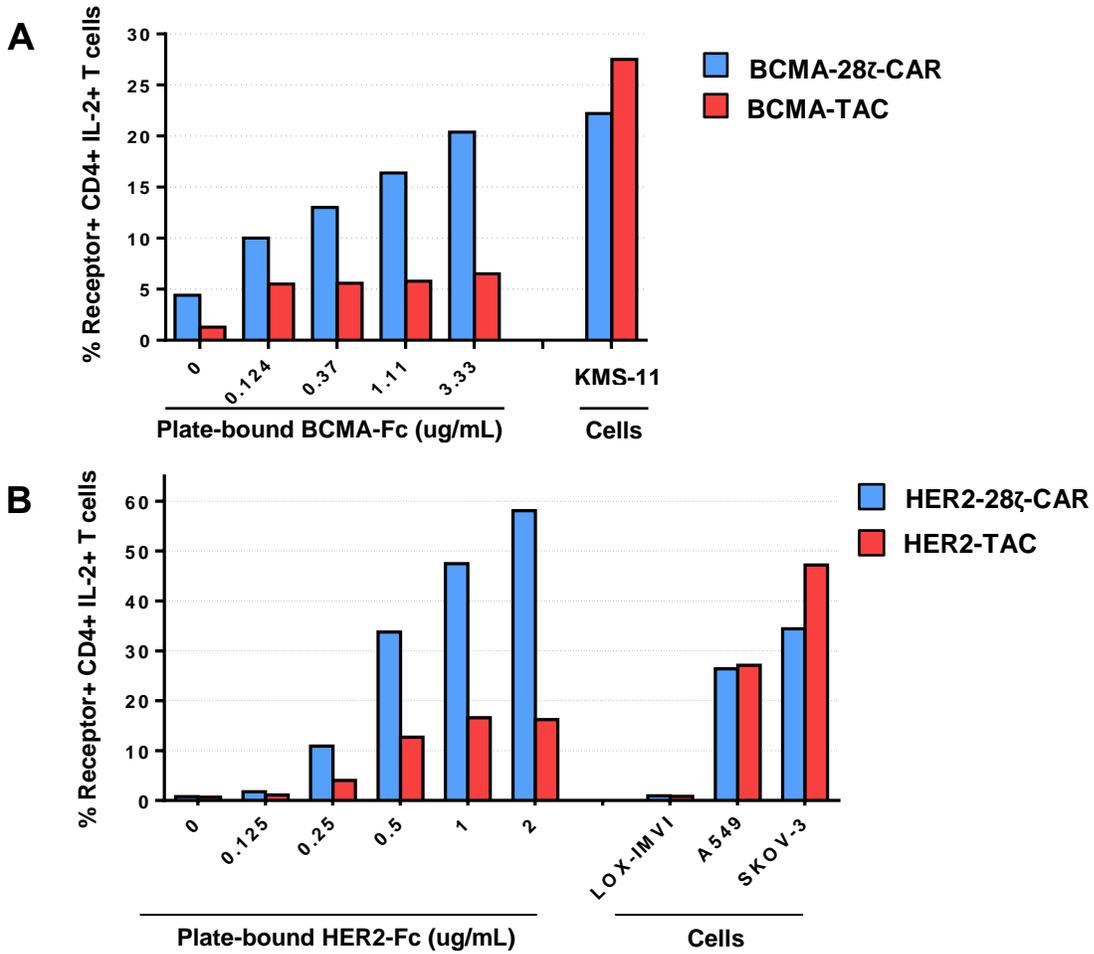
### 3.2.2 Lack of proliferation against antigen-loaded beads is independent of co-stimulation and IL-2 production

CARs deliver two signals in a fixed stoichiometry; a TCR-based activating signal, and a costimulatory signal in the form of CD28 in our current anti-BCMA scaffold. We sought to determine whether the lack of proliferation towards bead-based stimulation in TAC T cells might be due to a lack of signal 2. The KMS-11 myeloma line used for our stimulations expresses CD86, which can provide native costimulatory signals for TAC T cells during activation (**Figure 10A**). To address this possibility beads were loaded with both BCMA-Fc and CD86-Fc and verified for dual presentation of both antigens (**Figure 10B**). Inclusion of CD86 on the beads failed to improve TAC proliferation (**Figure 10C**), indicating that the lack of stimulation by the antigen coated on polystyrene beads is not due to lack of signal 2 but rather an aspect of the TAC receptor biology that relies upon the context of the antigen presentation (i.e. cell-bound versus cell-free).

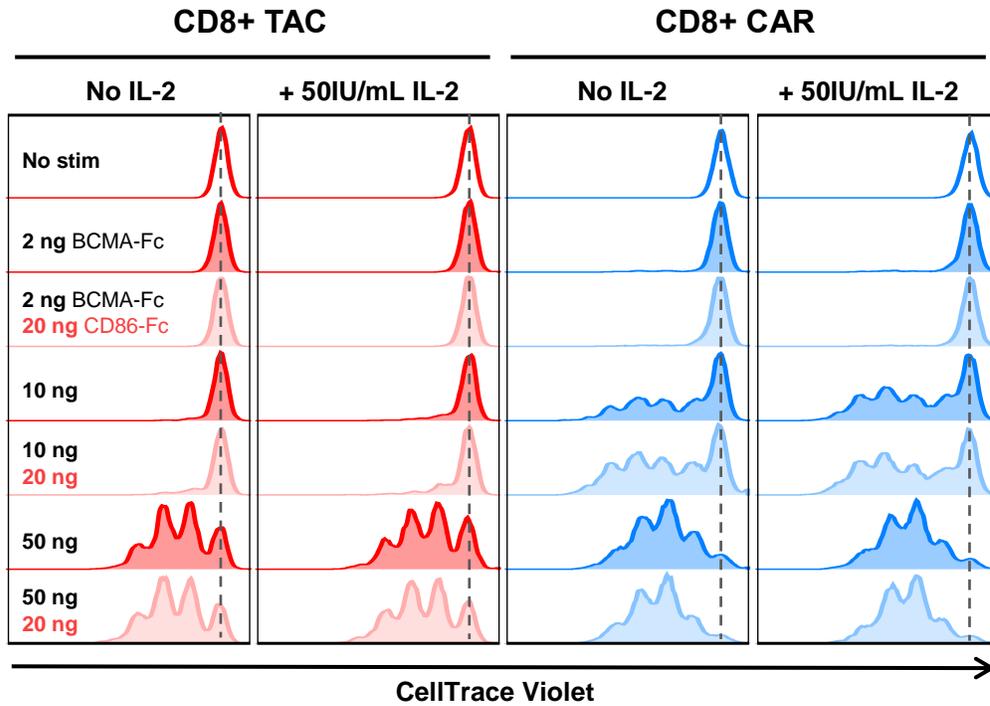
Examination of IL-2 production by TAC T cells and CAR T cells revealed that, while both cells produced equivalent IL-2 following cell stimulation, IL-2 production by TAC T cells was severely attenuated relative to CAR T cells following stimulation with antigen-coated beads. This outcome was true for both BCMA-TAC T cells (**Figure 11A**) and HER2-TAC T cells (**Figure 11B**). Given the importance of IL-2 production for T cell proliferation, we posited that the TAC T cells did not proliferate as well as CAR T cells due to a lack of IL-2. To address this hypothesis, the culture medium of proliferation assays was supplemented with half the concentration of IL-2 used during T cell expansion (**Figure 12**). However, this did not improve TAC T cell proliferation towards antigen-loaded beads, further reinforcing that tumour cells may be providing a collection of signals, rather than antigen alone.



**Figure 10.** Effects of co-stimulation are negligible for BCMA-TAC T cell proliferation. (A) Expression of BCMA, and costimulatory receptors CD80, CD86, and 4-1BBL on HEK 293T (control), K562, and KMS-11 cells by flow cytometry, shown as histograms. (B) BCMA-Fc and CD86-Fc are efficiently coated onto Protein G polystyrene beads. Beads were incubated overnight with Fc-conjugated proteins at the total protein amounts indicated per million beads. Beads were blocked with rat serum prior to flow analysis using anti-BCMA and anti-CD86 antibodies. (C) Addition of CD86-Fc onto BCMA-Fc-coated polystyrene beads does not influence proliferation of CD8+ BCMA-TAC or CAR T cells as determined using CellTrace Violet dilution by flow cytometry. Duration of stimulation, and effector:target ratios were the same as previously described. Dotted lines indicate peak fluorescence intensity of non-stimulated, parent population. Data is representative of three donors from two independent experiments.



**Figure 11.** BCMA- and HER2-TAC T cells exhibit lower frequencies of IL-2+ T cells when stimulation with beads. Percentage of CD4+ T cells expressing IL-2 following stimulation with plate-bound BCMA-Fc, or BCMA+ cells (A), or plate-bound HER2-Fc, or HER2+ cells (B), as measured by intracellular staining by flow cytometry. Data are each representative of one experiment.



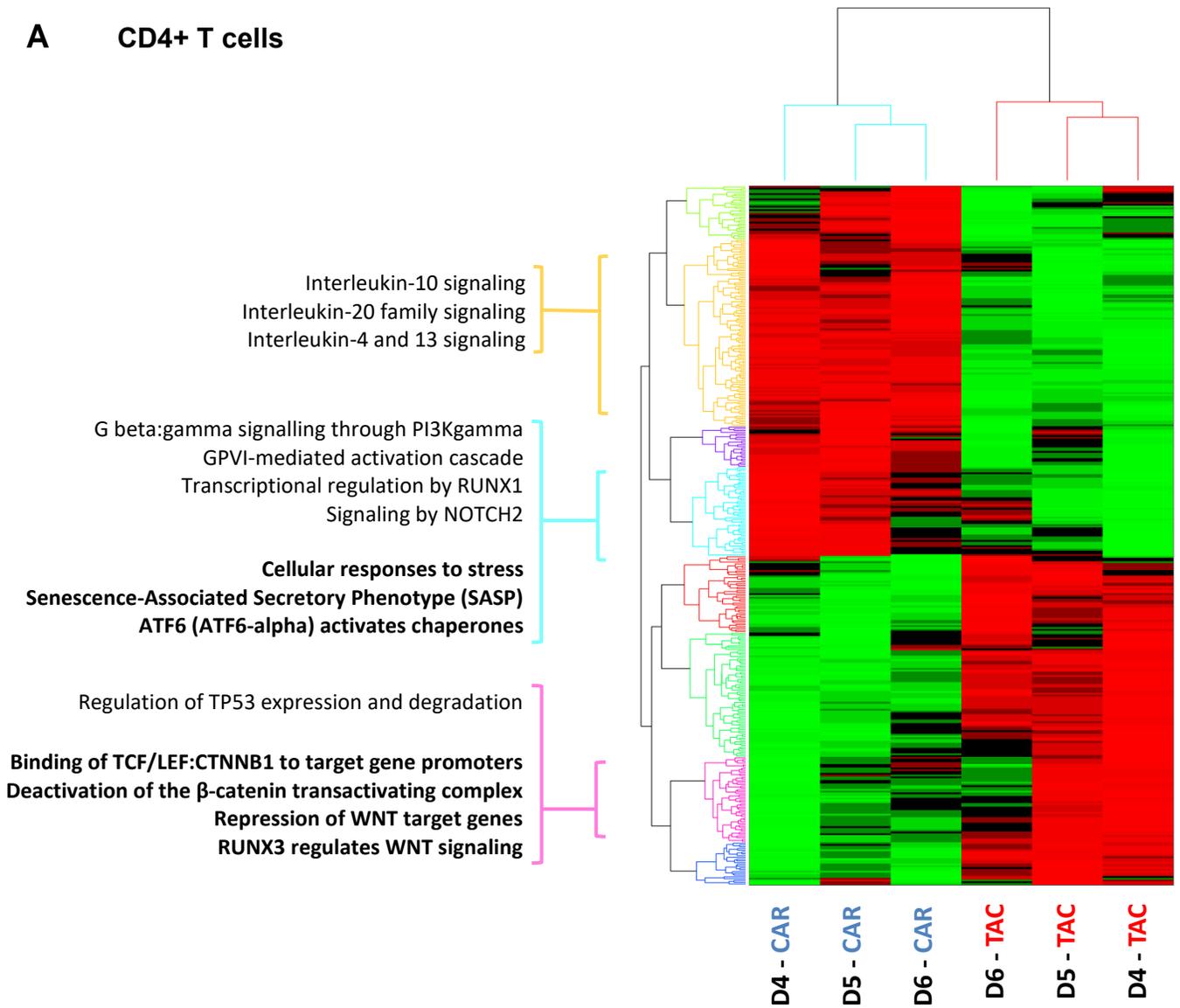
**Figure 12.** Proliferation of TAC T cells cannot be induced through exogenous IL-2 supply. (C) Comparison of BCMA-TAC and BCMA-CAR T cells stimulated with beads coated with BCMA-Fc, and with or without addition of CD86-Fc, for each individual column of histograms. Values represent quantity of each recombinant protein loaded per million beads. Side-by-side columns represent co-incubation of T cells in the presence or absence of exogenously supplemented IL-2. Dotted lines indicate peak fluorescence intensity of non-stimulated, parent population. Data is represented of two donors from one experiment. Populations were gated on CD4+NGFR+ or CD8+NGFR+ for all data shown.

### 3.2.3 Gene transcriptional profiling indicates that TAC and CAR T cells diverge following activation

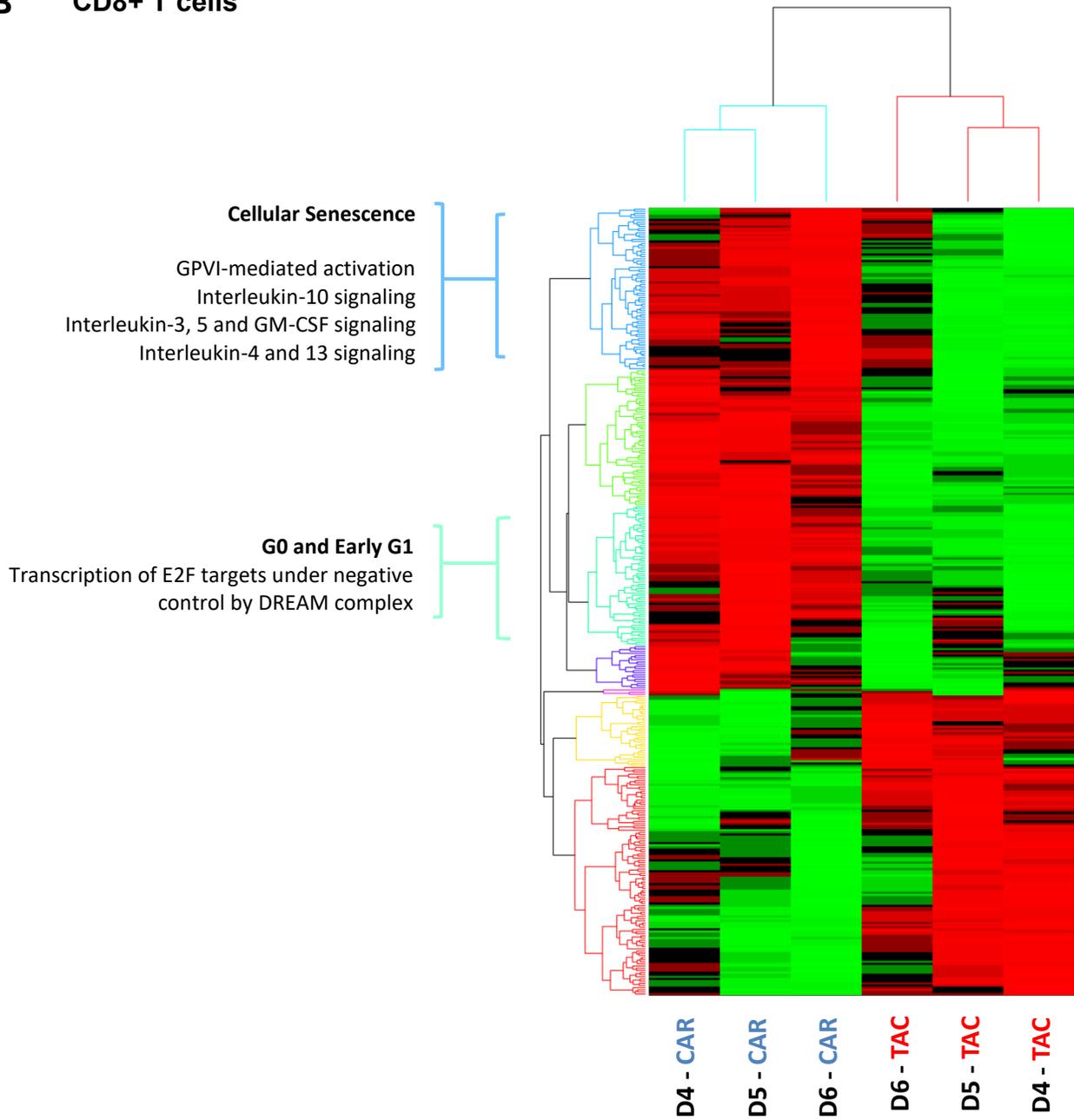
To begin to understand the difference in signaling pathways that lead to gene transcription in CAR and TAC T cells, BCMA-28 $\zeta$ -CAR and BCMA-TAC T cells were stimulated with BCMA-Fc-coated beads. RNA was isolated from T cells, separated into CD4<sup>+</sup> and CD8<sup>+</sup> populations, at 1 hour and 4 hours post-stimulation.

Transcriptome differences were analyzed for the 4-hour timepoint, as hierarchical clustering revealed subsets of genes that were upregulated in CAR, but not TAC, and vice versa, independently in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (**Figure 13A & 13B**). Gene ontology analysis was performed to search for biological relevance within gene clusters. Manually curated gene clusters (shown as different colours within the left dendrogram) were selected from parent clusters, automatically generated using NMF clustering. In CD4<sup>+</sup> T cells, CARs more strongly upregulated cytokines compared to TAC T cells, as well as genes related to cellular senescence and stress. CD4<sup>+</sup> TAC T cells downregulated Wnt/ $\beta$ -catenin-associated genes, which is a signaling pathway that is associated with T cell differentiation.<sup>176</sup> Downregulation of *Tcf7* and *Lef1*, which are central Wnt pathway genes, occurs following antigen-dependent stimulation of T cells, and may be indicative of memory formation.<sup>177</sup>

**A CD4+ T cells**



**B CD8+ T cells**

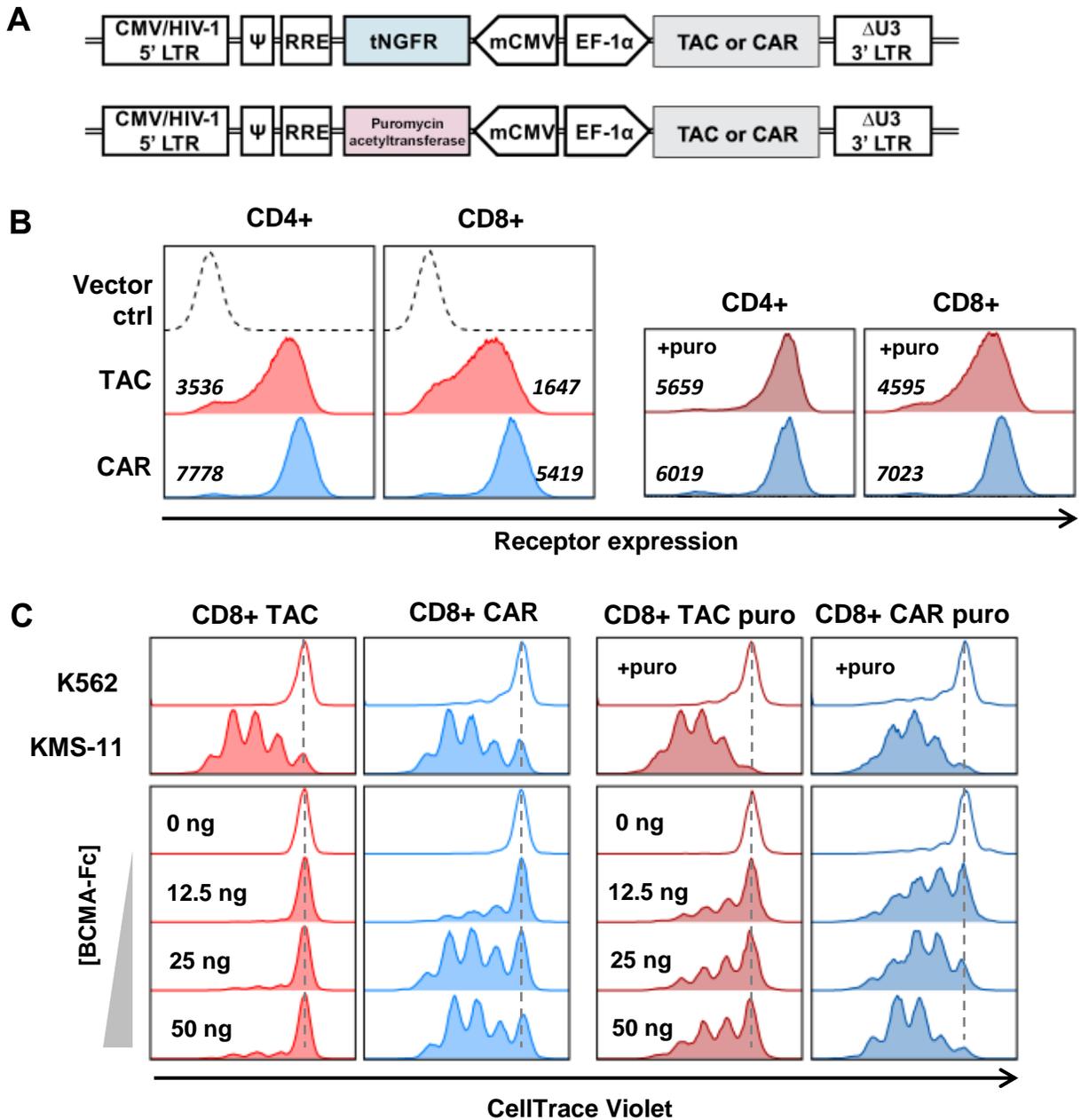


**Figure 13.** Transcriptional analysis of differentially expressed genes between (A) CD4+, and (B) CD8+ BCMA-TAC and BCMA-CAR T cells at 4-hours post-stimulation, clustered first by sample (top dendrogram), then by individual genes (left dendrogram). Red represents upregulation of genes, and green represents downregulation, relative to all samples. Coloured clusters were manually curated, and pathways of interest from pathway enrichment of genes within each cluster were selected. Transcriptome data are generated from three donors per experiment, indicated as D4, D5 and D6.

### 3.2.4 Higher surface expression of TAC does not fully restore proliferative capacity compared to CARs

Due to lower surface expression of BCMA-TAC T cells compared to BCMA-CAR T cells, we employed antibiotic selection of T cells transduced with a lentiviral vector encoding for puromycin acetyltransferase, in place of tNGFR under the promoter mCMV (**Figure 14A**). To increase receptor density, T cells were selected by puromycin 48 hours after transduction, and relative receptor levels was measured by flow cytometry (**Figure 14B**). Non-antibiotic-treated T cells (hereafter referred to as conventional T cells) were sorted for tNGFR<sup>+</sup> by cell sorting on Day 7. Puromycin selection significantly increased the surface expression of TAC, as the median fluorescence intensity was nearly doubled in both CD4<sup>+</sup> and CD8<sup>+</sup> TAC T cells.

We repeated the same stimulations using KMS-11 cells, or BCMA-Fc-coated beads as previously described for conventional T cells and measured proliferation via CellTrace Violet. Cell-based stimulation led to similar patterns of proliferation between TAC and CAR T cells (**Figure 14C**). Puromycin-selected T cells had a greater response compared to conventional T cells, where nearly 100% of both the TAC and CAR population divided at least once. Both puromycin-selected TAC and CAR T cells were more responsive to lower levels of BCMA-Fc on beads compared to conventional T cells. The highest concentration of BCMA-Fc on beads led to significant proliferation of conventional CAR T cells, as expected. However, the same level of antigen on beads did not trigger the same response in puromycin-selected TACs. This data reinforces that the conditions required for TAC proliferation are more reliant on context than abundance, compared to CAR T cells.

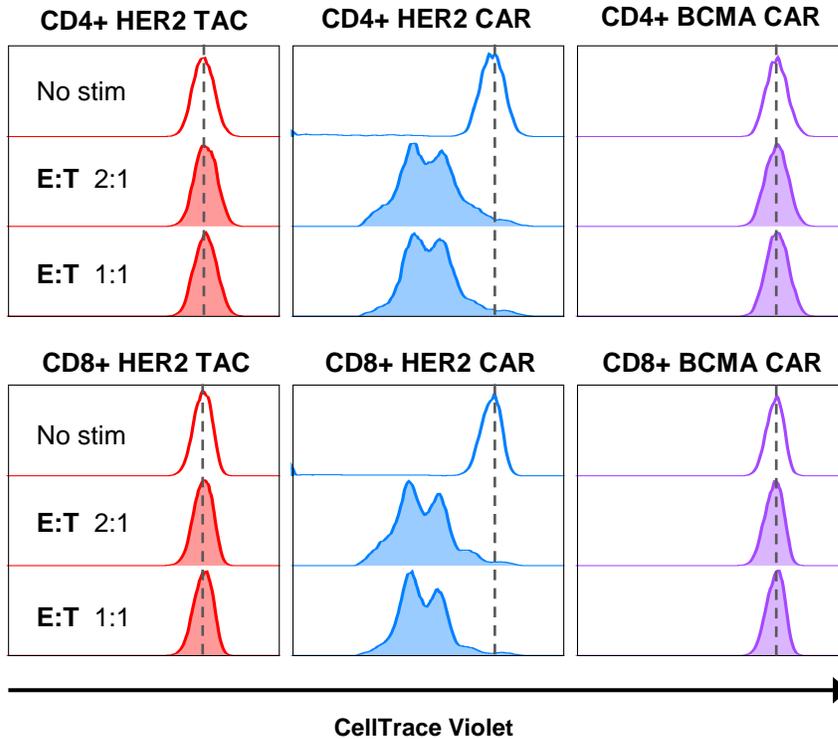


**Figure 14.** Artificial selection of higher BCMA-TAC receptor expression on T cells increases sensitivity to low antigen levels. (A) Puromycin-treated T cells utilize the same lentivirus transfer vector, with puromycin acetyltransferase transgene replacing tNGFR under minimal CMV promoter. (B) Histogram plots comparing endogenous TAC receptor and CAR expression on primary T cells transduced with TAC or CAR-encoding lentiviruses. Surface receptor expression is detected by labelling with recombinant Fc-conjugated BCMA, and a secondary PE-conjugated anti-human IgG Fc antibody. Dotted black lines represent vector control. (C) Proliferation of CD8<sup>+</sup> TAC and CAR T cells with cell-based (upper panels) or bead-based (lower panels) stimulation, tracked by CellTrace Violet 4 days after co-culture. T cells were stimulated at an effector:target ratio of 2:1. Vertical dotted lines indicate peak fluorescence intensity of non-stimulated, parent population. Non-puromycin T cell cultures are gated on CD4<sup>+</sup>NGFR<sup>+</sup> or CD8<sup>+</sup>NGFR<sup>+</sup> for all plots shown. Puromycin-treated T cells are gated only by CD4 or CD8 positivity. Data are representative of three donors from three independent experiments.

### 3.2.5 HER2-CAR T cells, but not HER2-TAC T cells, respond to stimulation by cells isolated from lungs of NRG mice

A major finding from our recent report on the TAC receptor was that in a HER2 xenograft murine model, HER2-28 $\zeta$ -CAR T cells were lethally toxic following infusion, in an off-tumour, off-target manner.<sup>158</sup> As previously mentioned, CAR T cells expressing an anti-HER2 DARPin binding domain accumulated in the lungs and heart of mice following adoptive cells transfer, and showed significant proliferation against an unknown antigen of murine origin. Conversely, TAC T cells bearing the same binding domain did not show any toxicities following infusion, and instead exerted regression of established HER2-positive tumours.

We sought to investigate whether HER2-TAC T cells were capable of proliferating against murine lung tissue in the absence of other *in vivo* factors. Lungs from 12-16-week-old NRG mice were perfused, harvested, and processed into single cell suspension using type IV collagenase. HER2-28 $\zeta$ -CAR and TAC T cells were labelled with CellTrace Violet, and co-cultured with cells isolated from murine lung tissue for four days, akin to proliferation experiments previously described. BCMA-28 $\zeta$ -CAR T cells were included as a negative control for the anti-HER2 DARPin. HER2-28 $\zeta$ -CAR T cells proliferated strongly; a very small number of T cells remained undivided at the end of the co-culture period. In contrast, neither TAC nor BCMA-28 $\zeta$ -CAR T cells proliferated following co-culture with murine lung cells (**Figure 15**) confirming that the proliferative response of HER2-28 $\zeta$ -CAR T cells was due to the combination of the HER-2 DARPin and CD28 $\zeta$ -CAR scaffold. Although multiplex immunohistochemistry staining previously showed that Ki-67+ co-localized primarily with CD4+ CAR T cells, CD4+ and CD8+ CARs were equally responsive to stimulation *in vitro*. These results support our *in vivo* observations of off-target reactivity by HER2-CAR T cells, in the absence of any proliferative response by TAC T cells. Further characterization of the murine cells isolated (i.e. identification of epithelial cells, endothelial cells, or fibroblasts) will aid in narrowing down what cell types cause CAR T cells to activate.



**Figure 15.** HER2-CAR T cells proliferate strongly when stimulated with cells isolated from lungs of NRG mice. HER2-28 $\zeta$ -CAR, HER2-TAC and BCMA-28 $\zeta$ -CAR T cells were labelled with CellTrace Violet and co-cultured with cell suspensions isolated from fresh lung tissue at the indicated effector:target ratios. Dotted lines indicate maxima of the undivided peak. Data are representative of three donors from three independent experiments, except for BCMA-28 $\zeta$ -CAR experiments which only have two donors from two independent experiments. Lung isolates were pooled from a minimum of two mice per experiment.

### 3.3 Consequences of stimulation, and characteristics of stimulated T cells

#### 3.3.1 CAR T cells experience faster activation kinetics, and stronger activation towards stimulation than TAC T cells

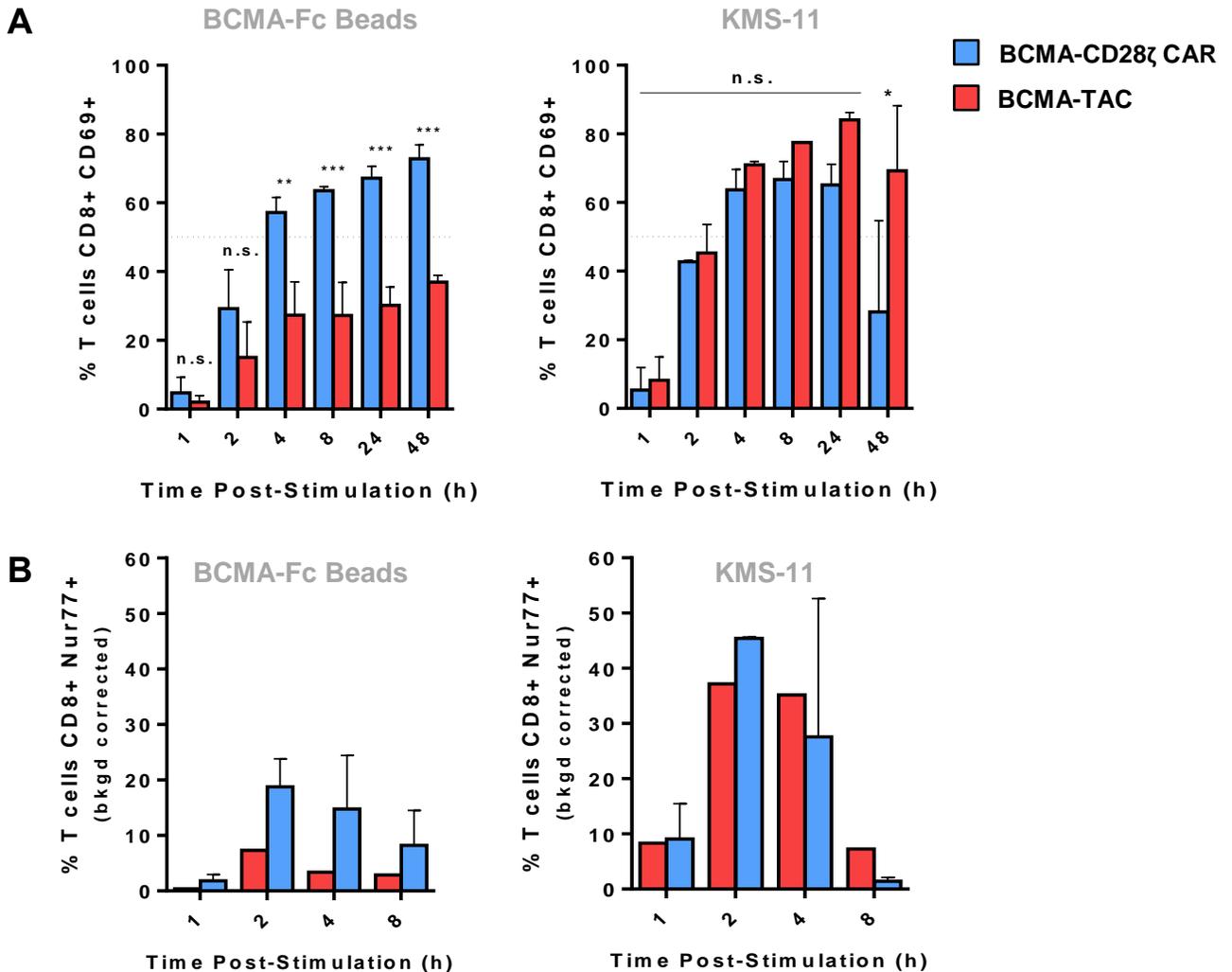
To gauge the immediate and early effects of signaling through the chimeric antigen receptors, we measured surface expression of CD69, a commonly used marker for T cell activation, and intracellular markers Nur77 and Ki-67 by flow cytometry. CD69 is the earliest activation marker upregulated by T cells that can be detected one to two hours following activation.<sup>178</sup> However, its expression can be regulated by multiple transcription factors, and therefore several different pathways can converge to induce CD69 upregulation.<sup>179</sup> In addition to CD69, we investigated Nur77 as a reporter of CAR- or TAC-mediated signaling. Nur77 is an orphan nuclear receptor belonging to the NR4A family, that binds DNA to activate gene transcription in several immune cell types.<sup>180</sup> In T cells, Nur77 functions as an immediate early gene reporter which is specific for TCR signaling and has been used recently to measure strength of TCR stimulation.<sup>181</sup> We further incorporated Ki-67 staining as a measure for cell cycle entry by T cells, as Ki-67 is expressed during all phases of cell division (G<sub>1</sub>, S, G<sub>2</sub>, mitosis), and absent from resting cells (G<sub>0</sub>).<sup>182</sup> As Nur77 is an intracellular stain, we combined Nur77 and Ki-67 during our timecourse to explore the link between strength of chimeric receptor triggering, and proliferation.

Recent reports have described CAR triggering to be much more potent, and rapid in kinetics, compared to conventional TCR triggering.<sup>157</sup> The percentage of CD69+ T cells following stimulation with KMS-11 cells, or BCMA-Fc-loaded beads, was determined by flow cytometry. CD69 was measured on TAC or CAR T cells at 1, 2, 4, 8, 24, and 48 hours following co-incubation with cells or beads at an effector:target of 2:1. For simplicity, results for CD8+ T cells are shown. Observations were consistent for both CD4+ and CD8+ compartments (complete dataset **Appendix I**). For bead-based stimulation, a significantly lower frequency of TAC T cells became CD69+ compared to CAR T cells (**Figure 16A**). Conversely, the number of CD69+ CAR T cells continued to accumulate past 4 hours, at each subsequent time point measured. When stimulated with KMS-11, peak CD69 upregulation by CAR T cells was much faster compared to TAC T cells. Maximal %CD69+ peaked at 8 and 24 hours for CAR and TAC T cells, respectively. In general, cell-based stimulation engaged more than 80% of CD8+ TAC T cells, indicating that the population is fully capable of responding, but does not do so when exposed to BCMA on beads alone. In contrast, both cells and beads engaged up to 70% of the CD8+ CAR population equally, suggesting that CAR T cells do not readily distinguish between the stimuli.

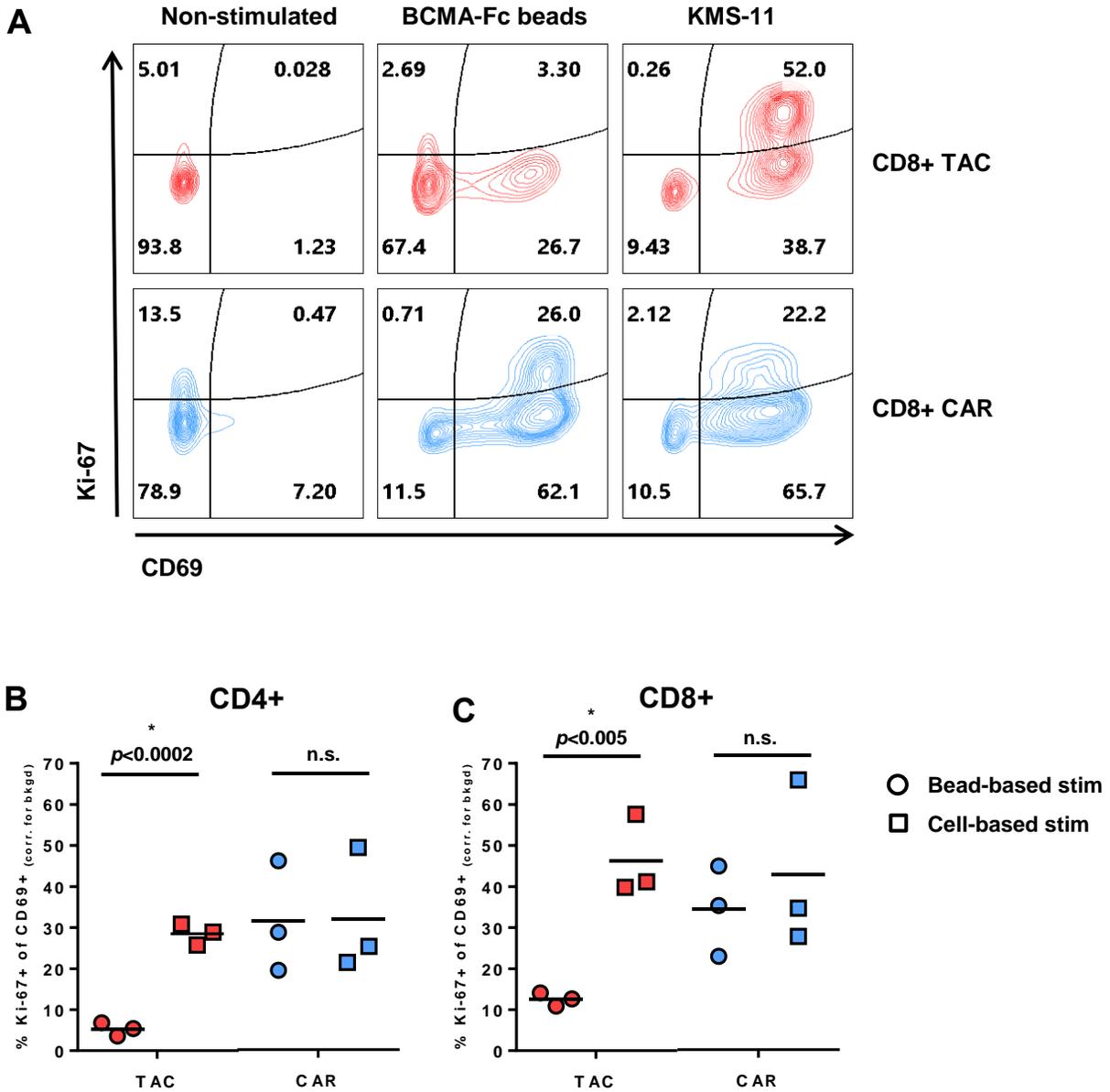
We measured intracellular expression of Nur77 and analyzed Nur77 upregulation 1-8 hours following stimulation (**Figure 16B**). Like CD69 induction, Nur77 was more highly expressed in CAR T cells than TAC T cells following bead-based stimulation, at roughly twice the frequency of Nur77+ CAR T cells compared to TAC at each time point following 1 hour. Cell-based stimulation resulted in significantly more Nur77+ T cells for both TAC and CAR T cells, at roughly equivalent frequencies. Unexpectedly, cell- and bead-based stimulation did not

result in similar frequencies of Nur77<sup>+</sup> CAR T cells. This confounded the earlier observation that roughly equal numbers of CAR T cells become CD69<sup>+</sup>, regardless of the stimuli. It is unclear whether signaling through the chimeric receptors leads to equivalent levels of Nur77 induction compared what was observed in literature, following TCR-based stimulation.<sup>181</sup> It is possible that other pathways, such as signal amplification by the CD28 costimulatory domain in CAR T cells can induce CD69 expression, but not Nur77.

Lastly, we measured Ki-67 expression as a marker for cell cycle entry, as we previously observed that cells, but not beads, strongly induces TAC proliferation. Although up to a quarter of the TAC population upregulated CD69 in response to bead stimulation, less than 10% of CD69<sup>+</sup> cells were also Ki-67<sup>+</sup> at 24 hours (**Figure 17A**). Stimulation with cells showed that TAC T cells were equally or more proliferative than CARs. CARs observed little difference in the proportion of Ki-67<sup>+</sup> cells between bead- and cell-based stimuli (**Figure 17B & 17C**).



**Figure 16.** CD69 and Nur77 serve as distinct activation markers for CAR- and TAC receptor-based signaling. (A) CD69 expression was analyzed following stimulation of CAR and TAC T cells with BCMA-Fc-loaded beads, or KMS-11 cells. Stimuli for each plot is indicated in grey text. Percentage of activated cells was determined by subtracting background CD69 stimulation in corresponding non-stimulated TAC or CAR T cells. Gating of CD69+ population was manually set according to non-stimulated engineered T cell samples. Dotted line is indicated at 50%. Statistical analysis was performed using two-way ANOVA per timepoint and corrected for multiple comparisons using Sidak method. n.s. = non-significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . (B) Nur77 expression was stained intracellularly following stimulation of CAR and TAC T cells with BCMA-Fc-coated beads or cells. Percentage of Nur77+ cells was determined by bisectonal gate on Nur77 histograms, using the non-stimulated T cell control as the negative for each time point. All data was gated for CD8+NGFR+ T cells and were from two donors from two independent experiments.



**Figure 17.** Ki-67 indicates cell cycle entry of CAR and TAC T cells stimulated with beads or cells. (A) Ki-67 and CD69 staining of BCMA-engineered T cells 24 hours after co-incubation with target cells or beads. Numbers indicate percentage of cells in each quadrant gate. Individual donors indicating percentage of Ki-67+ cells within the CD69+ population are plotted within (B) CD4+ and (C) CD8+ T cell. All plots are gated for CD4+NGFR+, or CD8+NGFR+ expression, unless otherwise stated. Statistical significance was determined using multiple *t*-tests for independent datasets. Data are representative of two donors from two independent experiments.

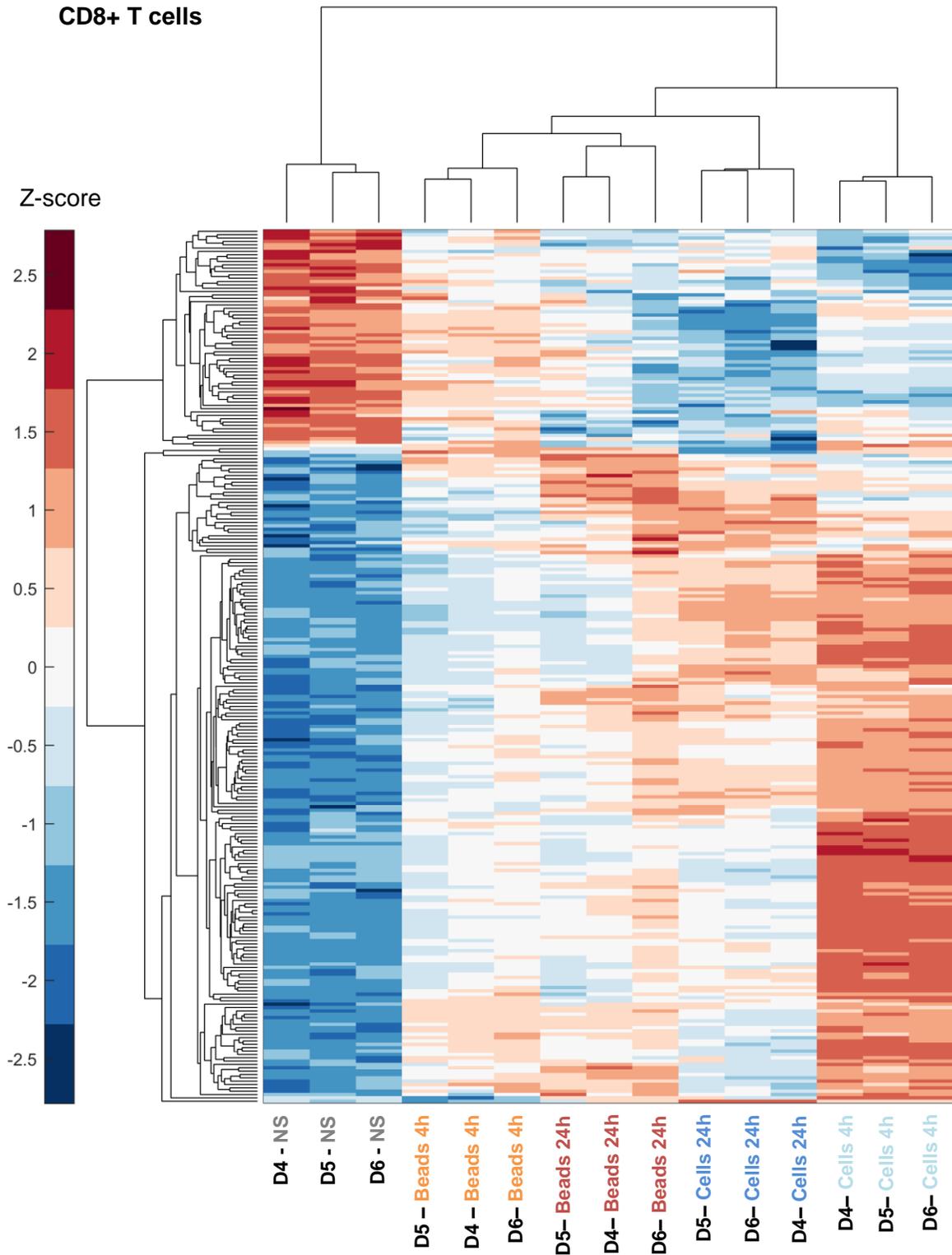
### 3.3.2 TAC T cells stimulated with beads exhibit low transcriptional activation, and lack activation of T cell signaling pathways

Following our observations that bead- and cell-based stimulation resulted in significantly different responses from TAC T cells, we performed RNAseq on differentially stimulated T cells to investigate whether unique pathways were activated. BCMA-TAC T cells from three individual donors were flow-sorted into NGFR+ populations on day 7 of culture. On day 14, purified T cell populations were stimulated with KMS-11 cells, or BCMA-Fc-coated beads at a density of 50ng BCMA-Fc per  $1 \times 10^6$  beads. The antigen density was chosen based on prior observations that this antigen density induces a sub-optimal proliferative response (see Fig. 8C as an example) compared to the robust response observed following cell-based stimulation. At 4 and 24 hours following stimulation, TAC T cells were separated into CD4+ and CD8+ via fluorescence activated cell-sorting, and RNA was collected for RNAseq.

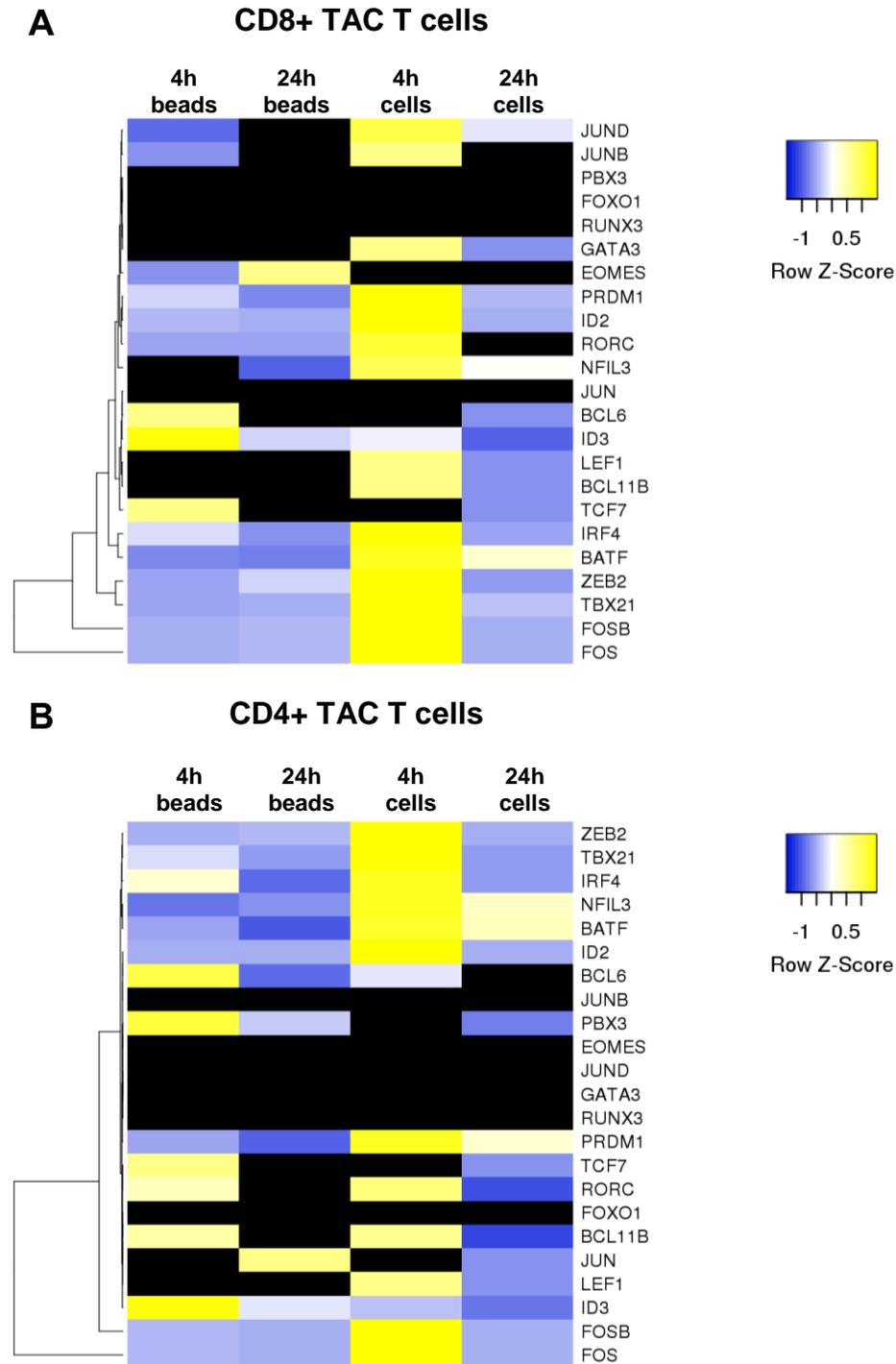
Heat maps were generated for differentially expressed genes compared to non-stimulated TAC T cell controls at a minimum fold-change of 2.0 or higher (**Figure 18**). Hierarchical clustering of the samples showed that TAC T cells stimulated with cells after 4 hours were most distantly clustered from non-stimulated control. Transcriptional changes observed at 4 hours resolved by 24 hours, but transcriptional patterns did not return to baseline levels of gene expression. Transcriptional patterns of TAC T cells stimulated with beads were similar between the 4 and 24 hour timepoints, suggesting that low level activation was sustained, but did not reach full activation as observed with cell-based stimulation. Dataset for CD4+ TAC T cells are shown in **Appendix II**.

TAC T cells stimulated with KMS-11 resulted in significant upregulation of several transcription factors, and at levels much higher than bead-based stimulation in CD4+ (**Figure 19A**) and CD8+ T cells (**Figure 19B**). Examples of transcription factors upregulated during cell-based stimulation but not bead-based stimulation include *GATA3*, *T-bet*, *JUND*, and *JUNB*, all of which have been associated with CD8 differentiation and cytotoxic T cell function.<sup>183–185</sup> *Bcl6*, *ID3*, and *Tcf7* were more highly upregulated in T cells exposed to beads for both CD4+ and CD8+ TAC T cells. Complete differential gene expression values are displayed in **Appendix III**.

We performed pathway analysis to determine whether cell- or bead-based stimulation diverged in their signaling pathways following activation. As expected, many pathways associated with cell division were not upregulated by TAC T cells stimulated with beads (**Table 3**). While some metabolic pathways could still be induced through bead stimulation, several other important pathways, such as nucleic acid biosynthesis and carbohydrate metabolism, were not upregulated at the 24-hour timepoint. Interestingly, apoptosis pathways were upregulated in bead-stimulated TAC T cells, but downregulated following cell-based stimulation. Taken together, the transcriptome data suggests that bead stimulation results in an understimulated state where TAC T cells fail to enter a proliferative state, which may render them susceptible to apoptosis in the absence of anti-apoptotic gene expression.



**Figure 18.** Transcriptome analysis of TAC T cells co-cultured with BCMA-Fc-coated beads or KMS-11 cells at 4h or 24h post-stimulation. Heat map was generated from differentially expressed genes at a fold-change cutoff  $>2.0$ . Donors are represented as “D4”, “D5”, or “D6”, followed by the type and duration of stimulation. Data are representative of three independent donors.



**Figure 19.** Heat map of transcription factors expressed by TAC T cells 4 or 24h post-stimulation. List of transcription factors were manually curated from literature. Black squares indicate non-significant fold-change value compared to control. Complete linkage method was used for hierarchical clustering of transcription factors. Dataset per sample type are representative of three independent donors.

**Table 3.** List of cell cycle, metabolism, signaling, and apoptosis pathways up- or down-regulated following bead- or cell-based stimulation of BCMA-TAC T cells.

	GO ID	CD4+ T cells				CD8+ T cells			
		Beads 4 hrs	Beads 24 hrs	Cells 4 hrs	Cells 24 hrs	Beads 4 hrs	Beads 24 hrs	Cells 4 hrs	Cells 24 hrs
<b>Proliferation and Cell Cycle Entry</b>	cell cycle			DOWN	UP		DOWN	DOWN	UP
	M phase			DOWN	UP		DOWN	DOWN	UP
	mitosis			DOWN	UP		DOWN	DOWN	UP
	nuclear division			DOWN	UP		DOWN	DOWN	UP
	DNA replication			DOWN	UP			DOWN	UP
	mitotic cell cycle checkpoint			DOWN	UP				
	regulation of mitosis			DOWN				DOWN	
<b>Metabolic Pathways</b>	positive regulation of macromolecule metabolic process	UP	UP	UP	UP	UP	UP	UP	UP
	regulation of cellular protein metabolic process	UP	UP	UP	UP	UP		UP	UP
	nucleic acid metabolic process			UP	UP			UP	UP
	polyamine biosynthetic process			UP	UP				
	L-serine biosynthetic process				UP				UP
	carbohydrate catabolic process				UP				UP
nucleobase biosynthetic process				UP			UP	UP	
<b>Signaling Pathways</b>	I-kappaB kinase/NF-kappaB cascade	UP	UP	UP		UP	UP	UP	
	JAK-STAT cascade			UP				UP	
<b>Apoptotic Pathways</b>	induction of apoptosis by extracellular signals				DOWN				DOWN
	regulation of apoptosis	UP	BOTH	BOTH	BOTH	UP	BOTH	UP	DOWN
	regulation of cell death	UP	BOTH	BOTH	BOTH	UP	BOTH	UP	DOWN
	positive regulation of apoptosis	UP	BOTH	BOTH	DOWN	UP	BOTH	BOTH	DOWN
	cell death	UP	BOTH	UP	DOWN	UP	BOTH	UP	DOWN
activation of pro-apoptotic gene products	UP	UP			UP				

#### 4.0 Discussion

Our current findings indicate that CAR and TAC T cells are fundamentally different in both activated and non-activated states. We initially aimed to understand the impact of the chimeric receptors on primary human T cells by measuring expression of inhibitory receptors, and memory-associated markers. We, and others, report that the extent of tonic signaling manifested by CARs varies according to the antigen-binding domain employed and intracellular signaling domains incorporated. Interestingly, although TAC T cells are designed to bind to the  $\epsilon$  domain of the TCR-CD3 complex, which might be expected to activate the T cells, tonic signaling in TAC T cells was minimal compared to levels observed in CAR T cells. Since scFv aggregation is one driver of tonic signaling in chimeric receptors, it remains plausible that the TCR sequesters TAC molecules away from one another on the surface of T cells, hence preventing formation of microclusters that could lead to spontaneous activation.

One consequence of tonic signaling is the promotion of T cell differentiation through continuous signaling, which we observed as a loss of CD45RA<sup>+</sup> CCR7<sup>+</sup> naïve T cells in the CAR T cell products. It is interesting to note that CD28-based and 4-1BB-based CARs promote alternate differentiation where CD28-based CAR T cells are enriched for central memory T cells, and the 4-1BB-based CAR T cells are enriched for central and effector memory T cells. Several groups have advocated for specific memory phenotypes to be generated, maintained, or isolated during the manufacturing of T cells for adoptive transfer.<sup>86,186</sup> Gattinoni *et al.* reported on a stem cell-like T cell population capable of self-renewal, which resulted in superior in anti-tumour responses.<sup>187</sup> Similarly, central memory CAR T cells have been purposely isolated from clinical preparations for their longevity and persistence over effector memory phenotypes.<sup>188</sup> Generation of these T cell subsets can be done using specific culturing conditions, such as supplying one of IL-15 or IL-7, or both.<sup>90,189</sup> Our current data, as well as reports by other groups<sup>190</sup>, have shown that lower IL-2 concentrations can also limit differentiation of CAR T cells during the culturing phase. Small molecule inhibition of targets such as Akt downstream of CD28 signaling has also been shown to prevent differentiation of CARs and TILs.<sup>191,192</sup> These strategies provide evidence that excessive CD28 signaling, either during the activation of PBMCs, or tonic signaling through the CAR, can lead to T cell differentiation *ex vivo*. For TAC T cells, tonic signaling is not exacerbated by culturing conditions (e.g. IL-2 concentrations) or antigen-binding domains, which may make the receptor much more amenable to a wider variety of binding domains and culturing conditions compared to CARs. Where some single chains undoubtedly induce debilitating tonic signaling within CAR scaffolds<sup>134</sup>, it would be worthwhile to explore whether the same scFvs can be used instead as TAC constructs.

We compared the sensitivity of CAR and TAC receptors against varying levels of antigen, which yielded surprisingly different results in proliferation and cytokine production. While CAR T cells were sensitive to low levels of antigen on both beads and cells, they did not appear to differentiate between the two types of stimuli. Conversely, significant proliferation and cytokine production by TAC T cells was only observed when stimulated with antigen in the context of a cell, indicating that triggering of TAC receptors is more contextually stringent compared to CARs. Many cell types, especially hematological tumour cells, naturally express

costimulatory receptors for T cells. For example, KMS-11 are myeloma cells that express CD86, which can provide costimulatory signals as well as other adhesion molecules that integrate to promote T cell receptor aggregation. Although CD86-Fc addition in our experiments did not improve TAC T cell proliferation, it is more likely that we did not adequately capture the collection of signals needed for complete TAC T cell activation. The similarity in the lack of response by HER2-TAC to HER2-Fc-loaded beads suggests that this effect is independent of the ligand binding domain of the chimeric receptor. In fact, loading of the same recombinant protein on K562 cells engineered to express high-affinity Fc $\gamma$ RI showed improved sensitivity towards lower antigen densities when present on a cellular surface, compared to bead-based stimuli.

One possible explanation for the difference in activation of TAC T cells when stimulated with cells or beads is the affinity of the interaction. We currently lack data for the affinity of the interaction between the scFv on the TAC receptor scaffold when bound to endogenous BCMA on tumour cells, compared to recombinant BCMA-Fc attached to protein G on beads. Previous studies have shown that low-affinity TCR interactions (albeit in the context of peptide-MHC) result in delayed expression of CD69 compared to high-affinity interactions.<sup>49</sup> Similar to our current observations, beads inefficiently induce early activation events in TAC T cells, and responses such as CD69 upregulation were delayed compared to strong signals such as cell-based stimuli. It is possible that the interactions between TAC receptors and bead-bound BCMA-Fc is much lower in affinity, due to steric hindrance, lower protein density, or rigidity of the beads, compared to interactions with BCMA on cells, which is presented on a fluid membrane.

In addition to proliferation, several other markers were used to measure the extent of activation in engineered T cells, ranging from early reporters, such as Nur77, or CD69, or late reporters, such as IL-2 production, or upregulation of checkpoint receptors. Nur77, used as a surrogate for TCR-dependent-signaling based on previous reports<sup>181,193</sup>, was strongly induced in TAC T cells following stimulation with KMS-11 cells. This translated to a strong proliferation signal, measured as Ki-67 expression 24-hours following stimulation. Although proliferation of CAR T cells was equivalent when stimulated with either cells or beads, Nur77 was weakly induced when stimulated with beads compared to cells. This hints to the possibility that CARs are more efficient at translating weak signals to full effector functions compared to TAC receptors.

Given that we often select antigen-binding domains based on their specificity to a ligand rather than selectivity against irrelevant ligands, each binding domain poses a risk for off-tumour toxicities which may not be predicted *in vitro*. Furthermore, tumour-specific targets are difficult to find, and often restricted to a subset of tumour types, and individual patients.<sup>194</sup> Most tumour targets are simply overexpressed in cancerous tissue, such as HER2, which is also lowly expressed on many healthy tissues.<sup>195</sup> Based on our current observations, CARs bearing cross-reactive binding domains can potentiate off-target toxicities due to its reactivity towards low abundance antigens. Because TAC T cells may require higher antigen densities, or a more stringent context for activation, the receptor itself may prove useful against a wider pool of tumour-associated antigens.

This potentially explains the observations from our HER2 xenograft model from our principle report on the TAC receptor.<sup>158</sup> In this model, the anti-HER2 DARPin used as the

binding domain on both HER2-TAC and HER2-28 $\zeta$ -CAR T cells. Infusion of equal number of HER2-TAC T cells and HER2-28 $\zeta$ -CAR T cells results in drastically different outcomes. HER2-TAC T cells expanded within the HER-2 expressing tumor whereas the HER2-28 $\zeta$ -CAR T cells expanded primarily in the lungs. Since the expansion in the lungs was found to be unique to HER2-28 $\zeta$ -CAR T cells, we predicted that the HER2-28 $\zeta$ -CAR T cells were cross-reactive with murine antigen expressed on lung cells. Indeed, it was confirmed that HER2-28 $\zeta$ -CAR T cells are reactive to mouse lung cells *in vitro* whereas HER2-TAC T cells do not react, despite carrying the identical antigen-binding domain. These results demonstrate a clear advantage of the differential biology of TACs and CARs.

The mechanism underlying this dichotomy between an off-tumour response by CAR T cell and an on-tumour response by TAC T cells can be explained by our current results demonstrating that activation of the TAC receptor is contextual. We hypothesize that while both receptors may recognize the same off-target antigen, TAC T cells will not proliferate against low levels of irrelevant antigen due to its dependence on context of stimulation. Furthermore, DARPins are well-structured proteins known for their stability and folding efficiency.<sup>196,197</sup> This minimizes the possibility that the structure of the HER2 DARPIn is significantly different on the surface of a CAR T cell, compared to a TAC T cell, especially since both receptors can still recognize HER2-positive targets. Rather, we are encouraged that the activation modalities between TAC and CARs are indeed fundamentally different. This supports our goal in generating genetically engineered T cells that may cause fewer side effects due to off-target toxicities.

Preclinical models for TAC T cells are limited to xenograft murine studies, due to the use of the anti-human CD3 $\epsilon$  binding domain which is critical for TAC function and only works with human T cells. Major limitations of xenograft models include the lack of interaction between human T cells and other functioning immune cells, and the lack of proper tumour microenvironment that contributes to tumour survival. In theory, a murine TAC receptor could be constructed using anti-mouse CD3 $\epsilon$  single chain antibody, derived from available monoclonal antibody clones, and a fully murine CD4 transmembrane and cytosolic domain; however, during the development of the TAC receptors, we learned that functionality is highly dependent upon the choice of anti-human CD3 $\epsilon$  binding domain.<sup>158</sup> Syngeneic models of orthotopic murine tumours have been suggested to better recapitulate disease progression in human patients.<sup>198,199</sup> However, models for treatment of B-cell acute lymphoblastic leukemia using anti-mouse CD19 CAR T cells did not reveal any potential lethal toxicities<sup>200</sup>, which was later observed in human CD19 CAR T cell trials.<sup>138</sup> One way to bridge the gap between xenograft models and syngeneic models is the use of humanized mice. Reconstitution of human hematopoietic stem cells in irradiated mice engineered to express a combination of human cytokines allowed for development of a complete human immune cell repertoire that is xenotolerant against murine tissues.<sup>201</sup> This type of model would be better suited to address TAC versus CAR T cell questions regarding T cell persistence, toxicity, and anti-tumour efficacy. As human CD19 CAR T cells were shown to induce significant cytokine release syndrome and neurotoxicity symptoms in this mice model<sup>202</sup>, it would be interesting to model whether we can predict clinical outcomes for patients treated with human CD19 TAC T cells currently under development.

## 5.0 Concluding Remarks

The development of engineered T cells has led to an exciting form of live medicine that is capable responding and adapting to its environment. T cells are markedly different from other forms of cancer immunotherapy, as they can be engineered to differentiate between healthy and cancerous cells. Arming them with the ability to expressly targets tumour cells has shown incredible promise against hematological cancers, and greater efforts will be needed to find strategies that are effective against solid tumours.

In terms of CAR and TAC T cells, we have placed relatively simple circuits in the form of a recombinant receptor into a complicated cell-type, one which we still do not fully understand. In some ways, these *in vitro* stimulations through a chimeric receptor can be viewed as an input signal into a T cell, which results in an output signal in the form of cytokine production, proliferation, etc.. The primary purpose of our experiments was to better grasp how an input antigen can result in output effector function by a T cell. Many reviews in the field attempt to apply contemporary knowledge of T cell signaling and activation to CAR T cells; perhaps the more interesting perspective is to reverse the viewpoint and employ chimeric receptors as a tool to understand T cells. For example, TAC T cells can serve as a model for the restriction point that segregates some effector functions from proliferation, as our data indicates that the same antigenic signal can result in contrasting T cell responses. CAR T cells, on the other hand, are representative of the maximal response that a T cell can produce and could be used as a model for upper physiological limits of antigen-dependent responses. At the crossroads between translational research and medicine, chimeric receptors are becoming equally valuable as both research tools and clinical products. Using one to inform the other, and vice versa, will be necessary for improvement and development of better cancer therapeutics in the future.

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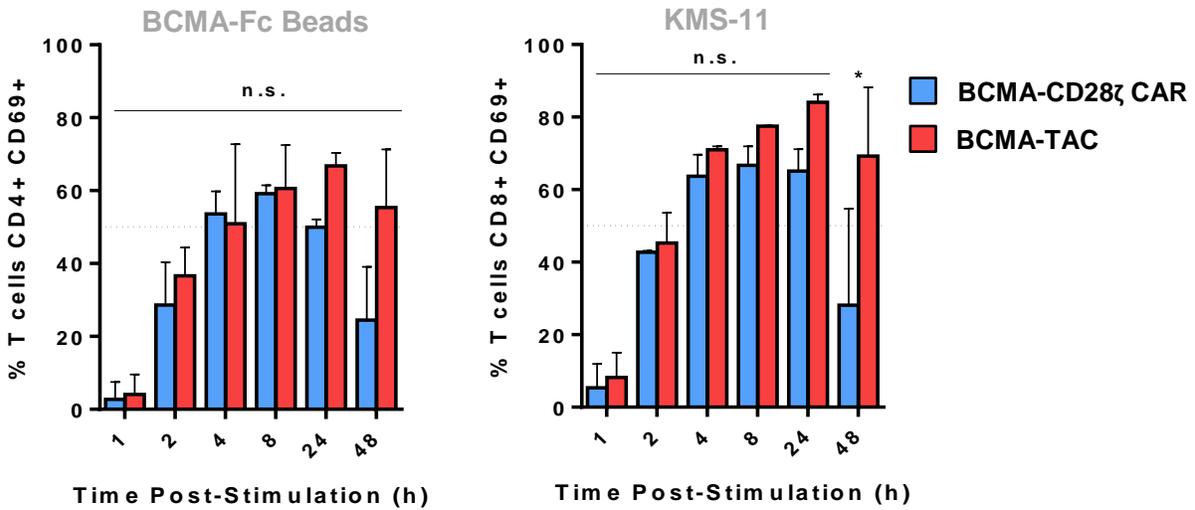
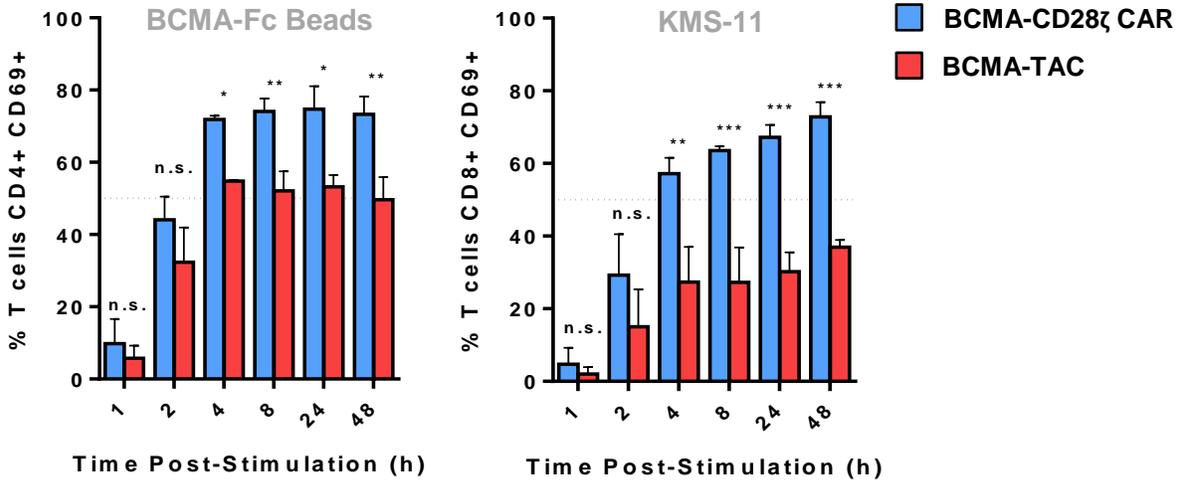
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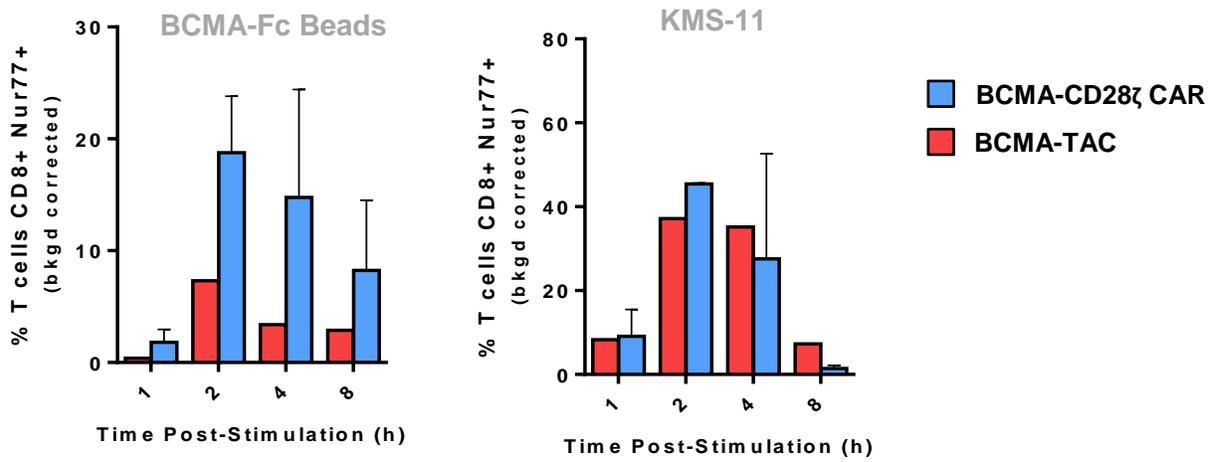
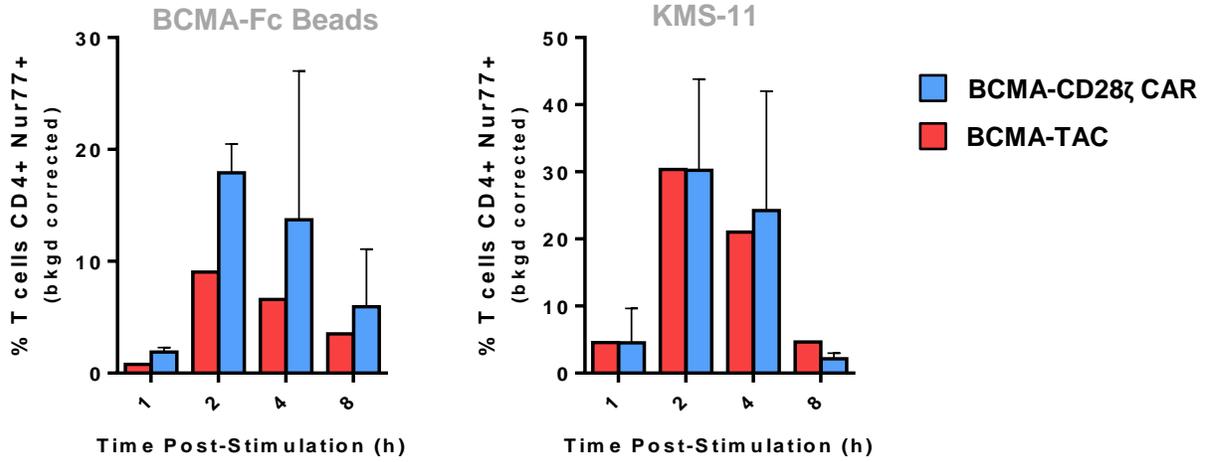
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7.0 **Appendix**

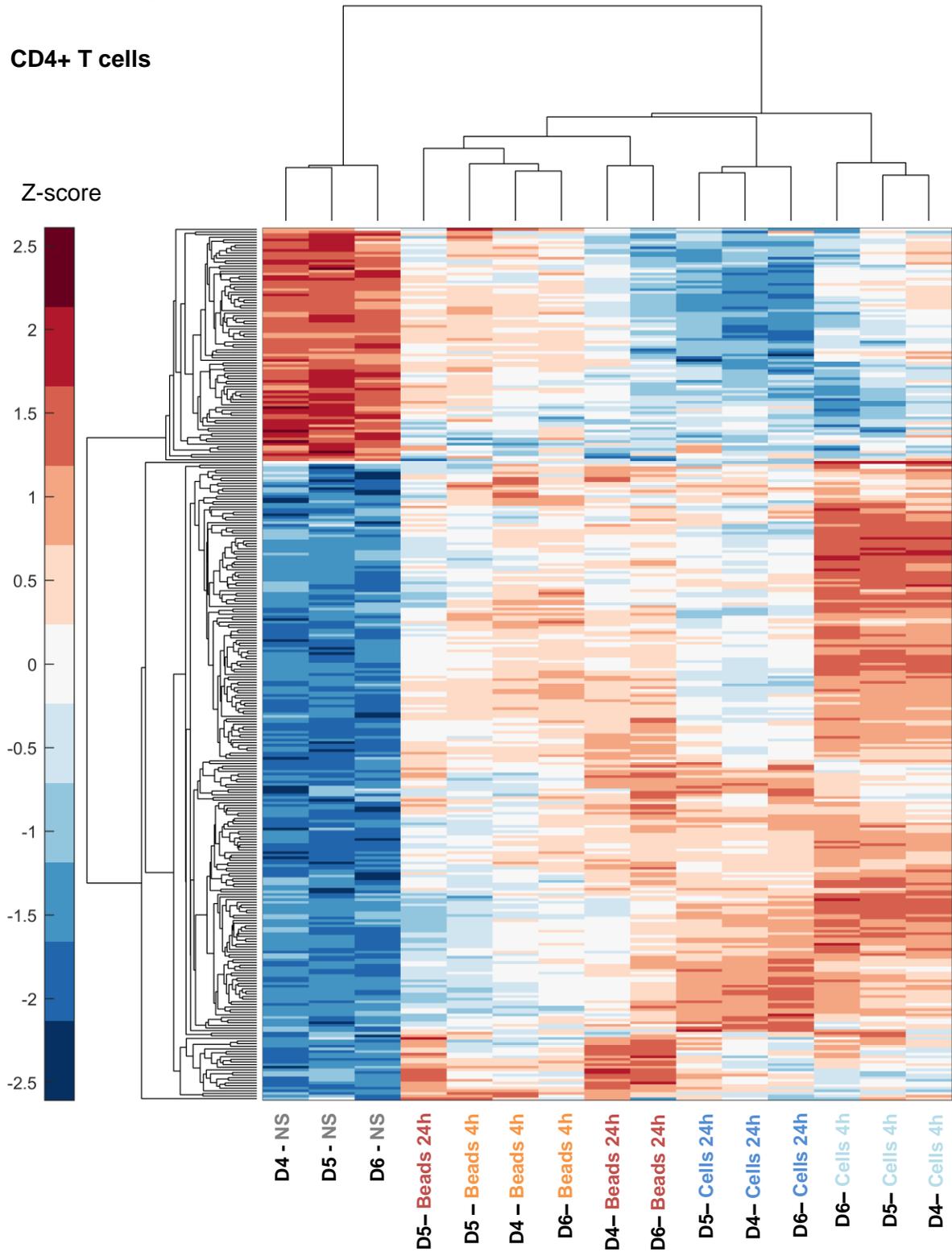
**Appendix I.** Extended graphs of CD69 and Nur77 expression by CAR and TAC T cells stimulated with BCMA-Fc-coated beads, or KMS-11 cells.

Refer to Figure 12 in main text.





**Appendix II.** Heatmap of transcriptome analysis of CD4+ TAC T cells stimulated with beads or cells. Refer to Figure 14 in main text.



**Appendix III.** Fold-change values from differential expression analysis of transcription factors in TAC T cells.

	CD4+ T cells; Beads 4hrs	CD4+ T cells; Beads 24hrs	CD4+ T cells, Cells 4h	CD4+ T cells, Cells 24h
<i>FOS</i>	99.85	29.1		
<i>FOSB</i>	27.09	14.68	419.47	11.58
<i>IRF4</i>	19.79	5.48	31.96	8.97
<i>TBX21</i>	11.28	4.81	35.4	4.61
<i>BATF</i>	7.75	4.55	19.31	13.95
<i>BCL6</i>	4.67	2.63	3.44	
<i>ZEB2</i>	3.42	4.48	31.56	3.94
<i>PRDM1</i>	3.14	1.98	8.47	5.5
<i>ID2</i>	2.17	2.22	4.99	2.19
<i>ID3</i>	2.14	-3.44	-4.18	-5.92
<i>RORC</i>	2.02		3.85	-5.29
<i>TCF7</i>	1.95			-6.42
<i>PBX3</i>	1.71	1.45		1.37
<i>NFIL3</i>	1.56	2.93	17.89	10.7
<i>BCL11B</i>	-1.41		-1.3	-2.55
<i>LEF1</i>			-2.49	-4.41
<i>JUN</i>		-1.6		-3.84
<i>FOXO1</i>				-1.65
<i>GATA3</i>			1.45	
<i>JUND</i>			1.67	
<i>RUNX3</i>			2.16	
<i>JUNB</i>			5.03	
<i>EOMES</i>		2.1		

	<b>CD8+ T cells; Beads 4hrs</b>	<b>CD4+ T cells; Beads 24hrs</b>	<b>CD8+ T cells; Cells 4hrs</b>	<b>CD4+ T cells; Cells 24 hrs</b>
<i>FOS</i>	12.36	14.71	387.25	3.59
<i>FOSB</i>	6.95	10.37	175.79	3.73
<i>IRF4</i>	9.74	3.28	32.3	4.38
<i>TBX21</i>	7.03	8.22	63.85	11.35
<i>BATF</i>	5.96	5.1	36.64	21.02
<i>BCL6</i>	2.79			-4.41
<i>ZEB2</i>	6.21	17.34	87.79	4.38
<i>PRDM1</i>	3.55	1.89	10.07	3.08
<i>ID2</i>	2.08	1.93	8.29	1.84
<i>ID3</i>	1.78	-2.27	-1.74	-3.96
<i>RORC</i>	4.06	4.09	6.48	
<i>TCF7</i>	2.14			-14.98
<i>PBX3</i>				
<i>NFIL3</i>		3.15	13.76	8.52
<i>BCL11B</i>			-3	-3.67
<i>LEF1</i>			-4.71	-6.08
<i>JUN</i>				-4.19
<i>FOXO1</i>				-1.87
<i>GATA3</i>			1.75	-1.45
<i>JUND</i>	1.5		1.85	1.64
<i>RUNX3</i>			2.15	
<i>JUNB</i>	2.1		5.62	
<i>EOMES</i>	2.89	5.42		