UNDERSTANDING T CELL DRIVEN TYPE II INTERFERON SIGNALLING IN SOLID TUMOURS

IFNγ IS REQUIRED FOR OPTIMAL THERAPEUTIC EFFICACY OF KIR-CAR ENGINEERED T CELLS

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
BCMA	B cell maturation antigen
B-ALL	B cell acute lymphoblastic leukemia
CAR	Chimeric antigen receptor
CAR T cell	Chimeric antigen receptor engineered T cell
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukemia
CRISPR	Clustered regularly interspaced short palindromic repeats
CRS	Cytokine release syndrome
crRNA	CRISPR RNA
CTV	CellTrace Violet
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DARPin	Designed ankyrin repeat protein
DNA	Deoxyribonucleic acid
DSB	Double stranded break
ELISA	Enzyme linked immunosorbent assay
FACS	Flow Cytometry
FBS	Fetal bovine serum
FDA	American Food and Drug Administration
GAF	Gamma activated factor
GAS	Gamma activated sequence
GBM	Glioblastoma Multiforme
GFP	Green fluorescent protein
gRV	Gamma-retrovirus
HIFI	High fidelity
HEK293	Human embryonic kidney 293 cells
HER-2	Human epidermal growth factor receptor 2
HSPC	Hematopoetic stem/progenitor cell
ICAM1	Intracellular adhesion molecule 1
ICS	Intracellular cytokine staining
IDO1	Indoleamine oxidase
IFN	Interferon
IFNG	Interferon Gamma (gene)
IFNγ	Interferon Gamma (protein)
IFNGR	Interferon Gamma Receptor (gene)
IFNγR	Interferon Gamma Receptor (protein)
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene

ISRE	Interferon-sensitive response element
ITAM	Immunoreceptor tyrosine based activation motif
IVIS	In-vivo imaging system
JAK	Janus kinase
KIR-CAR	Killer immunoglobulin-like receptor-chimeric antigen receptor
KIR-CAR T cell	Killer immunoglobulin-like receptor-CAR T cell
KO	Knockout
LAG-3	Lymphocyte activation gene 3
LOD	Limit of detection
LV	Lentivirus
MCA	Methylcholanthrene
MHC	Multi-histocompatibility complex
MFI	Mean fluorescence intensity
mRNA	Messenger RNA
MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa B
NGFR	Nerve growth factor receptor
NHEJ	Non homologous end joining
NHL	Non hodgkins lymphoma
NK Cell	Natural killer cell
NRG	NOD.Cg-Rag1tm1MomIl2rgtm1Wjl/SzJ
PAM	Protospacer adjacent motif
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PD-L2	Programmed death ligand 2
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative PCR
RAG	Recombination-activating gene
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RsLuc	Red shifted luciferase
RT-qPCR	Reverse transcription quantitative PCR
scFv	Single chain variable fragment
sgRNA	Single guide RNA
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TAC	T cell antigen coupler
TCR	T cell receptor
TIM-3	T-cell immunoglobulin and mucin domain-containing protein 3
TME	Tumour Microenvironment
TNFa	Tumour necrosis factor alpha

TRAC	T cell receptor Alpha Constant
tracrRNA	Trans-activating CRISPR RNA
Treg	Regulatory T cell
WT	Wild type

Declaration of Academic Achievement

This document was independently authored by Chris Silvestri, with editorial support from Dr. Jonathan Bramson. The studies described were designed, conducted, and analyzed by Chris Silvestri with guidance from Dr. Jonathan Bramson. Chris Silvestri was the primary researcher for all described experiments, with assistance from colleagues and collaborators as outlined below:

- All mouse work was performed by Chris Baker
- Allyson Moore generated all LV constructs used in this thesis.

1 Introduction

1.1 Cancer: Biological and Societal Burden

In their lifetimes, 1 in 2 Canadians will develop some form of cancer, and 1 in 4 will experience premature death as a result[1]. Globally, oncological research totalled over $253 billion in 2024, up nearly $200 billion from a decade prior[2]. Despite immense funding and the attention of hundreds of thousands of scientists and clinicians worldwide, cancer continues to be a leading cause of death in the global north[1].

The slow development of cancer treatments can be attributed to the highly variable nature of the disease. Tumours form as a result of accumulated mutations within a cell that allow it to circumvent processes naturally in place to regulate cell development and proliferation [3–5]. Genomic alterations eventually lead to the generation of cells able to grow and proliferate without regulation, and their aggregation eventually leads to tumour formation. Tumours grow independently from the surrounding tissue, containing their own internal environment known as the tumour microenvironment (TME)[5]. The rapid growth of neoplastic cells deprives the surrounding healthy tissue of oxygen and nutrients, leading to loss of function and eventual death of the affected tissue.

The causes of mutagenesis leading to cancer formation vary significantly from individual to individual, and from cancer to cancer, contributing to the difficulty of treatment [6–9]. Certain individuals may be pre-disposed to malignant cell formation due to heritable genetic factors, while in others, cancer may arise due to lifestyle choices or purely stochastic cellular mutations[6-9]. The result is a disease with an etiology unique to each cancer type and patient[7,8]. Even within patients of the same cancer type, subgroups exist with unique mutations and properties where some tumour subgroups respond to treatment while others do not.[9]

This raises the question of what is the most effective way of treating a disease characterized by profound inter- and intratumoural heterogeneity? Cancerous cells must be completely eradicated in order to ensure the cancer does not relapse after treatment is complete. However, as cancer cells are derived from healthy cells, conventional treatments have limited selectivity for cancer cells over healthy cells. With all current treatment regimes, healthy cells are inevitably affected, resulting in serious adverse effects for the patients[10,11].

1.2 Classical T cell biology and function

Immune responses in mammals are roughly divided into two classes: innate, and adaptive. It is evident that interplay between both classes of immunity is required for effective anti-tumour response. During an anti-tumour response, innate cells, such as macrophages and NK cells, work to create an environment inconducive to tumour growth, while signalling to adaptive cells, such as T and B lymphocytes, to infiltrate the TME[12]. Carrying out a diverse range of functions, T cells are an essential component of an anti-tumour immune response. Indeed, a wealth of research implicates T cell presence and function in the TME as an important prognostic factor for tumour clearance and long-term

survival, and as such T cells have been a focus in the development of anti-cancer therapies[12–15].

While lymphocyte precursors are generated from hematopoietic stem cells in the bone marrow, the bulk of T cell development occurs after their egress and subsequent arrival at the thymus. It is in the thymus that naïve T cells undergo VDJ recombination to generate a T cell receptor (TCR). The TCR is encoded in the genome as multiple segments, termed variable (V), diversity (D), and joining (J). During VDJ recombination, these gene segments are stochastically recombined by the RAG1/RAG2 enzymes to create unique T cell receptors (TCRs). This process generates extraordinary receptor diversity (with ~10^{15} possible combinations), enabling recognition of countless antigens. Conventional T cells bear an αβ TCR -composed of a TCRα chain formed by VJ recombination and TCRβ chain formed by VDJ recombination- which necessarily forms a complex with the multi-chain CD3 co-receptor. Containing multiple immunoreceptor tyrosine-based activation motifs (ITAMS) - conserved sequences of amino acids integral for signal transduction through the CD3-TCR complex in T cells - CD3 complexing with the TCR is required for T cell activation[16]. Ultimately, each αβT cell generates a TCR with unique specificity, creating a library of T cells effective at defending against a vast array of antigens. A secondary, rare population of T cells exists with TCRs composed of γ and δ chains (formed through separate VDJ recombination events), but their biology and function are not well understood.

During their development in the thymus, T cells undergo a process known as thymic selection, consisting of two phases: positive and negative selection. Positive selection occurs first, during which T cells are presented MHC bound with peptide antigens derived from host cells (often referred to as "self" antigen)[16]. The population of T cells that react moderately to these self-antigens receive survival signals to continue maturation, resulting in a selected population with functional TCRs with appropriate MHC restriction. During this time, T cells also commit to either CD4 or CD8 lineage, depending on whether they interact with MHC II or I respectively[16]. Subsequently during negative selection, these cells are again presented self MHC-peptide complexes. Cells that are strongly self reactive undergo activation induced cell death, those that are modestly self-reactive become regulatory T cells, while non-self-reactive cells continue to develop and ultimately enter circulation where they will survey tissues for their MHC/peptide target[16].

Conventional T cells are distinguished based on the expression of surface proteins CD4 and CD8. CD4+ T cells, generally termed 'helper' T cells, participate broadly in induction of immune response via release of cytokines like tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ), activating and recruiting other immune cells to the site of infection. Within the CD4+ population, further phenotypic diversification occurs in response to microenvironmental cues, generating specialized subsets that coordinate anti-tumour immunity synergistically with CD8+ T cells[16]. In contrast, CD8+ cytotoxic T lymphocytes (CTLs) directly eliminate target cells, which include virus-infected cells, tumour cells, or other stressed cells presenting aberrant peptides via MHC I. CTLs

mediate killing through two main mechanisms. These include receptor/ligand interactions (e.g., Fas-FasL) that trigger apoptotic pathways, and the exocytosis of cytotoxic granules containing perforin and granzyme, which disrupt target cell membranes and induce programmed cell death via cleavage of specific intracellular proteins respectively[16]. This targeted destruction ensures the removal of compromised cells while minimizing collateral damage to healthy tissue. The synergistic action of these T cell compartments, integrating helper functions with direct killing capacity, is essential for effective anti-tumour responses [17]. Beyond this, all T cells exhibit various phenotypes and functional states. The primary T cell subsets include effector T cells, which exist in a highly active and short-lived state specialized for rapid production of cytokines and cytolytic mediators to mount immediate immune responses upon antigen recognition, and memory T cells, which exist in a poised, long-lived state with a notable capacity for self-renewal, making them the cornerstone of long-term protection. The relative prevalence of these phenotypes in T cell centric immunotherapies has been shown to drastically alter their efficacy. For example, cell products dominated by terminally differentiated effectors may at first glance appear effective due to their rapid and potent cytotoxic capacity, however their lack of ability to persist and expand *in-vivo* ultimately leads to poor long term tumour control. Instead, increased proportion of memory cells has frequently been associated with better prognosis as a result of their capacity to persist and self renew, correlating with clinical regression and relapse prevention[18].

The two-signal hypothesis provides a fundamental framework for understanding T cell activation, explaining how the immune system balances sensitivity to foreign or aberrant antigens with tolerance to self. This model arose to address a critical biological paradox: how T cells achieve robust responses to pathogens and tumours while avoiding harmful reactivity against healthy tissues. At its core, the hypothesis proposes that naive T cells require two distinct signals to become fully activated. The first signal is delivered through TCR-CD3 complex engagement with peptide-MHC, which ensures antigen specificity. However, this recognition alone is insufficient to trigger activation, serving as a safeguard against inappropriate responses to innocuous antigens. The second signal comes from costimulatory receptors like CD28 interacting with their ligands (CD80/CD86 or 4-1BBL) on antigen-presenting cells. These costimulatory signals only occur when antigen-presenting cells have been activated by danger signals, typically in the context of infection or tissue damage. This dual requirement creates an effective biological checkpoint - T cells only mount productive responses when they encounter their cognate antigen in a dangerous context. When both signals are present, T cells undergo clonal expansion and differentiation into effector cells. In contrast, TCR engagement without costimulation leads to anergy or apoptosis[17]. The logic of this system explains why T cells remain unresponsive to self-antigens under normal conditions while being capable of vigorous responses during infection or malignant transformation. In the tumour microenvironment, this paradigm has particular relevance, as cancer cells often present antigens but frequently fail to provide proper costimulation, rendering tumour-specific T cells anergic and contributing to immune evasion.

1.3 Cancer and the Immune System

Cell growth and development are tightly regulated. Cell intrinsic mechanisms such as cyclin-dependent cell cycle checkpoint regulation offer an initial line of protection against the generation of neoplastic cells[19]. However, these mechanisms are imperfect and malignant cells inevitably escape and generate tumours[19,20]. At this point, the responsibility of tumour control and survival falls to the subject's immune system. Prior to the 21st century, cancer was widely considered a cell-intrinsic condition, whereby malignancies arose exclusively as a result of the failure of regulatory pathways within the malignant cells[21,22]. Despite this theory, evidence to suggest immune involvement in cancer progression can be found as early as the 19th century, when William Coley observed tumour regression in cancer patients who acquired severe bacterial infections. Indeed, his subsequent experiments whereby intentional infection of cancer patients led to increased frequency of regression, are often credited as the first examples of modern cancer immunotherapy[23]. Despite these observations, little progress was made over the first half of the 20th century, leading to a prevailing sentiment "that immune mechanisms probably will be of little use in the control of this disease"[24]. Concrete evidence of a direct link between the immune system and cancer progression would not be elucidated until 1957, when Prehn and Main would describe tumour-specific immune responses against methylcholanthrene (MCA)-induced sarcomas in an effort to confirm and expand upon Coley's findings. Indeed, these MCA induced tumours generated specific immune responses that did not cross-react with spontaneous autologous tumours, indicating that tumours may possess distinct antigenic molecules [24]. The manuscript closes with the passage "These results suggest that immunization against some types of carcinogenesis, using tumour tissue as antigen, may be feasible", almost serving as a hint for what would eventually evolve into the modern concept of cancer immunotherapy[24]. Crucially, development of genetic engineering technologies to produce murine knockout models paved the way for what is now known as the concept of *immunosurveillance*. By generating *in-vivo* models deficient in specific immune compartments, most notably $RAG^{-/-}$ mice unable to generate in T, B and NKT cells, alongside more targeted interruptions of important immune cytokine signalling pathways like $IFNGR^{-/-}$ and $STAT1^{-/-}$, researchers were able to confirm robust interplay between the immune system and tumour formation. Indeed, across multiple *in-vivo* knockout models, including $RAG^{-/-}$, $Perforin^{-/-}$, and $IFNGR^{-/-}$, cancer immunosurveillance was impaired, resulting in both increased spontaneous and carcinogen-induced tumour development, confirming an essential role for the immune system in preventing malignancies [25–28].

Pioneered by Schreiber et al., the current accepted model of cancer development is based on the "three Es", collectively describing the process of immunoediting: Elimination, Equilibrium, and Escape, each representing one of three possible outcomes[29]. Elimination denotes a theoretical process where cancerous cells emerge on a regular basis, but they are completely eliminated by the immune system, preventing *bona fide* tumour formation. Should the elimination phase fail to purge all malignant cells, tumour development shifts to equilibrium: a steady state between elimination and outgrowth. During this period, which may continue for years, the tumour is subjected to

constant, heavy immune pressure, keeping expansion and elimination of malignant cells balanced. Ironically, this sustained immune pressure drives immunoediting, wherein a subset of malignant cells acquires changes that enable immune evasion. These adaptations include upregulation of inhibitory ligands like PD-L1 and CD80/CD86 that dampen T cell activity, secretion of immunosuppressive molecules such as IDO1, and loss of immunodominant antigens that would otherwise mark tumour cells for destruction. Collectively, these mechanisms allow poorly immunogenic clones to escape immune surveillance, ultimately leading to progressive tumour outgrowth - even when the immune system initially responds effectively[29,30]. Furthermore, as they arise from "self", most tumour cells lack distinct surface features to differentiate them from healthy ones. As a consequence of thymic selection, T cells that naturally target neoplastic cells are uncommon, and thus mounting a successful endogenous anti-tumour immune response is difficult, especially in well established tumours.

However, recent advances in the generation of engineered T cells aim to overcome these barriers. In contrast to traditional techniques like radiation and chemotherapy that target fundamental biological processes in neoplastic cells (such as DNA replication and cell division), cancer immunotherapy leverages components of the endogenous immune system to selectively recognize tumour tumour cells based on expression of specific molecular markers, like tumour associated antigens [31,32].

1.3 Adoptive Cell Therapies using Engineered T cells

Given their natural ability to mediate destruction of malignant cells, T cells evidently possess the capabilities to be potent anti-tumour agents[33]. However, as a consequence of thymic selection, naturally occurring tumour specific T cells are rare and often incapable of mounting durable anti-tumuor responses. Adoptive cell transfer (ACT) involving engineered T cells aim to overcome this barrier. Adoptive transfer in the context of T cell therapies is simple in principle: T cells are removed from patients, expanded to large numbers ex-vivo, and then re-introduced to the patient to mediate tumour destruction[34]. While this approach effectively multiplies the endogenous tumour-reactive T cell population, the scarcity of naturally occurring tumour-targeted T cells, tumour immunoediting, and the vast intratumoural heterogeneity of antigen expression limits their efficacy. Engineering T cells to express synthetic receptors with specificities for antigens differentially expressed by tumour cells circumvents this limitation by enabling the generation of large quantities of T cells with customizable specificities and resistances, thereby enhancing potency and breadth of the anti-tumour response.

Chimeric antigen receptors (CARs) combine the ability of the TCR-CD3 complex to activate T cells with the MHC independent, highly specific antigen recognition of immunoglobulins[35–37]. Structurally, CARs consist of an extracellular antigen recognition domain and at least one intracellular domain responsible for T cell activation. The antigen binding portion of the CAR utilizes a single chain antibody (or single chain variable fragment; scFv) comprised of the heavy and light chains of an antibody linked via a peptide spacer, allowing its target to be highly customizable, while simultaneously nullifying the requirement of MHC restriction[38,39]. Some scFvs have also been generated

to target antigens that cannot be presented by MHC, like glycolipids or carbohydrate chains, further expanding the library of potential targets[40,41]. First generation CARs utilized a single CD3ζ domain linked via a hinge region to the extracellular scFv[42]. Lack of sufficient IL-2 production – which has been shown to be critical for maximal T cell expansion - by T cells upon CAR engagement prompted addition of one or more co-stimulatory intracellular domains like 4-1BB or CD28, to second and third generation CAR design, enhancing potency, persistence and function. No one costimulatory domain has been selected as most optimal, with both CD28 and 4-1BB demonstrating unique strengths and weaknesses, and both continue to be used in pre-clinical and clinical CAR T cell products[43–45].

The evolution of CAR-engineered T cells (CAR-T cells) over the past decades has led to impressive clinical efficacy[46]. CD19, a B-cell specific surface antigen, is an effective CAR T cell target due to its restriction to the B cell lineage and the fact that patients can tolerate B cell aplasia with appropriate supportive care. After unprecedented success in clinical trials, four CD19 directed CAR-T cell therapies have been approved for clinical use against various B-cell malignancies by the FDA, including B-cell acute lymphoblastic leukemia (B-ALL), non-Hodgkin's lymphoma (NHL), and chronic lymphocytic leukemia (CLL)[47–49]. Recently, clinically approved offerings have expanded to include two B cell maturation antigen (BCMA) targeted products, indicated for use in the treatment of multiple myeloma[50,51]. However, despite efficacy in liquid tumour models, CAR T cell therapies have displayed limited clinical activity against solid tumours[52].

While highly efficacious against hematological malignancies, CAR-T cell therapies are also associated with significant toxicities. Perhaps the most immediate toxicity is cytokine release syndrome (CRS), a systemic storm of inflammatory cytokines that can escalate to severe organ dysfunction. In some patients, immune effector cell-associated neurotoxicity syndrome (ICANS) is observed, causing a wide range of neurological pathologies, likely as a consequence of systemic inflammation caused by CAR T-cell treatment[53,54]. Hematological toxicities like cytopenias are also common, leading to increased susceptibility to infection among other risks. While neutralizing antibodies, like the IL-6 receptor antagonist, tocilizumab, can be used as treatment for cytokine induced toxicities, it would be preferable to avoid these toxicities altogether.

The synthetic nature of the CAR and has been linked to basal, or tonic, signalling and overactivation of the engineered T cells, ultimately rendering the engineered T cell dysfunctional[55]. To address this concern, several groups have developed strategies that redirect the endogenous TCR, including the TAC receptor developed by the Bramson lab. In general, T cells engineered with TCR-directed receptors display reduced tonic signaling and improved therapeutic outcomes relative to T cells engineered with CARs[56–58].

An alternate synthetic antigen receptor, known as the KIR-CAR, takes inspiration from the concepts of traditional CAR design, but contains unique structural and signalling components[59]. Employing the backbone of the KIR2DS2 receptor, a killer cell

immunoglobulin-like receptor found primarily on NK cells, the KIR-CAR is directed by an antigen specific scFv in the same manner as a traditional CAR. However, instead of associating with CD3 for signal transduction, the KIR-CAR signals through DAP-12, a transmembrane signal transducing protein containing a single ITAM. In contrast, traditional CARs using the CD3ζ domain possess three ITAMs. Multiple groups have shown that the number of ITAMs present in a CAR is directly tied to the strength of activation, with 3 ITAM CARs often exhibiting high degrees of tonic signalling and exhaustion[60,61]. It has also been shown that CARs bearing only one functional ITAM exhibited enhanced persistence and equitable anti-tumour efficacy in comparison to three ITAM CARs in pre-clinical work.

Both the TAC receptor and the KIR-CAR have proven to be highly efficacious. *In vivo,* in comparison to second generation CAR T cells directed against same target, TAC T cells exhibited better tumour penetration, improved tumour clearance, and reduced toxicity i[56]. *In vivo,* KIR2DS2 KIR-CARs directed against the tumour associated antigen mesothelin were more effective than their CD3ζ CAR counterparts[62].Within the Bramson Lab, KIR-CARs directed towards CD133, IL13Rα2, and HER2 have been efficacious in recognizing and eliminating tumour cells bearing their target antigen. Both *in vitro* and *in vivo,* the KIR-CARs display high levels of cytotoxicity, including comparable performance to their TAC counterparts.

1.4 IFNγ: Canonical Signalling

One common measure of T cell functionality is the production of pro-inflammatory cytokines upon antigen stimulation In general, immune cell mediated tumour clearance relies on the release of a wide array of cytokines, particularly IFNγ, TNF-α, and IL-2. IFNγ directly inhibits tumour proliferation while upregulating MHC class I expression, TNF-α induces cytotoxic apoptosis via death receptor pathways, and IL-2 drives T cell expansion and NK cell activation. Indeed, high intratumoural expression of cytokines like IL-2, TNFα and IFNγ correlate with increased tumour immune infiltration and thus are often linked to better patient outcomes[63–66]. Alterations in cytokine signaling pathways within the tumour microenvironment are frequently associated with resistance to immunotherapies and poorer clinical outcomes. However, the directional relationship between these phenomena remains complex, as dysregulated cytokine networks may be both a cause and consequence of immune evasion mechanisms. Characterizing cytokine expression patterns within tumours has proven valuable for predicting treatment responses and patient outcomes. Further, therapeutic approaches that modulate cytokine signaling continue to be an active area of research with promising clinical implications for cancer treatment.[67–70].

Among the numerous pro-inflammatory cytokines expressed during anti-tumour response, IFNγ is of particular interest in the context of the TME. Interferons (IFNs), categorized into types I, II and III, are highly effective immune regulators with a diverse range of functions. As a family, IFNs exhibit a number of similar immunomodulatory effects, primarily during anti-viral responses. However, IFNγ, the sole type II interferon, is of particular interest as an immunotherapeutic target due to its direct impact on T cell

function and potency[71]. Structurally defined as a dimeric glycoprotein, IFNγ is a pleiotropic cytokine produced primarily by activated T cells, and NK cells [72]. Its production is regulated by the secretion of cytokines such as IL-12 and IL-18 from APCs, linking the innate and adaptive immune responses [73]. As a pro-inflammatory cytokine that is believed to be important for anti-tumour responses, the secretion of IFNγ by tumour-specific T cells is often used as a proxy marker for functionality in the context of adoptive therapies.

Biologically active IFNγ exists as an anti-parallel dimer and acts on the IFNγ Receptor (IFNGR), which is a heterodimer composed the IFNGR1 and IFNGR2 subunits and expressed ubiquitously across human tissues[74,75]. Though the IFNGR1 chain is the major binding subunit, the IFNGR2 chain plays an important role in stabilizing the receptor complex, and is required for signal transduction[76]. While constitutively expressed, the level of expression of the IFNGR2 chain is more tightly regulated than IFNGR1, and thus frequently acts to limit IFNγ sensitivity in T cell populations[77,78]. Neither chain has intrinsic kinase/phosphatase function, however the IFNGR1 chain contains binding motifs for Janus-activated Kinase 1 (JAK1) and signal transducer and activator of transcription (STAT1), while IFNGR2 contains a motif for JAK2, allowing the receptor to utilize the JAK-STAT pathway for signalling[76,79].

Functionally, IFNγ binds to the IFNγR1 subunit, complexing two IFNγR1 chains to the ligand and recruiting two IFN R2 chains to form the oligomeric complex. The formation of this complex brings the nonreceptor tyrosine kinases found at the ends of the carboxy termini, JAK1 and JAK2, into proximity with each other, which subsequently phosphorylate the receptor. This phosphorylation event creates a binding site for STAT1, where it is subsequently phosphorylated. Phosphorylated STAT1 forms a homodimer, known as gamma activated factor (GAF), and translocates to the nucleus, where it modulates gene expression at sites known as gamma activated sequences (GASes). The signalling cascade is negatively regulated by SHP phosphatases and suppressor of cytokine signalling (SOCS) family proteins[71,80,81].

IFNγ contributes to both innate and adaptive immune responses. Originally named macrophage activating factor, IFNγ is important in promoting the anti-tumour M1 phenotype in macrophages associated with greater direct tumouricidal capacity through increased phagocytic capabilities [82]. It also promotes induction of cytotoxic T cell responses via increasing production of pro-inflammatory cytokines, including TNFa and IL-12, alongside upregulation of costimulatory molecules CD80 and CD86[83,84]. Furthermore, aids in recruiting NK cells to the TME via cooperation with chemokines such as CXCL9,10 and 11[85,86].

Distinct from its immunostimulatory effects, IFNγ also potentiates numerous anti-tumour functions upon direct interaction with IFNγR on the tumour cell surface. Perhaps the most important contribution to the anti-tumour response is its ability to upregulate expression of MHC I molecules[87]. Heightened expression of MHC I and antigen presentation machinery by IFNγ on both APCs and tumour cells leads to more effective activation of endogenous CD8+ CTLs in the TME. Evidence of this function date as far

back as 1988, when an increase in MHC I expression was observed in multiple cell lines after treatment of with IFNγ. A second report in 1994 confirmed this observation, where it was shown fibrosarcoma cell lines with defective IFNγR signalling grew better and were harder to eliminate in mice, on account of decreased MHC I expression[26].

IFNγ also mediates suppression of tumour cell proliferation and promotes apoptosis in tumour cells. The cyclin-dependent kinase inhibitor, p21, is an important regulator of the cell cycle and implicated in the early arrest of tumour growth. Tumour cell lines deficient in IFNγ signalling showed no reduction in growth when incubated with the IFNγ. However, lines sensitive to IFNγ signalling showed increased p21 mRNA and a reduction in growth, indicating IFNγ to be an important regulator of early tumour progression [88]. IFNγ mediated STAT1 activation is also implicated in upregulation of caspases 1,3, and 8, sensitizing tumour cells to apoptosis[89]. These anti-proliferative effects have been verified thoroughly in melanoma cell lines, supporting the growth inhibitory functions of the IFNγ-STAT1 signalling axis[71,72,80,90–92].

Beyond its direct anti-tumour effects, IFNγ signaling appears to be a fundamental component of the biological mechanisms underlying certain cancer immunotherapies. Cancer immunotherapies that block immune checkpoint receptors and ligands, such as PD-1, PD-L1, and CTLA-4, have shown promising results in patients with late stage and hard to treat cancers[66,93].In all cases, patients with higher intratumoural expression of IFNγ regulated genes responded better than those without [94]. It has also been reported that impairment of the IFNγ signalling pathway through disruption of the IFNGR1 gene renders tumour cells resistant of CAR T cell therapy[94,95]. Although the mechanism of resistance remains to be full elucidated, it has been suggested that IFNγ induced ICAM1 expression plays a critical role in CAR mediated tumour clearance[93,96]. Tumours with impaired IFNGR signalling failed to upregulate ICAM1, and were resistant to T cell killing and sensitivity to killing could be restored via forced overexpression of ICAM1. Furthermore, IFNγ sensitive tumours lacking ICAM1 also resisted T cell attack, indicating the IFNγ-ICAM axis to be essential to CAR T cell function. Interestingly, this dependency on IFNγ signalling was observed only in solid tumour models, and did not hold true in liquid tumour models, highlighting the complex systems at play in the TME[96].

1.6 IFNγ promotes adaptive resistance in cancer cells

Mounting evidence indicates that IFNγ acts as a double-edged sword, promoting both tumour clearance and immune evasion. Prior work from our lab demonstrated that IFNγ induces rapid adaptation of tumours, blunting the efficacy of vaccination [97]. Treatment of mice bearing B16F10 melanoma with an adenovirus vaccine expressing dopachrome tautomerase resulted in modest response by CD8+ tumour infiltrating lymphocytes until day 5 that rapidly decreased by day 10. Transcriptional analysis of tumours revealed many immunosuppressive genes that were rapidly expressed following vaccination, including checkpoint receptors LAG-3, TIM-3 and PD-1, among others. IFNγ was required for expression of a significant portion of these genes, and blockade of IFNγ signalling resulted in abrogation of immunosuppression, suggesting its presence in

the TME was responsible for the loss of function of TILs. Anti-tumour efficacy of the vaccine was abolished when IFNγ was depleting revealing that despite its role in driving adaptive resistance in the tumour, IFNγ remained critical for therapeutic efficacy. Findings from multiple groups corroborate IFNγ mediated induction of PD-L1 expression[98,99].

Immunoediting, primarily the escape phase, has also been linked to IFNγ exposure In the CT26 colon cancer carcinoma model, downregulation of immunodominant tumour antigen gp70 upon immune attack was attributed directly to IFNγ secretion by CD8+ TILs[90]. In melanoma models, expression of checkpoint receptor CTLA4 has been correlated with levels of IFNγ expression, and loss of IFNγR signalling in tumour cells has been shown to promote anti-CTLA-4 treatment resistance[100]. A comprehensive study from Takeda *et al.* in 2017 suggested IFNγ produced by CD8+ CTLs was essential to genetic instability in multiple tumour models[101]. Increased copy number alterations were seen as a direct response to IFNγ signalling in mammary carcinoma, lymphoma, and fibrosarcoma cell lines, as well as loss of tumour antigen expression in both the mammary carcinoma and lymphoma lines[101]. Other mechanisms of adaptive resistance facilitated by IFNγ include induction of indoleamine 2,3-dioxygenase (IDO), a rate limiting enzyme required for tryptophan metabolism. IDO is directly involved in the suppression of NK and T cells, is upregulated by IFNγ in lung cancer, prostate cancer, and myeloid leukemia models, and promotes the accumulation of regulatory T cells (Tregs)[72,102–104.] Accumulation of immunosuppressive Tregs in the TME has also been suggested to occur due to $CD8^+$ CTL infiltration and IFNγ signalling[102].

1.7 IFNγR signalling plays a multifaceted role in T cell function

While the relationship between IFNγ and tumour cells has been intensely researched, the effects of IFNγ on the T cells from whence it is derived is less understood. T cells express IFNγR allowing them to sense and react to IFNγ in an autocrine/paracrine fashion[78]. Similar to how it executes two seemingly contradictory roles in tumour cells (both pro/anti tumour), IFNγR signalling in T cells contributes to both their expansion, and contraction.

Initial IFNγ exposure triggers activation programmes in both CD4+ and CD8+ CTLs, driven by expression of numerous IFNγ regulated genes. In CD4+ T cells, this signalling is required for polarization to the Th1 effector phenotype. In CD8+cells, IFNγ exerts particularly potent effects, simultaneously enhancing cytotoxicity via upregulation of perforin and granzyme, promoting cellular motility to allow rapid migration to the site of infection[105.]. Containing a GAS in its promoter sequence, master transcription factor interferon regulatory factor 1 (IRF1) is preferentially activated in response to IFNγ signalling via pSTAT1 homodimers. Early in the immune response, IRF1 is responsible for initiating transcription of genes associated with proliferation, cytokine production, and cytotoxicity[106–108]. Soon after activation, T cells also rapidly upregulate the suppressor of cytokine signaling 1 (SOCS1), leading to downregulation of both IFNγR subunits, rendering the T cells hyposensitive to IFNγ. This may allow T cells to avoid the regulatory effects of IFNγ, namely activation induced cell death, during the T cells'

expansion phase[109]. Over time, the IFNγR subunits are re-expressed. Prolonged exposure to IFNγ ultimately leads to activation induced cell death, resulting in the contraction of the effector T cell population and resolution of the immediate immune response[109–111]. This autocrine/paracrine regulation of T cell expansion and contraction is essential for controlling the kinetics of T cell responses within the setting of infection and the development of memory. In the context of anti-tumour therapy, where T cells within the tumour will be activated for days/weeks leading to chronic IFNγ production, the negative regulatory effects of IFNγ on T cells may hinder anti-tumour efficacy. IFNGR1 knockout in mature CD8+ OT1 T cells (a widely used transgenic, ovalbumin specific T cell line) greatly enhanced survival, proliferation, and reduced exhaustion in a variant of the B16 mouse melanoma model that expresses ovalbumin[112]. Modulation of specific downstream ISGs has also shown to significantly impact T cell function. The transcription factor interferon response factor 2 (IRF2) is associated with exhaustion programming in T cells, and is potently induced by IFN signalling. When knocked out in CD8+ P14 transgenic T cells, adoptively transferred IRF2KO T cells exhibited resistance to exhaustion and sustained functionality in the TME[113]. This supports the hypothesis that while IFNγ is beneficial in the early stages of an anti-tumour response, prolonged signalling through the IFNγR pathway leads to exhaustion and contraction of the T cell population.

Taken altogether, IFNγ acts directly on T cells in a modulatory fashion, controlling both their expansion and contraction. While this regulatory feedback mechanism is necessary during a typical immune response to prevent continued activation beyond the resolution of infection, it seems to act detrimentally during an anti-tumour response, leading to exhaustion and contraction of anti-tumour T cells prior to clearance of the tumour. Evidently, it may be possible through gene editing methods to render desired T cell populations resistant to IFNγ induced dysfunction, though this has yet to be explored in depth in human T cells. It is becoming increasingly evident that IFNγ plays a multifactorial role in anti-tumour responses, both anti and pro tumourigenic in nature. It is thus more important than ever to understand where IFNγ fits into the field of cancer immunotherapy. In doing so, we can learn how best to modulate its function, and harness it in the development of novel therapeutics.

1.8 CRISPR Gene editing to enhance engineered T cells

The ability to efficiently, accurately, and safely manipulate the human genome opens up a plethora of possibilities for cancer treatment. Technologies that enable genome editing have undergone remarkable evolution over the past three decades. Starting with zinc finger nucleases in the late 90s, followed by transcription activator like effector nucleases (TALENs) and then the CRISPR Cas9 system in recent years, the repertoire of gene editing technologies continues to expand[114]. Of the available technologies, the CRISPR-Cas9 system offers the ability to easily target and induce double stranded breaks at specific loci.

The acronyms CRISPR and Cas9 are derived from clustered-regularly-interspaced-palindromic-repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), respectively. The CRISPR/Cas9 system was discovered initially as an adaptive immune

system in bacteria. By targeting Cas proteins capable of cleaving double stranded DNA to a bacteriophage target sequence via a complementary guide sequence stored in the CRISPR locus of the bacterial genome after a previous bacteriophage challenge, bacteria are able to efficiently defend themselves from viral infection. This system has been adopted for use in human cells, requiring only the design of a guide RNA (gRNA) sequence to target the Cas9 protein. Guide sequences are RNA complexes consisting of two parts: a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). The crRNA contains a sequence 20bp long that is complementary to the region of DNA targeted for cutting, and responsible for targeting the Cas9 to the correct sequence. The tracrRNA is responsible for complexing with the Cas9 protein to form the ribonucleoprotein (RNP) complex that will facilitate the double stranded break . Guides must be located within close proximity to a protospacer adjacent motif (PAM) sequence, which is recognized by the Cas9 protein to induce a double stranded break. Upon recognition of the PAM site, the Cas9 enzyme will induce a double stranded break three bp upstream of the PAM[114]. The PAM sequence for *streptococcus* pyogenes Cas9, the most commonly used variant, is 5'-NGG-3'; other members of the Cas family employ other PAMs.

Cell machinery repairs double stranded breaks in two ways. Non-Homologous End Joining is the cells predominant repair mechanism, executed through addition or removal of bases surrounding the double strand break[115]. As addition or deletion of a base will shift the reading frame and disrupt proper protein synthesis, targeting Cas9 to induce DNA repair via non-homolgous end joining in coding regions of target genes has emerged as a highly effective strategy for gene knockout.

Integrating CRISPR/Cas systems into cell engineering has led to marked developments in next generation therapeutics. Perhaps its most impressive application yet is in the treatment of sickle cell disease, where Casgevy™ became the first FDA approved CRISPR therapy. Briefly, patients with defective adult hemoglobin (HbS) have HSCs extracted from their blood or bone marrow, which are then CRISPR edited *ex-vivo* at the BCL11A locus to re-activate fetal hemoglobin (HbF) production as compensation for the lack of HbS. Existing diseased HSCs in the patient's bone marrow are cleared via chemotherapy prior to reintroduction of the CRISPR edited HSCs, which are reintroduced to the patient, engrafting to fill the bone marrow niche with HbF producing cells. While no commercial engineered T cell product incorporates CRISPR technology yet, its use is already widespread among the research community, with numerous groups designing products with enhanced abilities to persist in the host, resist exhaustion, or minimize the risks of graft vs host disease (GvHD)[113,116,117]. As the field of cancer immunotherapy evolves, new uses for CRISPR systems continue to emerge. The capacity to efficiently edit multiple genes simultaneously, coupled with improved specificity, can ultimately enhance personalized medicine strategies, allowing for treatments tailored to individual patient genetic profiles and disease states. These advancements not only promise to improve the efficacy of existing therapies but also introduce entirely new therapeutic modalities, pushing the boundaries of what is possible in gene therapy and cell-based treatments.

1.9 Thesis Scope and Content

The objective of this MSc thesis research was to investigate the complex and paradoxical role of interferon gamma (IFNγ) in T cell-mediated cancer immunotherapy, particularly within the context of engineered synthetic antigen receptors. This work aimed to elucidate how IFNγ contributes to both the efficacy and failure of adoptive T cell therapies by modulating tumour cell susceptibility, driving adaptive resistance, and influencing T cell exhaustion. By employing CRISPR gene editing to disrupt IFNγ or its receptor in engineered T cells, we sought to determine whether therapeutic efficacy could be preserved or improved by uncoupling these cells from IFNγ-driven immunosuppression.

This research builds on foundational work from the Bramson and Wan labs exploring IFNγ and its role in adaptive resistance in the context of ACT, and introduces a targeted gene-editing strategy to probe the functional implications of IFNγ signaling in engineered T cells in the context of solid tumours.

2.0 Research Objectives

This thesis broadly aims to enhance the efficacy of adoptive T cell therapies by modulating IFNγ signaling in engineered T cells. This work is organized around two primary research objectives, discussed in detail in Chapter 3:

- **Objective 1**: Evaluate the functional impact of IFNγ deletion on T cell cytotoxicity, expansion, and tumour killing in vitro and in vivo. Assess the contribution of IFNγ to tumour cell immunoediting via STAT1 signaling and the expression of immunosuppressive genes such as PD-L1 and IDO1, and determine whether IFNγ-deficient T cells can reduce adaptive resistance while retaining therapeutic efficacy.
- **Objective 2**: Investigate the effects of IFNγ receptor (IFNGR1) knockout in engineered T cells to assess the impact of disrupting autocrine IFNγ signaling. Examine whether IFNGR1-deficient T cells exhibit enhanced persistence, reduced exhaustion, and improved anti-tumour activity by escaping IFNγ-driven negative feedback within the tumour microenvironment.

Results

3.1.1 *Optimizing RNP formulation*

IFNγ has been described as a double-edged sword in the tumour microenvironment, exhibiting both anti-tumour and tumourigenic effects. To better understand the impact of IFNγ on immune responses in the TME, we sought to generate a T cell product incapable of producing IFNγ upon antigen recognition, via Neon electroporation of IFNG targeted CRISPR-Cas9 RNPs. While many protocols for CRISPR/Cas9 editing of primary Human T cells exist, editing efficiency can be impacted by a multitude of factors. Thus, we began by testing four RNP formulations, and two different Cas9 enzymes to determine optimal reagent conditions for editing. We utilized pre-validated guides for the TRAC locus to control for any sgRNA specific effects. The TRAC locus was selected because homozygous knock-out of TRAC leads to loss of surface TCR expression, which can be accurately measured by flow cytometry. IDT AltR HIFI Cas9 and IDT gRNA were complexed at molar ratios of 1:2, 1:2.5, and 1:3 at a final quantity of 20pmol Cas9 per electroporation, and one condition at a ratio of 1:2.5 given a final quantity of 50pmol Cas9 per electroporation. Pulse parameters and culture conditions were previously optimized and kept constant across all conditions. A ratio of 1:3 Cas9:gRNA at 20pmol Cas9 provided the greatest TCR knockout in both CD4 and CD8 cells compared to 1:2 and 1:2.5 (Figure 1A). The 50pmol condition provided similar results to the 1:2 condition at 20pmol(Figure 1B**)**. Thus, a ratio of 1:3 Cas9:gRNA at 20pmol Cas9 per electroporation was selected going forward. Knockout efficiency by FACS was similar between both enzymes, thus we selected the IDT AltR HIFI Cas9 for further experiments (Figure 1C).

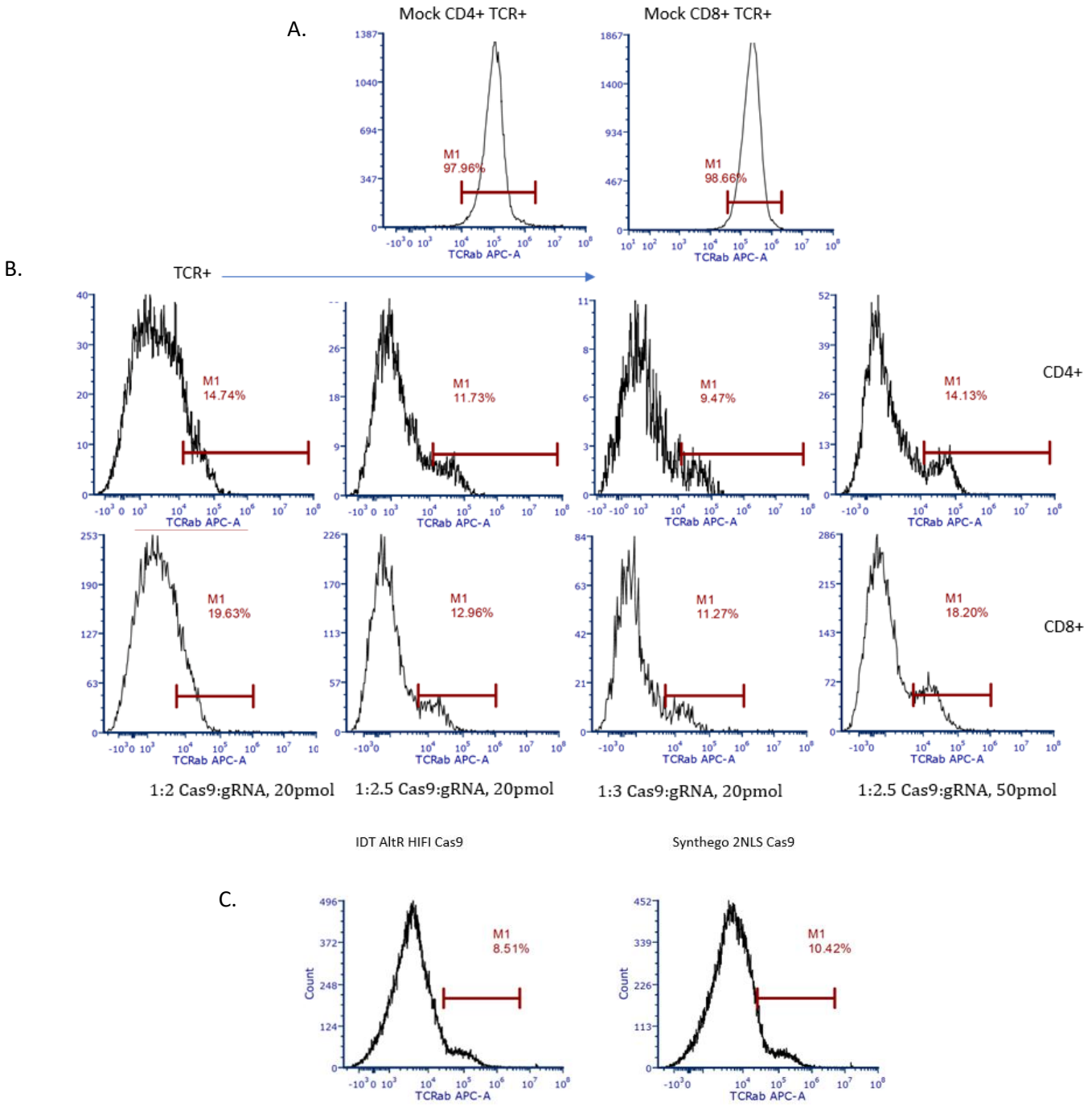
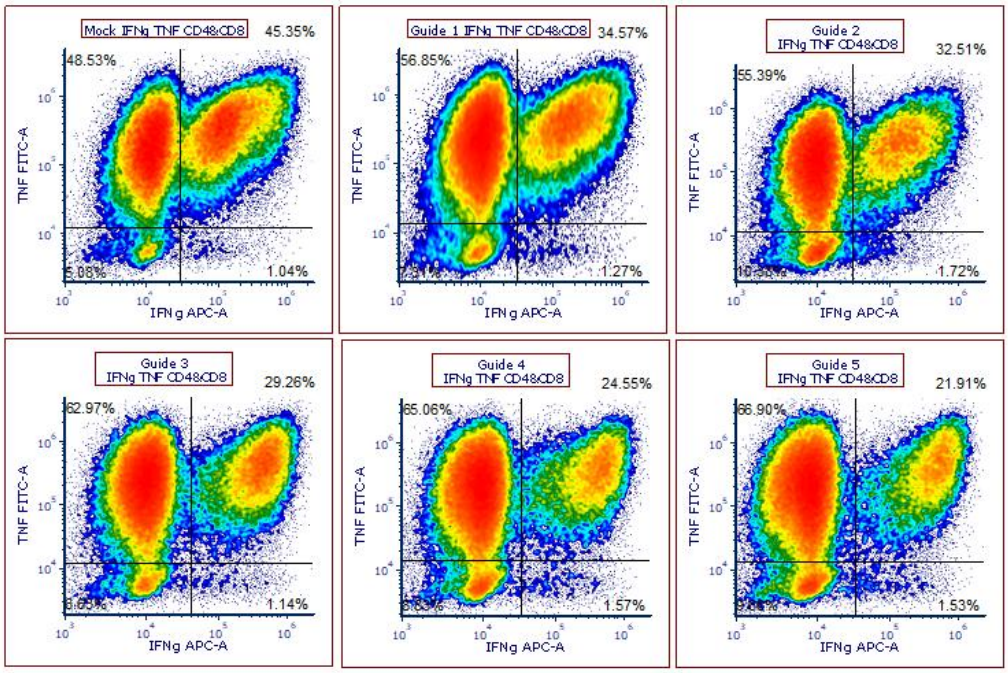


Figure 1. Flow cytometric analysis of TRAC gene knockout. A. Non-transduced T-cells were electroporated on day 3 after activation with RNPs containing Synthego TRAC KO multi-guide pool sgRNA and IDT AltR HIFI Cas9. RNPs were formulated using Cas9:gRNA in molar ratios of 1:2, 1:2.5, 1:3 at a final dose of 20pmol Cas9 per electroporation. A 1:2.5 condition with a final dose of 50pmol Cas9 was also tested. Knockout is stratified into CD4+ and CD8+ subpopulations. A Mock is included for comparison. Flow cytometry was run on day 6 of culture, 3 days after electroporation. B. Cells were edited on day 3 after activation using RNPs formulated with Synthego multi-guide pool TRAC sgRNAs and either Synthego 2NLS or IDT AltR HIFI Cas9. Flow cytometry was performed on day 6 of culture.

3.1.2 *Optimizing sgRNA sequences*

It is well understood that guide design has significant impacts on the efficacy of gene editing. PAM site, GC content, sgRNA length, and location of cut site are all factors contributing to editing efficiency. With this in mind, we designed and tested 5 sgRNAs for IFNG gene knockout (sgRNA sequences can be found in Table 1 of methods). The CHOPCHOPv3 software and Invitrogen TrueDesign Genome Editor tool were used to design and rank guides based on predicted efficiency and potential off-target binding. The IFNG gene consists of 4 exons; guides were designed targeting exons 1-3 (1 in exon 1, 3 in exon 2, 1 in exon 3) to determine the most effective cut site. Guide sequences were ordered as a crRNA and complexed with a tracrRNA to create gRNA. In non-transduced primary human T-cells, Guide #5 targeting exon 1 was the most efficient at reducing IFNγ expression in response to PMA/Ionomycin stimulation (Figure 2A.), where we observed a relative reduction of 52% in the fraction of cells expressing IFNγ. Literature indicates much higher knockout efficiency should be achievable with CRISPR/Cas9, thus, we examined other avenues for increasing knockout. Pooling of multiple guide sequences in a single exon to increase probability of large indel formation is an effective method for increasing knockout efficiency. As such, a multi-guide pool with 3 guides targeting the first exon of the IFNG locus was ordered from Synthego (now EditCo). A two-guide pool of previously evaluated guides 4 & 5 was also tested. A proprietary electroporation enhancer from IDT was also included in the electroporation milieu. The enhancer alone increased editing efficiency for a single guide significantly, causing a relative reduction of 72%, compared to 52% without enhancer (Figure 2B**)**. Use of the multi-guide pool from Synthego achieved greatest IFNγ reduction (w>95%; Figure 2B).

A.



B.

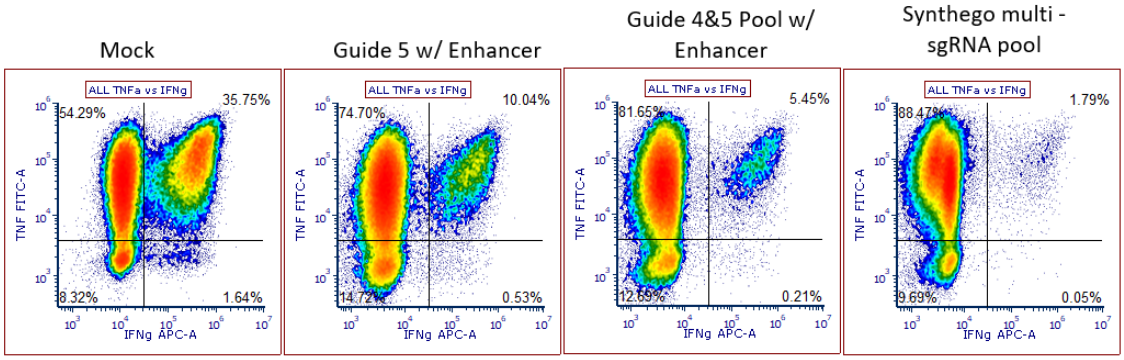


Figure 2. Cytokine expression patterns after electroporation with IFNγ KO guides. PBMCs were activated day 0 with Immunocult CD3/CD28 soluble activator, and electroporated on day 3. 6 days later, cells were stimulated with PMA/Ionomycin for 4 hours and then permeabilized and stained intracellularly; Flow cytometry data was collected on day 10. **A**. IFNγ expression is plotted against TNF-α for guides 1 through 5 targeting the IFNG locus. **B**. IFNγ expression is plotted against TNFα for guide 5 w/ enhancer, pooled guides 4&5, and Synthego multi-sgRNA pool.

3.1.3 *Optimizing Engineered T Cell Manufacturing for CRISPR Workflows*

With the ability to efficiently knock-out IFNG in non-trandsuced primary human T cells, we next sought to combine our established T cell manufacturing protocol, whereby healthy human PBMCs are activated with CD3/CD28/CD2 tetramers, are virally engineered with a TAC receptor, and subsequently expanded for 14 days prior to cryopreservation. Gammaretroviruses (gRV) have shown high transduction efficiency within the context of the TAC, thus, we started by engineering T cells to express two BCMA TAC constructs using gRV to determine whether retrovirally engineering our cells with a TAC receptor had any impact on knockout efficiency. T cells were transduced 48 hours post activation with gRV, and CRISPR edited 24 hours later. 7 days post activation, cells were stimulated via PMA/Ionomycin for 4 hours in the presence of GolgiPlug, and stained for FACS analysis to assess receptor transduction and IFNγ production. Notably, transduction for both constructs was lower than expected given historical data using gRV for each construct (16 and 36% vs 50 and 70% respectively) (Figure 3A). Both constructs showed robust cytokine profiles in response to stimulation. However, reduction in IFNγ production in comparison to controls varied between the two constructs (Figure 3B**)**. Given the variable knockout efficiencies between constructs and poor transduction, we decided not to pursue gRV engineering further.

We then evaluated the compatibility of lentiviral engineering with our CRISPR editing workflow. Lentiviral engineering is an attractive option as transduction occurs earlier post T cell activation in comparison to gRV, potentially allowing for a longer period of contact between virus and T cells. T cells were transduced 20 hours post activation with the LV equivalent of the previously tested gRV BCMA TAC. 72 hours post activation, T cells were CRISPR edited. 7 days post activation, T cells were collected and stimulated using either PMA/Ionomycin. Sub optimal transduction was observed with efficient knockdown of IFNγ production (Figure 3A&B).

Due to refocusing of the lab on the KIR-CAR synthetic antigen receptor design, further optimization employed a CD133 targeting KIR-CAR. We noted consistently reduced transduction in cells that had been CRISPR edited than expected from past data with the CD133-KIR-CAR construct (~50% vs ~75%). We hypothesized this to be due to the wash step involved in editing, limiting the transduction window to 24 hours in comparison to 48 hours with the standard protocol. To confirm, we compared cells that were washed and electroporated 24 hours post transduction to those that were allowed to transduce for 48 hours. Optimal transduction was observed when cells were transduced for 48 hours prior to electroporation where the electroporation did not impact transduction efficiency (Figure 3C). Previously, we showed T cells can be CRISPR edited up to 96 hours post activation with little impact on KO efficiency, which would allow for a longer transduction period. Thus, moving forward all CRISPR edits were done 72 hours post activation

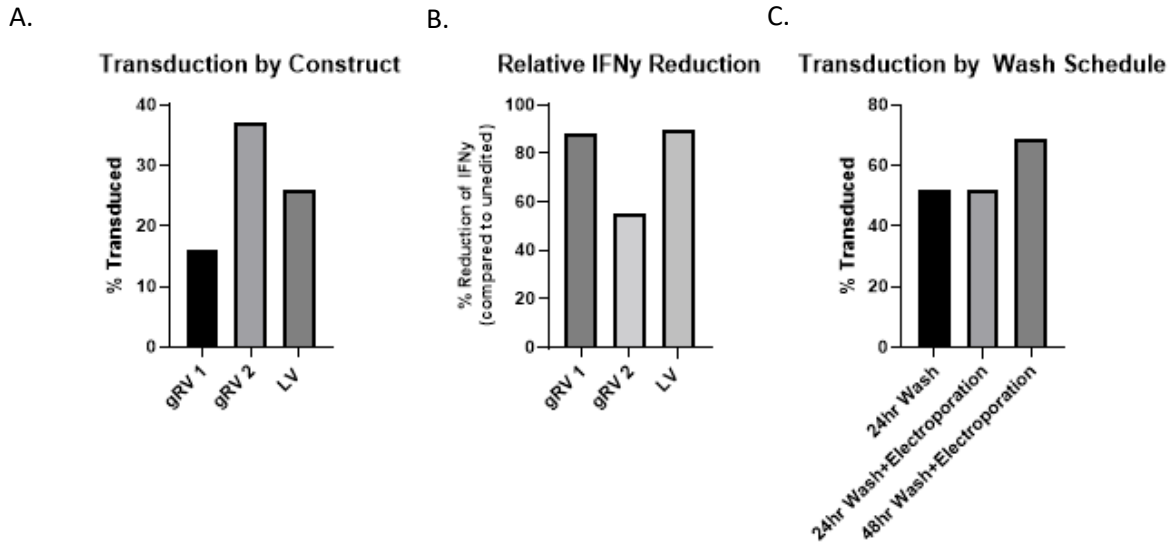


Figure 3. Optimization of Viral Engineering for CRISPR editing workflows. gRVs 1*(BCMA-TAC-Furin-T2A-TNFR1-GITR-GITR)* and 2 *(dCD34-T2A-C11D5.3 BCMA scFv huUCHT1 Y177T)* were compared to LV *(anti-GRP78 scFv-CAR-NGFR)* for A. transduction efficiency and B. compatibility with CRISPR editing via Neon Electroporation. Relative IFNγ reduction was assessed in comparison to an un-edited Mock. C. Timing of wash steps was investigated for impact on transduction of a CD133 KIR-CAR construct. Cells were stained for transduction marker NGFR and assessed by flow cytometry. n=1.

Finally, we validated our manufacturing workflow by conducting a large-scale manufacturing of Mock and IFNGKO CD133 KIR-CARs, and evaluating KO efficiency and receptor transduction through genomic analysis, flow cytometry, and ELISA. Receptor transduction remained comparable between Mock and IFNGKO groups (Figure 4A.). We used the Synthego ICE analysis tool to determine indel percentage at the genomic level. Genomic DNA was extracted from CD133 KIR-CAR Mock and KO T cells at D10 of manufacturing, PCR amplified across the first Exon, and sequenced. ICE analysis of the resulting chromatograms indicated 87% indel frequency, confirming a high degree of knockout at the genomic level (data not shown). These results were corroborated via stimulation of CD133 KIR-CAR Mock (electroporated without payload) and IFNG KO T cells with HCT116 tumour cells bearing the CD133 antigen for 4 hours. FACS analysis revealed a 90% relative reduction in IFNγ production in the KO sample when compared to the Mock (Figure 4B). As many cytokine pathways are heavily interlinked, we also included TNFa as a marker in our analysis. Importantly, the proportion of TNFa producing cells remained unchanged by IFNGKO. Finally, coculture supernatants collected from the same experiment were analyzed via ELISA for IFNγ and TNFa. As expected, we observed near complete loss of IFNγ production in the cultures with the KO T cells relative to Mock T cells (Figure 5A) with no impact on the production of TNFa (Figure 5B)

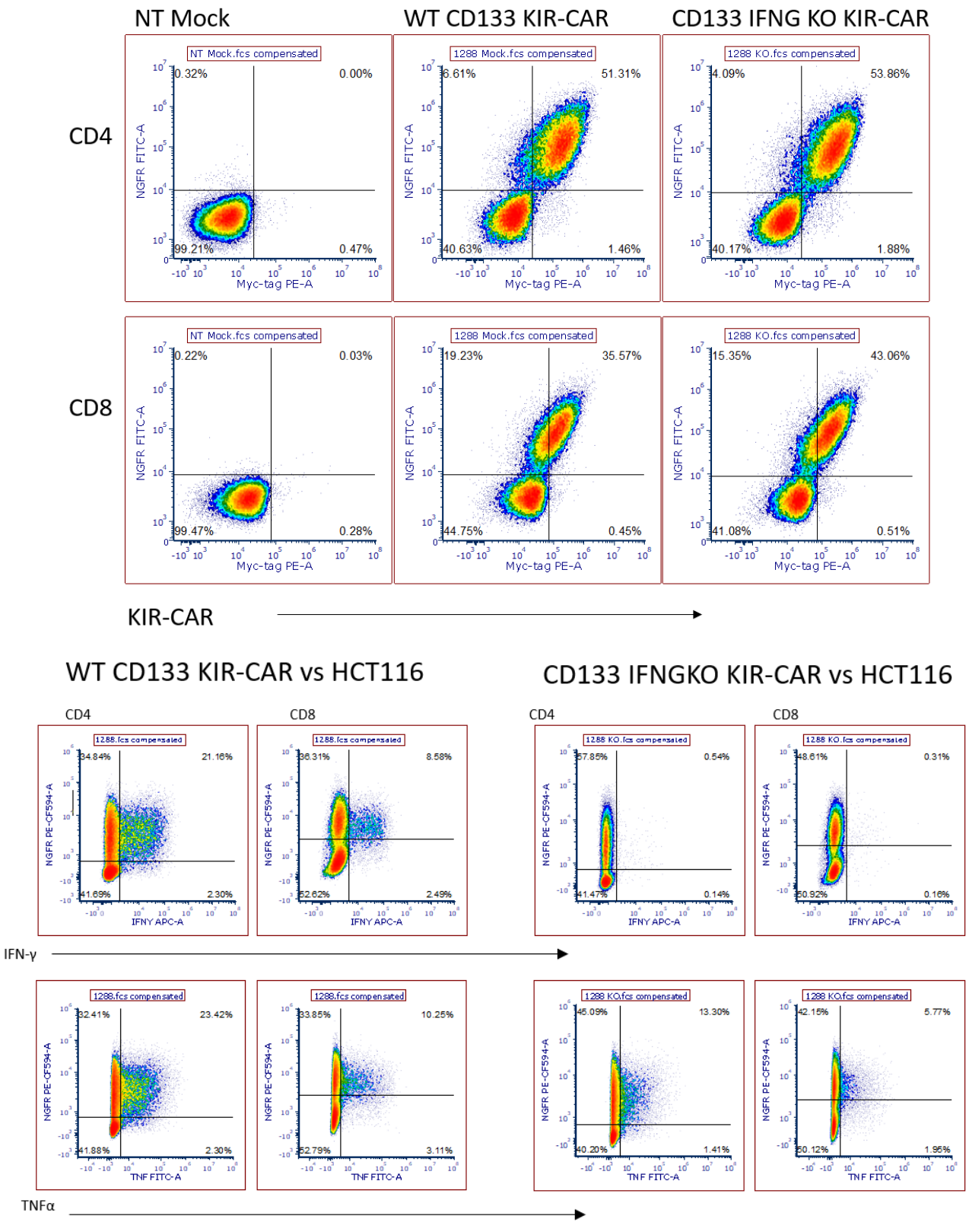


Figure 4. Lentiviral transduction is compatible with CRISPR editing in T cells. T cells were engineered with CD133 KIR-CAR LV and CRISPR edited via Neon Electroporation to knock out IFNγ. A. The CD133 SAR construct 1288 is highly expressed on the cell surface in both MOCK and IFNGKO conditions. Cells were infected with LV bearing the CD133 KIR-CAR post activation, and collected and stained for flow cytometry on day 7 post activation. Stratified by CD4/CD8 expression. **B.** Cytokine production by Mock and IFNGKO CD133 KIR-CAR T cells was assessed CD133 bearing HCT116 cells. Golgiplug was included in coculture to prevent cytokine release. After 4 hours, cells were permeabilized and stained intracellularly prior to quantification via FACS.

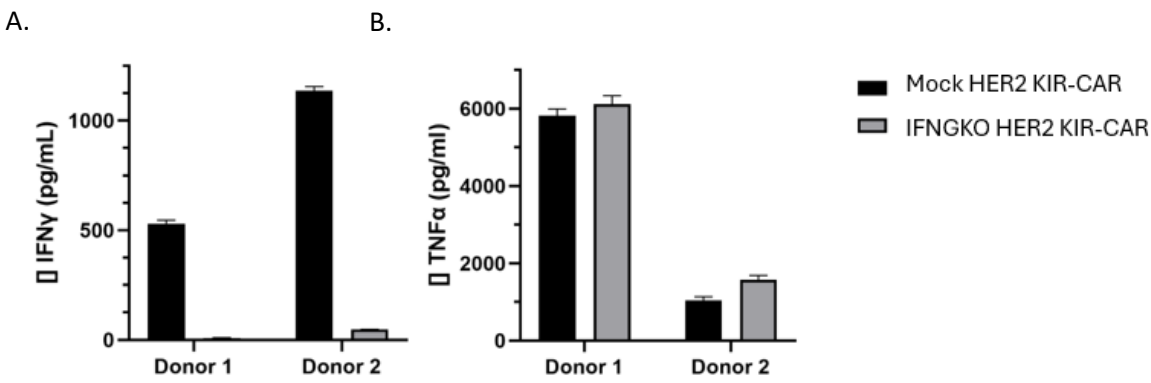


Figure 5. IFNG KO results in functional reduction of IFNγ but not TNFa in cocultures. Mock or IFNGKO HER2 KIR-CAR T cells were cocultured for 4 hours with HCT116 target cells. Supernatants were collected at 4 hours and analyzed via ELISA for A. IFNγ and B. TNFa.

3.2.1 *IFNGKO T cells exhibit comparable expansion but reduced cytotoxicity in-vitro*

To evaluate cytotoxicity in the absence of IFNγ, we generated HER2-targeted KIR-CAR T cells and conducted in-vitro cytotoxicity assays using fluorescence-based live-cell imaging. HER2 was selected as the target due to its high expression across multiple well-characterized and accessible tumour lines (HCT116, U251, SKOV3, A549). Co-cultures were established with HER2-KIR-CAR-engineered T cells—either Mock or IFNG KO—and HER2-positive tumour cells at varying effector-to-target (E:T) ratios (Figure 6A). IFNG KO T cells exhibited significantly reduced cytotoxicity compared to Mock T cells, particularly at lower E:T ratios.

To determine whether IFNγ was necessary for in-vitro cytotoxicity, we repeated the assay using Mock T cells in the presence or absence of an IFNγ-neutralizing antibody (Figure 6B). The concentration of anti-IFNγ was optimized using PD-L1 expression as a readout for IFNγR signaling. HCT116 cells were treated with 50 ng/ml recombinant human IFNγ (rhIFNγ), exceeding the IFNγ levels produced by T cells in co-culture, and anti-IFNγ was titrated to identify the concentration required to fully suppress PD-L1 expression. We selected a dose twice this required concentration (4 μg/ml). Neutralization of IFNγ markedly impaired Mock T cell cytotoxicity, confirming IFNγ's essential role in T cell-mediated killing (Figure 6D).

To verify that impaired cytotoxicity in IFNG KO T cells was due specifically to the absence of IFNγ, we repeated the cytotoxicity assay with IFNG KO T cells in the presence or absence of recombinant human IFNγ. The amount of rhIFNγ used was determined via ELISA from supernatants collected 24 hours after co-culturing HCT116 cells with Mock HER2-KIR-CAR T cells (Figure 6C). Notably, adding rhIFNγ (50 ng/ml) restored the cytotoxicity of IFNG KO T cells to levels comparable to those of Mock T cells (Figure 6E). Together, these findings demonstrate that IFNγ production by engineered HER2-KIR-CAR T cells is essential for maximal cytotoxic function.

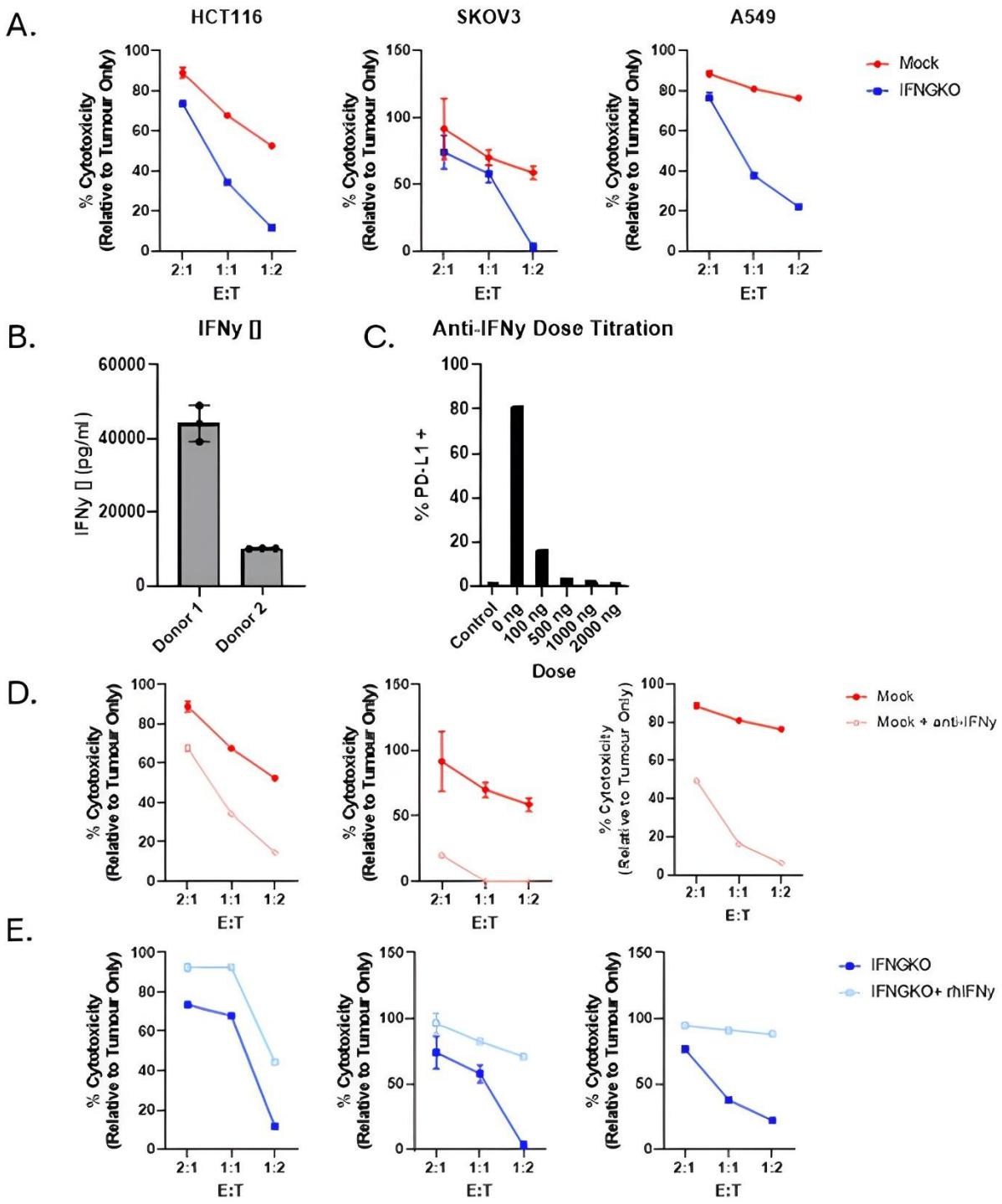


Figure 6. IFNγ deletion reduces cytotoxicity of KIR-CAR-T cells. A. Cytotoxicity plots for Mock and IFNGKO HER2 KIR-CAR T cells across varying E:Ts and three tumour lines, measured via green fluorescence via Incucyte. B. IFNγ concentration in HCT116/Mock HER2 KIR-CAR cocultures after 24 hours, measured via ELISA. C. PD-L1 expression in HCT116 cells after coculture with 50ng/ml rhIFNγ and escalating dose of anti-IFNγ neutralizing antibody, measured via FACS. D Representative cytoxicity plots from MOCK cells in the presence or absence of 3ug IFNγ neutralizing antibody. E. Representative cytotoxicity plots from IFNGKO cells in the presence or absence of 50ng/ml rhIFNγ.

Given the role of IFNγ in the activation, expansion and motility of CD8+ T cells, we hypothesized that the observed loss of efficacy may be due to a defect in expansion post activation. To test, we performed an *in-vitro* dye dilution assay, where HER2 KIR-CAR T cells were stained with Cell Trace Violet (CTV) (Figure 7A, right panel), cocultured 1:1 with HCT116 cells, and then collected for analysis 3 days later. Three donors were evaluated for their ability to enter division (percent divided; Figure 7A, right-hand panel) and the total T cell yield at the end of the three-day period.No significant differences were observed between Mock and KO cultures (Figure 7). To confirm that the role of IFNγ in T cell cytotoxicity is not limited to the HER2-KIR-CAR, we repeated these experiments with T cells engineered with the CD133 targeted KIR-CAR. As with the HER2-KIR-CAR, knock-out of IFNG in T cells engineered to express the CD133-KIR-CAR impaired their cytotoxicity (Figure 7B) without impacting their proliferative capacity (Figure 7C).

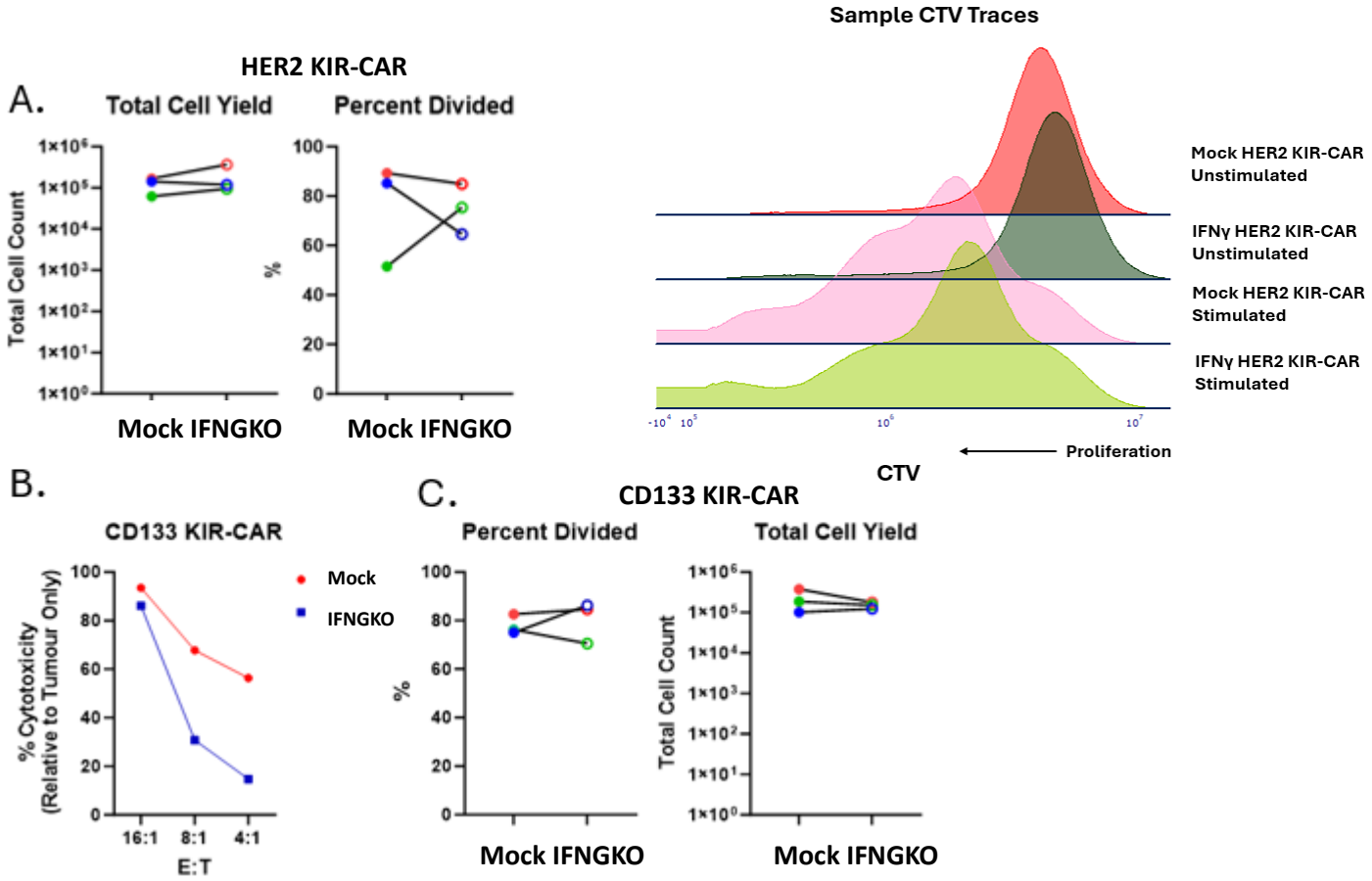


Figure 7. IFNγ deletion reduces cytotoxicity of KIR-CAR-T cells. A. Representation of Mock and IFNGKO HER2 KIR-CAR T cell proliferation against HCT116 target cells after a 3 day CellTraceViolet Dye Dilution assay, reported as total cell yield and percentage of total cells entering division. Each colour represents a unique donor. B. Cytotoxicity plot for Mock and IFNGKO CD133 KIR-CAR T cells across varying E:Ts. C. Representation of Mock and IFNGKO CD133 KIR-CAR T cell proliferation against HCT116 target cells after a 3 day CellTraceViolet Dye Dilution assay, reported as total cell yield and percentage of total cells entering division. Each colour represents a unique donor.

3.2.2 *IFNGKO T cells reduce adaptive resistance in-vitro*

Our prior data, and reports from other labs, support a role for T cell-derived IFNγ to be directly responsible for driving upregulation of immunosuppressive pathways within malignant cells. Upon observation of reduced cytotoxic capacity within our IFNγKO T cells, we sought to determine whether the absence of IFNγ was also significant enough to impact expression of immunosuppressive genes regulated by IFNγ in tumour cells. IFNγR binding induces STAT1 phosphorylation and homodimerization into GAF via JAK1 and JAK2, thus we sought to quantify pSTAT1 to visualize the impact of IFNGKO on immediate downstream signalling within tumour cells. To verify that IFNγ is capable of inducing STAT1 phosphorylation in HCT116 cells, we exposed them to increasing concentrations of recombinant human IFNγ (rhIFNγ) up to 5 ng/ml, the amount known to be produced by our engineered T cells, as shown in Figure 6B. STAT1 phosphorylation was observed at concentrations as low as 75 pg/ml, with mean fluorescence intensity (MFI) increasing in a concentration-dependent fashion (Figure 8A). To determine the timepoint to best capture IFNγ induced STAT1 phosphorylation, HCT116 cells were co-cultured with Mock and IFNGKO HER2 KIR-CARs for six hours, with supernatants collected at hourly intervals. These supernatants were analyzed via ELISA, revealing that IFNγ first became detectable at the two-hour mark and exceeded the upper detection limit of 600 pg/ml by three hours (Figure 8B). Based on this, we determined that three hours was the optimal time point for assessing initial STAT1 phosphorylation in response to IFNγ signalling. To assess STAT1 phosphorylation in tumour cells, we co-cultured HCT116 cells with either Mock or IFNγKO HER2 KIR-CAR-T cells at a 1:1 effector-to-target (E:T) ratio for three hours, then collected and stained the tumour cells for phosphorylated STAT1 (pSTAT1). Unexpectedly, we observed no reduction in pSTAT1 MFI in the IFNGKO T cells compared to the Mock T cells, suggesting that either IFNGKO did not affect STAT1 phosphorylation via IFNγR or the small amount of IFNg produced by the T cells (<100pg/ml) was sufficient to trigger STAT1 phosphorylation (Figure 8C). To further investigate the involvement of T cell-derived IFNγ in STAT1 phosphorylation, we incorporated an anti-IFNγ antibody into the co-culture to assess the specific contribution of IFNγ to STAT1 phosphorylation. Notably, IFNγ blockade reduced but did not completely eliminate pSTAT1 expression in the IFNγKO group, while having no detectable effect on the Mock condition (Figure 8D).

As other factors like type I interferons can also induce STAT1 phosphorylation, we decided to probe for transcriptional differences between Mock and IFNG KO treated groups further downstream in the IFNγR pathway. To do so, we selected Interferon regulatory factor 1 (IRF1), a master transcription factor activated by pSTAT1 homodimers, as well as two downstream hallmark immunosuppressive genes known to be upregulated by IFNγ PD-L1 and IDO1, for qPCR analysis. As before, HCT116 cells were cocultured with Mock or IFNG KO HER2 KIR-CARs 1:1 for 3 hours before washing off T cells and processing RNA for RT-qPCR. While relative expression of IRF1 was no different between the co-cultures with Mock and IFNG KO T cells, PD-L1 and IDO1 expression were significantly higher in the co-cultures with Mock T cells treated

than co-cultures with IFNG KO T cells (6.83 vs 3.22 and 87.06 vs 7.58 respectively), confirming IFNG KO did reduce tumour adaptive resistance in T cell: tumour co-cultures (Figure 9).

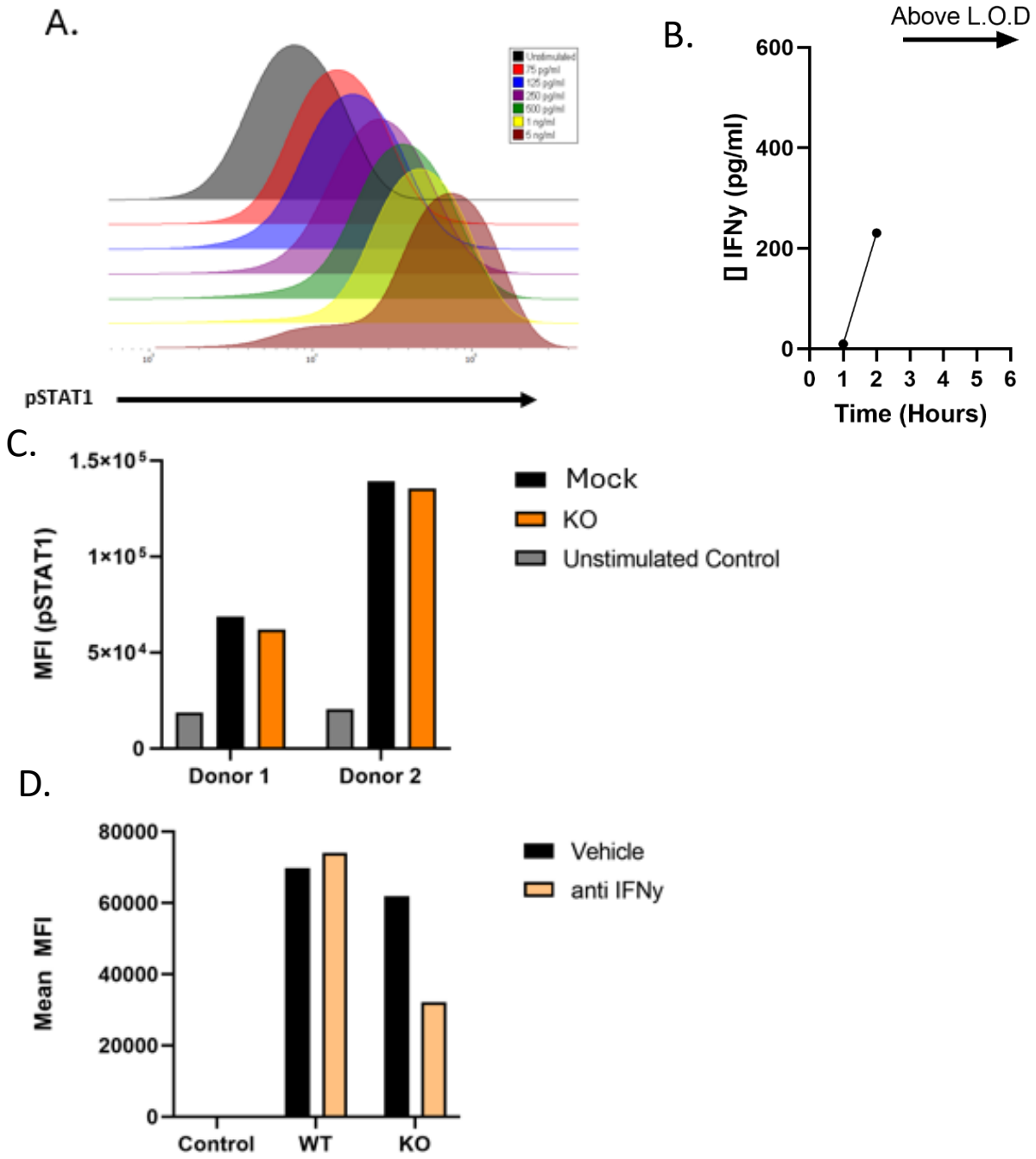


Figure 8. Impact of IFNγ on STAT1 Phosphorylation in HCT116s. A. HCT116 tumour cells were stimulated with escalating doses of rhIFNγ for 1 hour, before fixation and staining for FACS analysis.B. ELISA measurements of supernatants collected from HCT-T cell cocultures across 6 hours. C. HCT 116 tumour cells were cocultured with either Mock or IFNγKO HER2 KIR-CAR T cells for 3 hours, before fixation and staining for pSTAT1 FACS analysis. D. HCT116 tumour cells were cocultured with either Mock or IFNγKO HER2 KIR-CAR T cells in the presence or absence of IFNγ neutralizing antibody for 3 hours prior to FACS analysis of pSTAT1.

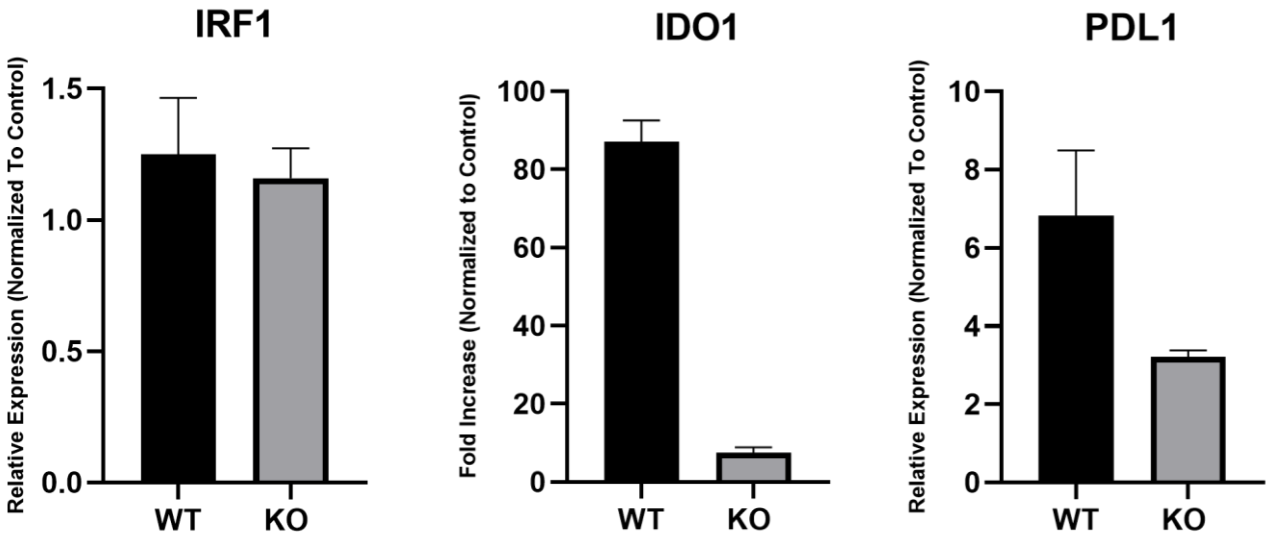
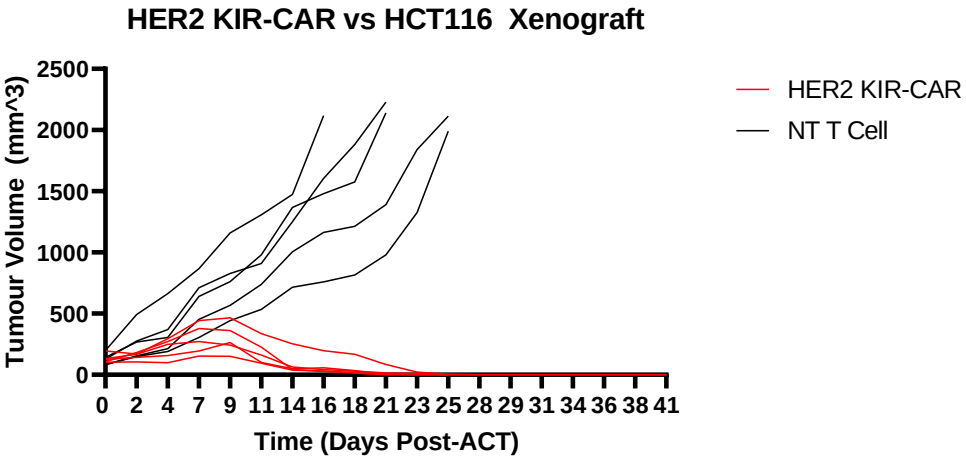


Figure 9. IFNγKO SAR T cells induce reduced levels of immunosuppressive markers in-vitro. WT and IFNγKO T cells were cocultured with HCT116 tumour cells for 3 hours. T cells were then washed off, and tumour cells collected. RNA extraction and RT-PCR were performed, followed by qPCR with TaqMan probes for IRF1, IDO1 and PDL1.

3.2.3 *Validating HER2 KIR-CAR Specificity in-vivo*

Acknowledging the limitations of in-vitro models in recapitulating the complexity of the solid tumour microenvironment (TME), we proceeded to confirm our findings using an HCT116 xenograft model in NRG mice. The HER2-KIR-CAR-T cells demonstrated potent anti-tumour effects (Figure 10A) without signs of toxicity. Additionally, these cells exhibited on-target reactivity in vivo, expanding selectively in HER2-positive tumours (Figure 10B, left) and failing to expand in HER2-negative tumours (Figure 10B, right).

A



B

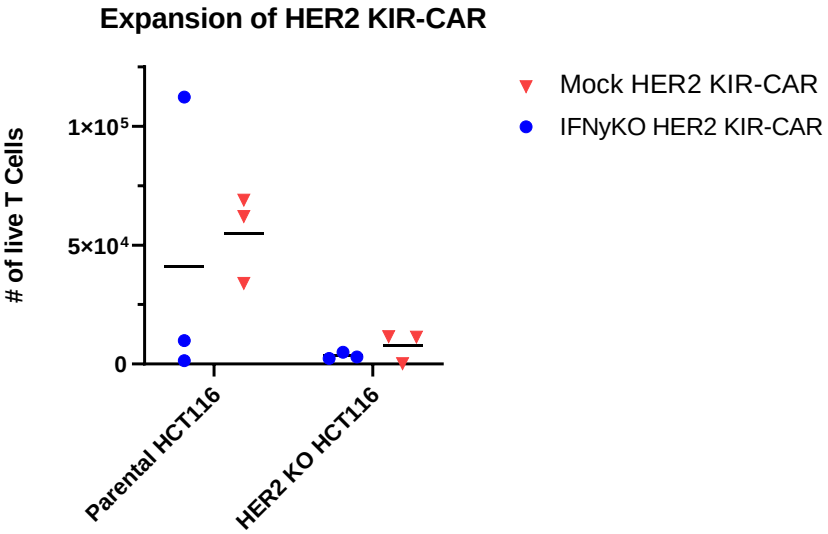


Figure 10. HER2 KIR-CAR T cells exhibit potent on target cytotoxicity. A. Survival plots for Mock HER2 treated mice in HCT116 model. Mice received $6x10^6$ T cells, N=5 per group. B. Quantification of T cells in spleens extracted from mice bearing parental or HER2 KO HCT116 tumours, treated with $6x10^6$ Mock or IFNGKO HER2 KIR-CAR T cells. N=3 per group.

3.2.4 *IFNGKO T cells reduce adaptive resistance in-vivo*

Based on our in-vitro findings, we hypothesized that knocking out IFNG in HER2-KIR-CAR-engineered T cells would similarly diminish adaptive resistance in treated tumours. To test this, we analyzed transcriptional changes linked to adaptive resistance in two xenograft models—HCT116 colorectal cancer and U251 glioblastoma—after treatment with HER2-KIR-CAR T cells. Mice bearing ~200mm³ tumours received 6×10^6 engineered T cells (either Mock or IFNG KO) or remained untreated (UT). Ten days post-infusion, tumours were harvested and analyzed for IFNγ signaling (via IRF1 expression) and hallmark adaptive resistance markers (PD-L1 and IDO1). Tumours treated with IFNG KO T cells exhibited a significant reduction in PD-L1 and IDO1 expression relative to tumours from Mock-treated mice (Figure 11A). This reduction in adaptive resistance was observed across both tumour models, reinforcing our in-vitro findings. To investigate adaptive resistance at a spatial level, we collected tumour sections from mice bearing HCT116 tumours treated with either Mock or IFNG KO HER2-KIR-CAR T cells. These sections were stained for human CD3 (to mark T cells), Cytokeratin (to identify tumour cells), Ki-67 (to measure local tumour and T cell proliferation), phospho-STAT1 (to assess IFNγ signaling), and PD-L1/IDO1 (to evaluate adaptive resistance). Tumours from IFNG KO-treated mice exhibited lower phospho-STAT1 expression (Figure 11B-C, Figure 12), indicative of reduced IFNγ signaling. Consistent with our qPCR results, tumours from IFNG KO-treated mice had a diminished presence of PD-L1$^+$ and IDO1$^+$ tumour cells compared to those treated with Mock T cells (Figure 11B).

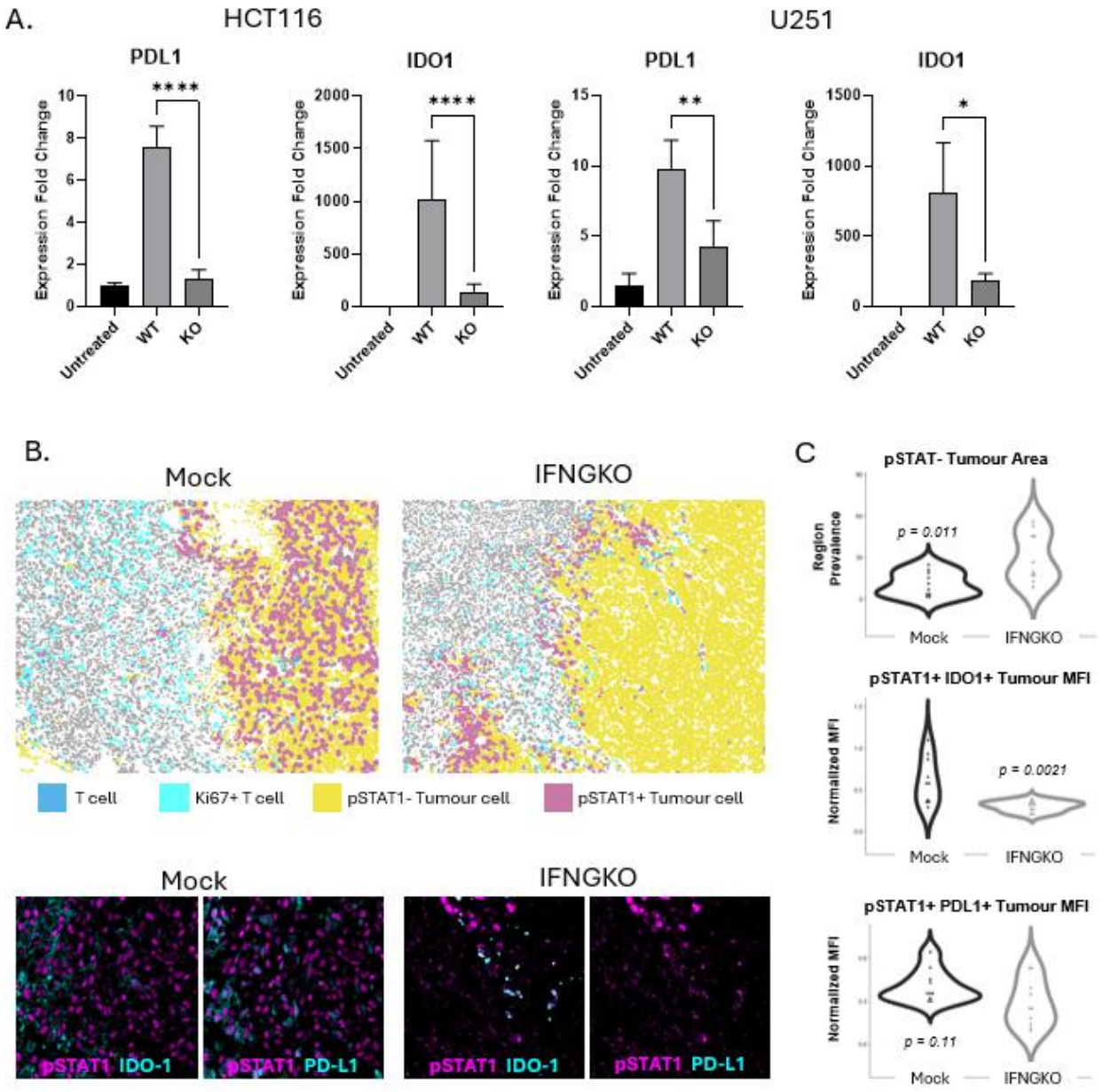


Figure 11. Evaluation of IFNG KO HER2 KIR-CAR T cells. A. Expression of markers of adaptive resistance, measured from tumour homogenates from WT/IFNGKO treated mice. ΔΔcT values reported as log2 fold change, 2 sided unpaired T test. B. IHC staining of HCT116 tumour slices taken from WT or IFNGKO treated mice. Representative images (left) and quantification represented as C. violin plots (right)

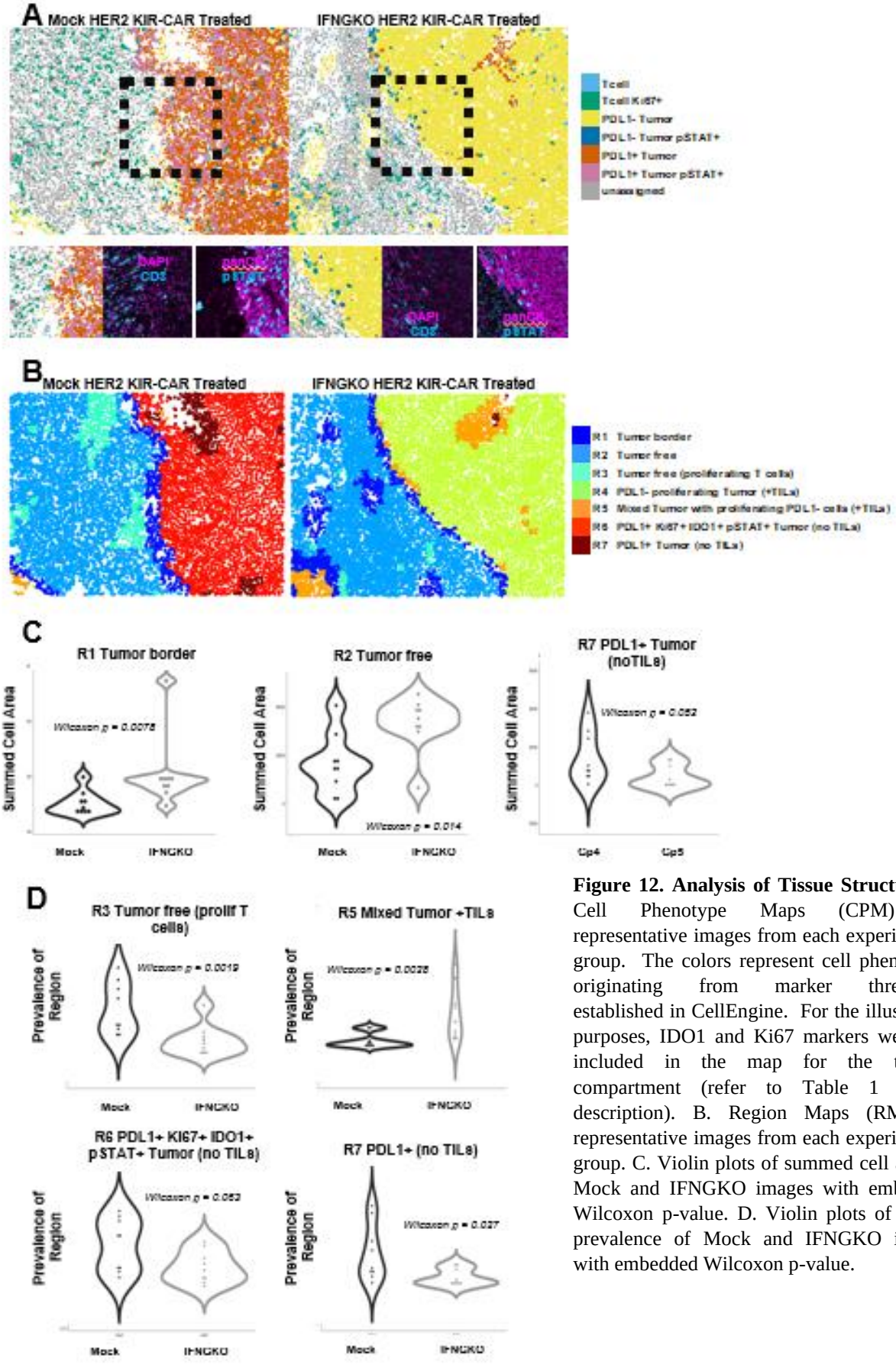


Figure 12. Analysis of Tissue Structure A. Cell Phenotype Maps (CPM) for representative images from each experimental group. The colors represent cell phenotypes originating from marker thresholds established in CellEngine. For the illustrative purposes, IDO1 and Ki67 markers were not included in the map for the tumour compartment (refer to Table 1 for a description). B. Region Maps (RM) for representative images from each experimental group. C. Violin plots of summed cell area of Mock and IFNGKO images with embedded Wilcoxon p-value. D. Violin plots of region prevalence of Mock and IFNGKO images with embedded Wilcoxon p-value.

3.2.5 *IFNGKO KIR-CAR T cells exhibit decreased efficacy in-vivo*

To determine whether the observed decrease in adaptive resistance conferred a survival advantage, we treated HCT116 and U251 tumour-bearing mice with either Mock or IFNG KO HER2-KIR-CAR T cells and monitored tumour progression and survival. Surprisingly, in both models, mice treated with IFNG KO T cells had reduced survival compared to those receiving Mock T cells (Figure 13), suggesting that although IFNG KO attenuated adaptive resistance, it impaired rather than enhanced anti-tumour efficacy. Given that in-vitro analyses showed no defect in T cell proliferation following IFNG KO, we sought to validate this in xenograft models. To enable in-vivo tracking, Mock and IFNG KO T cells were co-transduced with lentiviruses encoding HER2-KIR-CARs and red-shifted luciferase (RsLuc) (Images in Figure 14A; Quantified data in Figure 14B). Mock T cells reached peak tumour-localized radiance seven days post-infusion, followed by a decline in radiance correlating with tumour regression. IFNG KO T cells exhibited a comparable peak in radiance, but their contraction within the tumour was delayed. Notably, IFNG KO T cells persisted for over three weeks, indicating that their reduced efficacy was not due to impaired tumour infiltration or expansion (Figures 14A-C).

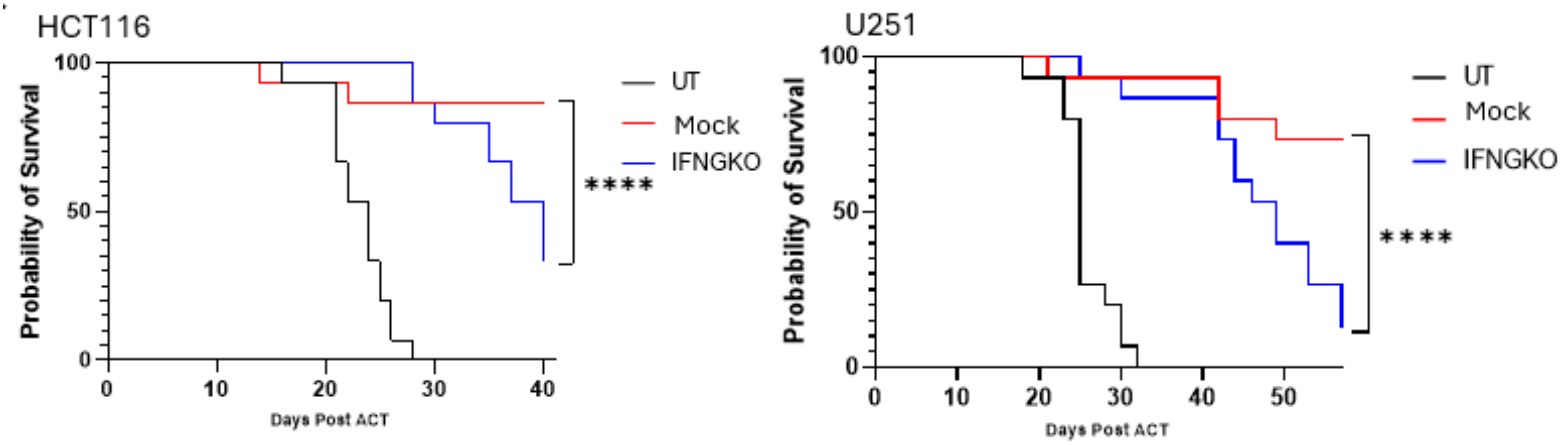


Figure 13. IFNGKO HER2 KIR-CAR T cells exhibit reduced tumour control in multiple tumour models. A. Survival plots for Mock/IFNGKO treated mice in HCT116 and U251 models. Mice received $6x10^6$ T cells, N=15 per group, 3 independent experiments.

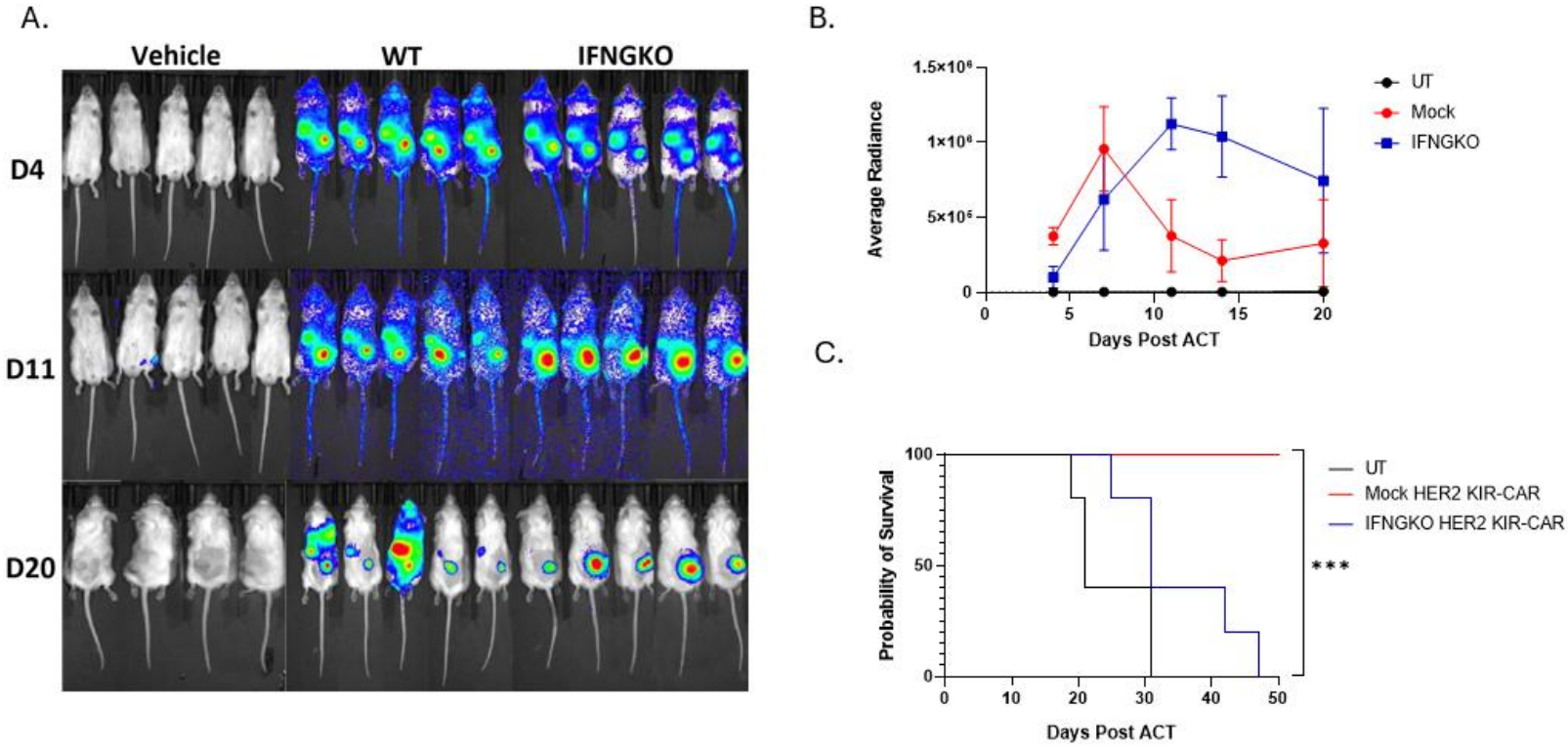


Figure 14. IFNγ deletion reduces cytotoxicity *in-vivo* and *in-vitro* without impacting T cell expansion. A, representative BLI images tracking T cell expansion over time in HCT116 xenograft bearing mice, n=5 . b, Quantification of radiance from a over time. c, Survival curves for cohorts (n=5) from a. d, Representation of Mock and IFNGKO HER2 KIR-CAR T cell proliferation against HCT116 target cells after 3 day CellTraceViolet Dye Dilution assay, reported as total cell yield and percentage of total cells entering division.

3.3.1 *Autocrine T cell IFNγR Signalling is dispensable for KIR-CAR Function*

While it has become clear that in this setting IFNγ is required for the maximal cytotoxic efficacy of our HER2 KIR-CAR T cells, within our current system it is unclear whether the loss of efficacy arises due to dysfunction within the T cell compartment caused by diminished autocrine IFNγR signalling, or if maximal cytotoxicity relies on paracrine IFNγR interactions directed towards the tumour cells. In fact, recent reports have indicated chronic IFNγ signalling may directly restrict CAR T cell expansion and persistence. To understand the importance of directionality within this system, we disrupted the expression of the IFNGR1 subunit using the CRISPR/Cas9 method, attenuating IFNγ signalling within our KIR-CAR T cell. Knockout was validated via flow cytometry (Figure 15A). To confirm IFNGR1 KO led to functional impairment of IFNγ signalling, we stimulated Mock or IFNGR1KO T cells with 50ng/ml rhIFNγ for 30 minutes, and measured STAT1 phosphorylation via FACS analysis. Indeed, a substantial decrease in pSTAT1 expression was observed in the IFNGR1 KO group, indicating reduced signalling via the IFNγR pathway (Figure 15B.).

To determine the directionality of IFNγR signalling, we repeated the cytotoxicity previously described, this time evaluating Mock and IFNGR1KO cell products against HCT116 via fluorescence based live cell imaging, in the presence or absence of either anti-IFNγ neutralizing antibody. Interestingly, IFNGR1KO T cells displayed cytotoxicity comparable to Mock T cells at all E:Ts, indicating IFNγR signalling in T cells may be dispensable for anti-tumour efficacy (Figure 15C.). While in the presence of neutralizing antibody, Mock and IFNGR1KO T cells exhibited a loss of efficacy at decreasing E:Ts similar to that observed in untreated IFNGKO cultures confirming that T cell derived IFNγ interacting with IFNγR on the tumour cell surface but not the T cell, is a critical interaction for T cell mediated cytotoxicity. Similar results were observed in the A549 and SKOV3 tumour cell lines (Figure 6D and Figure 15D.). We also confirmed that T cell expansion was not impaired by IFNGR1KO through a 3 day proliferation assay where Mock or IFNGR1KO T cells were cocultured with multiple tumour lines at a 1:1 E:T. We observed no difference in cell count after coculture across, and validated this in multiple donors (Figure 15E).

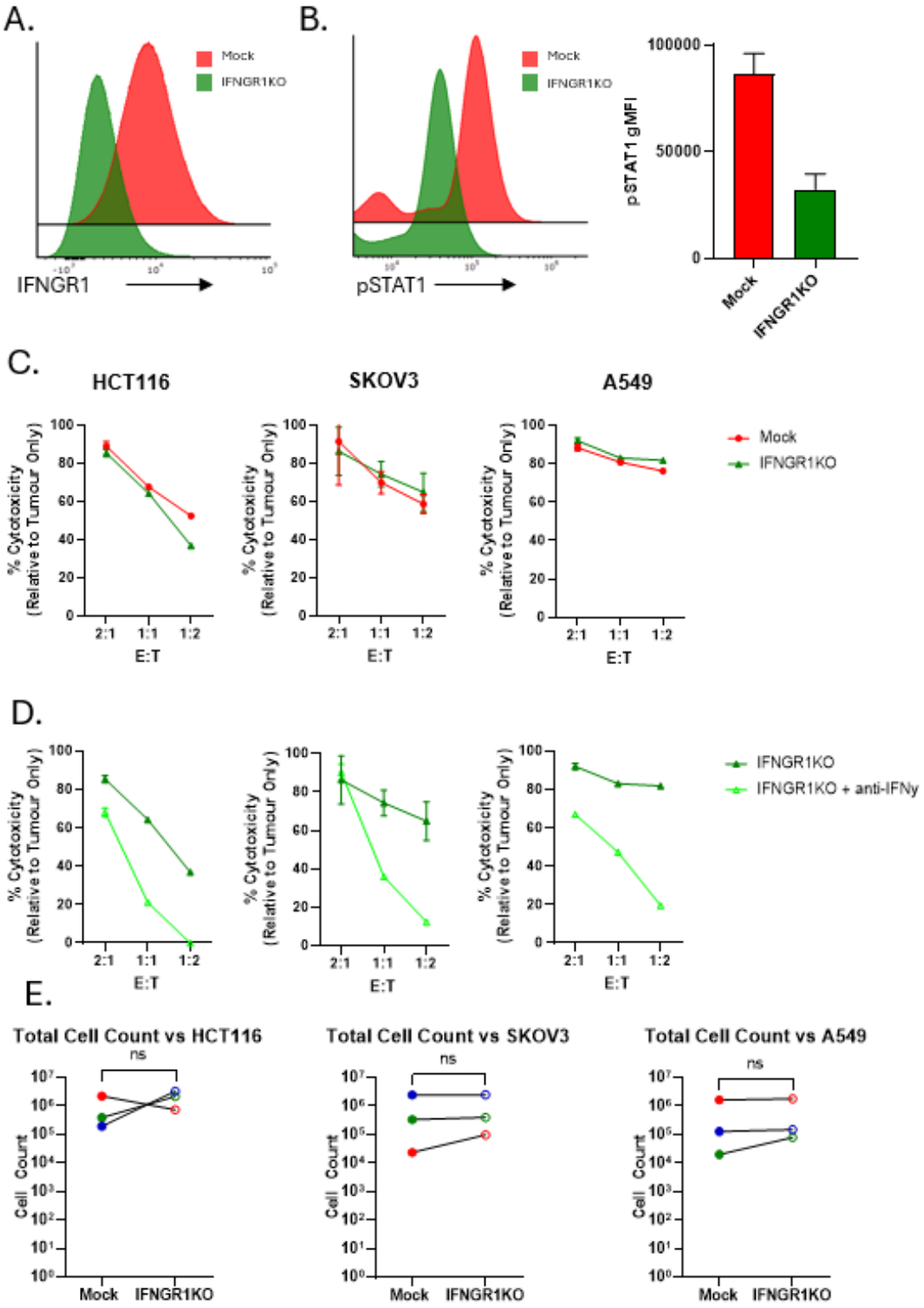


Figure 15. T cells do not require autocrine IFNγR1 signalling for efficacy A. Histogram indicating successful knockout of IFNGR1 via FACS analysis. B. Left: Representative histograms of pSTAT1 expression following stimulation with rhIFNγ for 30 minutes. Right: graphical quantification of gMFI of IFNGR1 after stimulation. C. Cytotoxicity plots for WT and IFNGKO and IFNGR1KO HER2 KIR-CAR T cells across varying E:Ts and three tumour lines, measured via green fluorescence via Incucyte. D. Representative cytoxicity plots from IFNGR1KO HER2 KIR-CAR T cells in the presence or absence of 4ug IFNγ neutralizing antibody E. T cell counts after 3 day coculture with HCT116 cells, quantified via FACS. Each colour represents a unique donor.

As a final confirmation, we conducted an *in-vivo* evaluation of our IFNGR1KO HER2 KIR-CAR T cells. Using the HCT116 and U251 xenograft models described previously, mice were treated with Mock or IFNGR1KO HER2 KIR-CARs. In agreement with our *in-vitro* data, we observed no loss of efficacy as a result of IFNGR1KO in either model, confirming that the impairment observed in our IFNGKO cohorts was not a result of T cell dysfunction stemming from decreased autocrine IFNγR signalling (Figure 16).

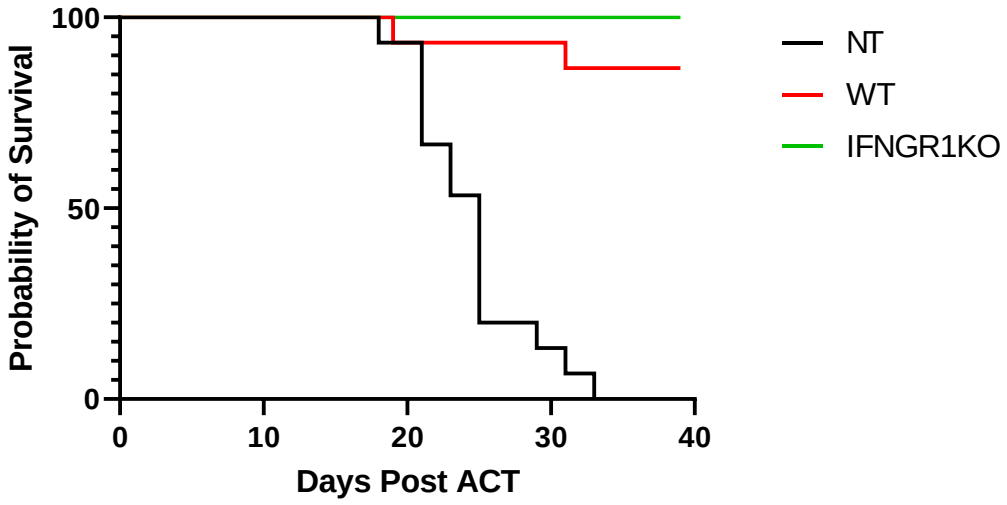


Figure 16. IFNGR1KO HER2 KIR-CAR T cells perform equitably to Mock *in-vivo*. Survival curves for cohorts treated with Mock or IFNGR1KO HER2 KIR-CAR T cells. (n=10).

3.3.2 *IFNGR1KO does not enable enhanced expansion in-vitro*

Recently, multiple reports have suggested that chronic exposure to interferon may restrict anti tumour T cell function. Therefore, we assessed the contribution of IFNg-mediated signals to T cell dysfunction in a recursive stimulation setting *in vitro*. Mock or IFNGR1KO HER2 KIR-CAR were cocultured with HCT116 cells at a 1:1 E:T for 72 hours, at which point they were phenotyped and quantified via flow cytometry before repeating the process until the cells failed to expand sufficiently for re-stimulation. Consistently, IFNGR1KO T cells expanded to similar or greater numbers than Mock T cells across multiple rounds of stimulation (Figure 17). Similar results were observed using alternate HER2 positive cell lines A549 and SKOV3 as stimulus. Thus we concluded IFNGR1KO did not enhance T cell function in the context of HER2-KIR-CAR-engineered T cells.

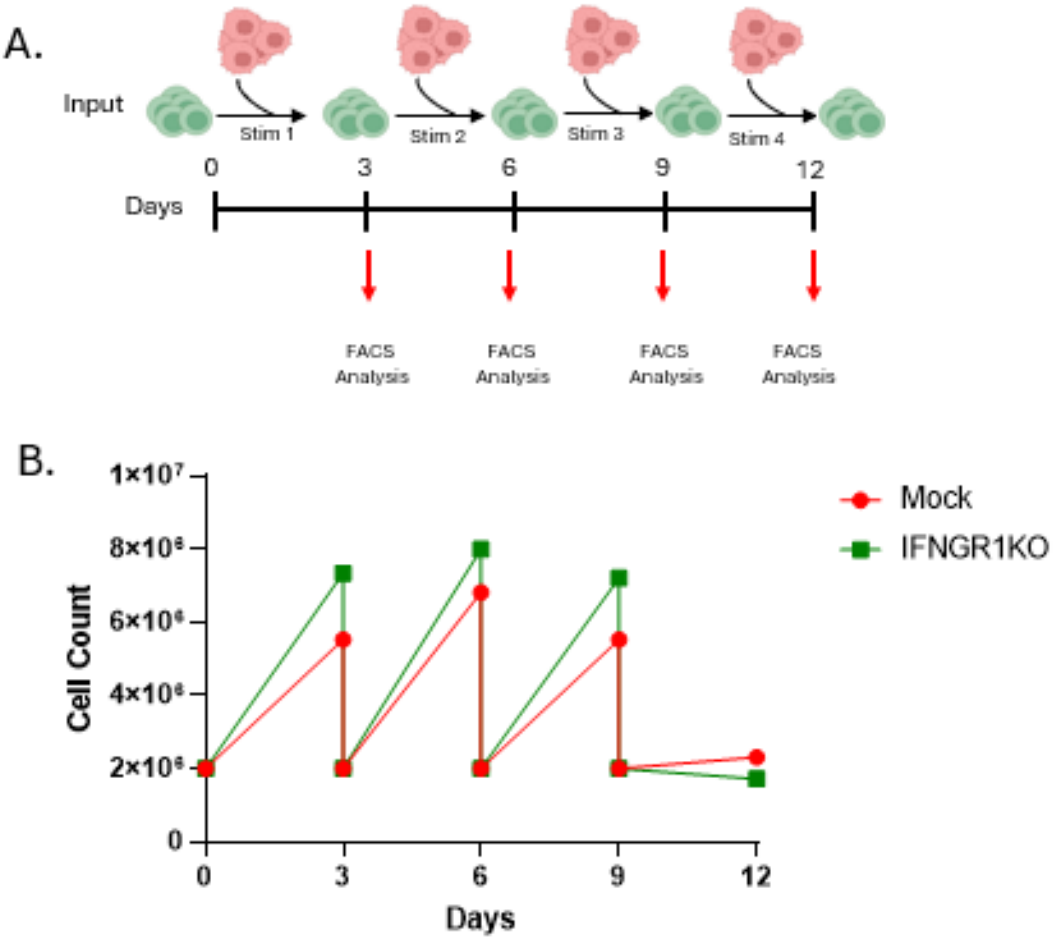


Figure 17. IFNGR1KO HER2 KIR-CAR T cells do not have enhanced ability to expand over multiple rounds of stimulation. A. Schematic representation of the recursive stimulation workflow. B. Cell counts quantified after each round of stimulation for Mock and IFNGR1KO T cells.

3.3.3 *IRF1 Expression is decreased upon activation in IFNGR1KO T cells*

Next, we sought to understand why IFNGR1KO in our KIR-CAR T cells had no impact on expansion or persistence. Notably, Matzet et al. performed IFNGR1KO in mature murine CD8 T cells, while our IFNGR1KO was performed on activated human PBMCs. The role of IFNγ signalling in CD8 T cell function is widely accepted, promoting CD8 differentiation and effector function. It is well known that interferon regulatory factor 1 (IRF1) is responsible for promoting numerous effector functions in T cells, and its expression is upregulated preferentially upon IFNγR signalling (although it also can be activated by type I interferon) as it contains a GAS in its promoter region. By knocking out IFNGR1 during manufacturing to prevent chronic IFNγR signalling, we may also be impacting the ability of CD8 T cells to become properly activated through IRF1, leading to the divergence in results between our work and Matzet et al's. To test this, we conducted a 24-hour timecourse assay, where Mock, IFNGKO, or IFNGR1KO T cells were stimulated with plate bound anti-CD3 for 24 hours, with cells being collected at 4 hour intervals. RNA was isolated from cell pellets for each timepoint, and RT-qPCR was conducted to quantify IRF1 expression. Baseline IRF1 expression was comparable between all conditions. However, in both the IFNGKO and IFNGR1KO conditions, IRF1 expression was visibly lower at 4 hours in comparison to Mock, and continued to decrease across the timecourse, indicating that the lack of IFNγR signalling did decrease IRF1 expression (Figure 18A). Importantly, IFNGR1KO did not result in a decrease in IFNγ production, as measured by ELISA of coculture supernatants collected at each timepoint (Figure 18B).

As we have shown IFNγ to be present at appreciable levels in stimulated cultures as early as 3 hours, we repeated the assay collecting timepoints every hour from 0-4hrs, to capture changes observed in IRF1 expression during intial IFNγR signalling events. Indeed, IRF1 expression was higher in the Mock cultures in comparison to the IFNGR1KO cultures at each timepoint, and significantly decreased at 4 hours in agreement with our previous experiment (Figure 18C).

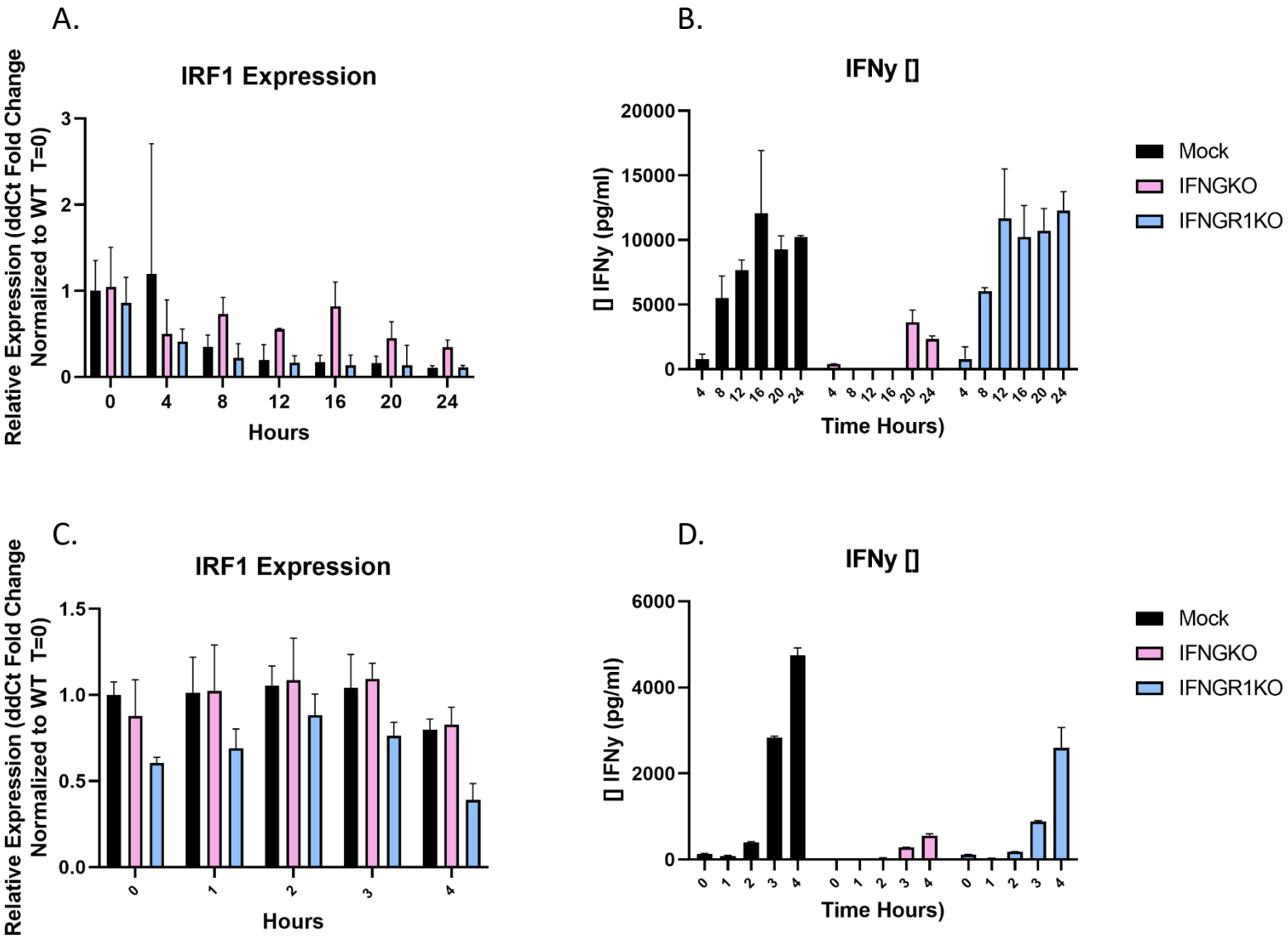


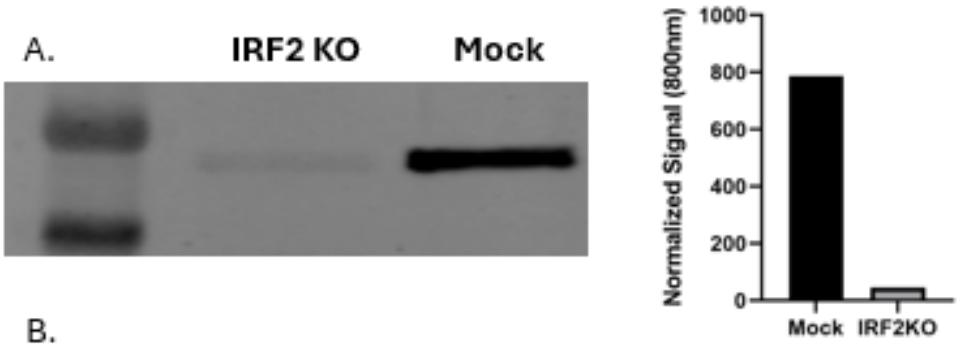
Figure 18. IRF1 expression is decreased upon activation in the absence of T cell IFNGR signalling. A. Changes in IRF1 expression across 24 hours in Mock, IFNGKO and IFNGR1KO T cells stimulated with plate bound anti-CD3. Cells were collected every 4 hours and IRF1 expression was measured via RT-qPCR. B. IFNγ [] in coculture across 24 hours, measured via ELISA of coculture supernatants from A. C. Changes in IRF1 expression across 4 hours in Mock, IFNGKO and IFNGR1KO T cells stimulated with plate bound anti-CD3. D. IFNγ [] in coculture across 4 hours, measured via ELISA of coculture supernatants from C.

3.3.4 *IRF2 KO does not enhance KIR-CAR Persistence or Expansion*

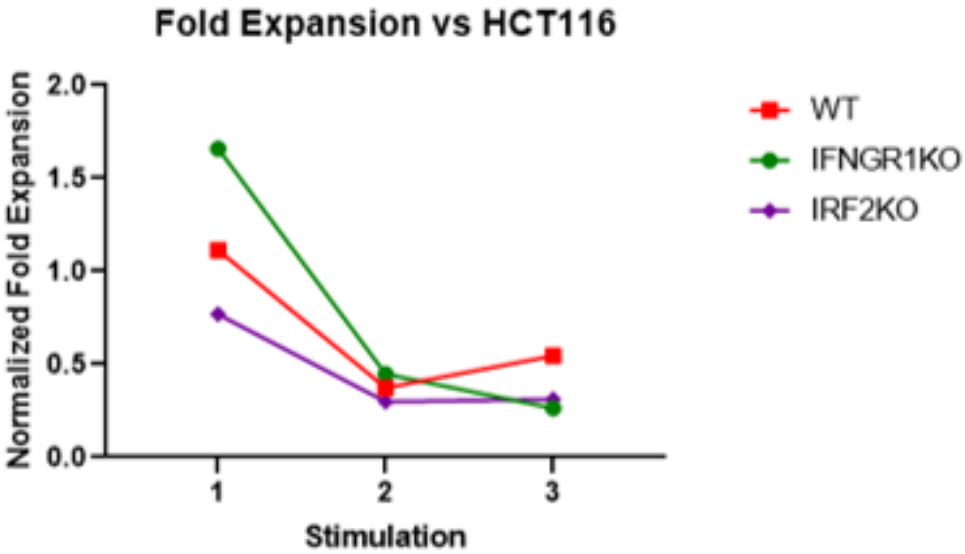
The transcription factor IRF2 is upregulated in response to both type I and type II interferon and competes for binding with IRF1. Recently, IRF2 was shown to promote expression of exhaustion machinery in T cells. We hypothesized that by knocking out IRF2, we could retain the upstream benefits of IFNGR1 signalling via IRF1 while preventing the IRF2-mediated exhaustion.

To test this hypothesis, we generated IRF2KO HER2 KIR-CAR T cells using the same CRISPR/Cas9 workflow previously described. Two sgRNAs targeting the second exon of the IRF2 gene, and knockout was confirmed via western blot to be >95% (Figure 19A). We then utilized the same recursive stimulation assay with HCT116 cells as previously described to evaluate their expansive capacity and long-term persistence. Across multiple rounds of stimulation, the IRF2KO T cells exhibited no advantage over Mock or IFNGR1KO T cells (Figure 19B). To interrogate expression of IRF2 across the conditions, cell lysates were collected at the end of each round of stimulation, and probed via western blot for IRF2 protein. While the IRF2KO T cells exhibited low levels of IRF2 at all timepoints as expected, we also observed no appreciable difference in IRF2 levels between the Mock and IFNGR1KO cultures, suggesting that there are other mechanisms, like type I interferon signalling, that drive IRF2 expression in the absence of IFNGR. Thus, IRF2KO is insufficient to prevent exhaustion driven by chronic IFNγ signalling.

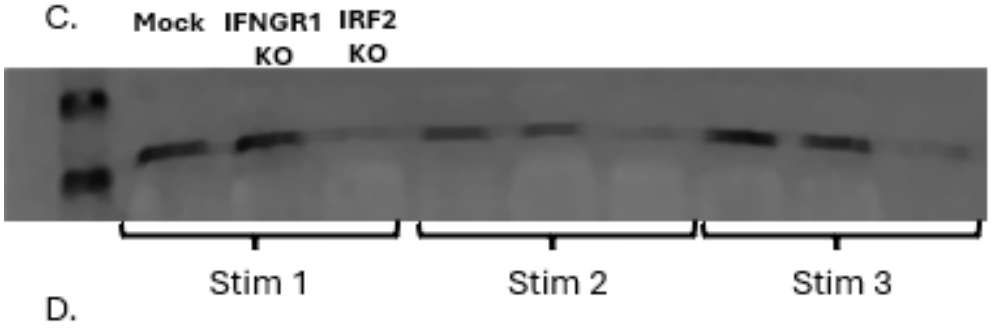
A.


IRF2 KO
Mock
Normalized Signal (800nm)
1000
800
600
400
200
0
Mock
IRF2KO

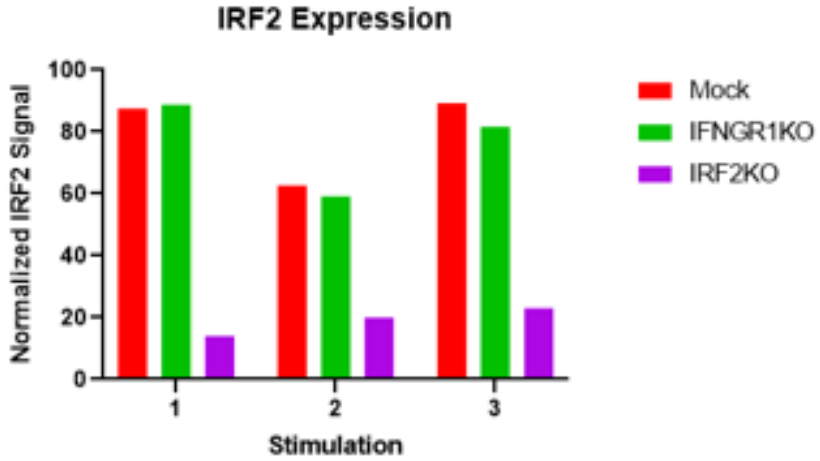
B.


Fold Expansion vs HCT116
Normalized Fold Expansion
2.0
1.5
1.0
0.5
0.0
1
2
3
Stimulation
WT
IFNGR1KO
IRF2KO

C.


Mock
IFNGR1 KO
IRF2 KO
Stim 1
Stim 2
Stim 3

D.


IRF2 Expression
Normalized IRF2 Signal
100
80
60
40
20
0
1
2
3
Stimulation
Mock
IFNGR1KO
IRF2KO

4.0 *Discussion*

The ability to produce IFNγ is commonly used as an indicator of functionality in engineered T cell therapies. Efficacious products generally demonstrate higher levels of IFNγ secretion following stimulation with tumour target, which correlates with enhanced cytotoxic activity and improved anti-tumour responses. Indeed, the presence of IFNγ within the tumour microenvironment and local lymphoid tissue can promote biological processes that enhance anti-tumour activity, including antigen presentation, Th1 differentiation, and optimal function of CD8+ cytotoxic T cells[110,118–120]. As a result, the IFNγ signalling pathway has been largely found to be essential for successful ACT in solid tumours, both in TCR and CAR driven models[94,96]. In striking contrast to its requirement for optimal anti-tumour immunity, IFNγ also enables adaptive resistance in tumour cells, allowing them to evade immune surveillance through upregulation of immunosuppressive gene sets[71,81,90,99,101]. Furthermore, IFNγ is a key driver of cytokine release syndrome (CRS), a severe systemic inflammatory response often observed in patients receiving engineered T cell therapies[121–123]. Thus, finding the optimal balance in IFNγ production—maximizing its anti-tumour benefits while minimizing its contribution to adaptive resistance and CRS—remains a critical challenge in advancing the therapeutic potential of engineered T cell therapies.

The DAP12-based KIR-CAR is an understudied synthetic antigen receptor in the field of ACT. Unlike conventional CARs, which signal through a CD3ζ chain fused to a co-stimulatory domain (generally CD28 or 41BB), KIR-CARs leverage DAP12, an adaptor protein integral to the signaling pathways of various activating receptors, including those found in NK cells[59,62,124]. One of the notable advantages of DAP12 over CD3ζ is its association with enhanced surface stability of the KIR-CAR. Traditional CARs often experience rapid internalization upon activation - likely as a result of the use of the CD3ζ domain - which can diminish their effectiveness over time[42,55]. As a result of these biological differences between conventional CARs and the KIR-CAR, we reasoned that the DAP12 based KIR-CAR may possess different requirements for activation and cytotoxicity, and thus KIR-CAR-engineered T cells may have altered reliance on IFNγ signalling in the context of anti-tumour immunity. Our data reveal that despite the biological differences between receptor platforms, suppression of IFNγ production from KIR-CAR T cells impairs their therapeutic efficacy through reduced cytotoxicity. This reaffirms the importance of IFNγ in the anti-tumour activity of adoptively transferred T cells and extends the universality of the prior observations in CAR and TCR driven platforms by suggesting that the requirement of IFNγ for optimal efficacy may be intrinsic to T cell immunotherapies as a whole.

Our attention to the role of interferon-gamma (IFNγ) in both anti-tumour immunity and tumour adaptive resistance presents a significant shift from previous reports which focused on either anti-tumour immunity or adaptive resistance. As predicted, we observed a bona-fide decrease in expression of immunosuppressive markers *in*-vivo in mice treated with IFNGKO T cells in comparison to Mock T cells, aligning with previous studies that have demonstrated how secretion of IFNγ by CD8 T cells can

induce the upregulation of immunosuppressive factors such as PD-L1 and indoleamine 2,3-dioxygenase (IDO) in the tumour microenvironment[102].

The impaired therapeutic activity observed following treatment with IFNGKO T cells despite reduced adaptive resistance suggests that upregulation of pathways such as PD-L1 and IDO are not a major impediment to adoptive T cell therapies. Increased IFNγ signature has been associated with enhanced responses to immune checkpoint blockade. Indeed, in our earlier report, we demonstrated that ACT in combination with an adenovirus vaccine produced enhanced tumour destruction relative to treatment with either agent alone, and this enhanced tumour destruction was associated with a corresponding increased in adaptive resistance[97]. These combined data enforce the concept that IFNγ induced adaptive resistance is not one of the primary obstacles facing adoptive T cell therapies. This may explain the modest clinical benefit of strategies that seek to simply block PD-1 in the context of adoptive therapies. While ICB therapies have shown promise in enhancing T cell responses against tumours, their efficacy can be highly variable between cancers. For example, ICB has demonstrated modest activity in treating epithelial ovarian cancer, evidenced by a median response rate of 10–15%[125]. Similarly, in the context of triple-negative breast cancer (TNBC), despite high levels of TILs and neoantigen expression, the immunological landscape often does not translate into robust clinical responses. Analysis of these tumour types frequently reveals variable levels of T and NK cell infiltration, with many non-responding tumours showing limited immune cell presence despite PD-L1 expression, indicating that simply inhibiting PD-1 is not enough to enable tumour control in these poorly infiltrated microenvironments[126]. Moreover, the effectiveness of ICB in combination with CAR T cells is often limited by immunosuppressive mechanisms beyond adaptive resistance pathways in the TME, representing distinct challenges that cannot be addressed by targeting PD-1/PD-L1 interactions alone; for example, the strong presence of regulatory T cells and myeloid-derived suppressor cells often observed in advanced solid tumours and during chronic inflammation, which dampen the overall efficacy of combined therapies[127]. Lack of endogenous immune cells in xenograft models may mask some of these shortcomings in pre-clinical models, explaining some of the disparity in efficacy between pre-clinical data and clinical results. Thus, while ICB has the potential to augment the action of CAR T cells through enhanced T cell activation and effector function, the single targeted nature of ICB therapies often results in therapeutic benefit being overshadowed by alternate tumour-induced immunosuppressive pathways.

As an alternative approach, redirecting PD-1 signaling through fusion of the PD-1 extracellular domain with intracellular costimulatory domains appear to have more robust therapeutic benefit. Inhibition of PD-1 does not inherently enhance T cell function, rather it prevents their inactivation via the PD-1/PD-L1 axis, thereby allowing other inhibitory pathways to compensate. However, redirecting PD-1 via switch receptor signalling not only blocks PD-1 induced exhaustion programming, but converts it into favourable signalling like co-stimulation, to enhance T cell function in the face of other immunosuppressive mechanisms. Pre-clinical studies using PD1-CD28 switch receptors have shown them to be efficacious in these settings, however little clinical data exists to

support their use in humans[128–131]. Other strategies, like engineering CAR T cells to secrete a PD-1-blocking scFv, have proved effective at neutralizing PD-1/PD-L1 signaling directly at the effector cell level, leading to enhanced anti-tumour efficacy in pre-clinical models[132]. Similar approaches using bispecific proteins to target multiple immunosuppressive pathways have also shown promise in preclinical models[133]. Future work should focus on strategies to redirect adaptive resistance rather than simply target one of many immunosuppressive pathways.

Despite a wealth of literature describing the importance of IFNγ in anti-tumour responses, particularly in solid tumours, the specific mechanisms through which it exerts its effects remain somewhat ambiguous. It is still inconclusive whether the requirement for IFNγ arises primarily from interactions between IFNγ and T cells, IFNγ and tumour cells, or a combination of both. Interestingly, Larson et al. found IFNγ to not be required for immunity against hematological malignancies in the context of CAR T cells[96]. In contrast, we and others have found opposite results in the context of solid tumours, where T cell production of IFNγ was required for maximal cytotoxicity[81,91,92,95,134–137]. In some cases, loss of IFNGR signalling in tumour cells renders them resistant to immune mediated destruction. At a first glance, this may seem contradictory to the observation that IFNγ promotes adaptive resistance and immune evasion. However, this apparent paradox can be resolved by considering the temporal dynamics and context-dependent effects of IFNγ signaling. The pro-tumour, immune evasive mechanisms induced by IFNγ (such as PD-L1 upregulation and IDO expression) represent compensatory feedback mechanisms that arise secondary to potent anti-tumour effects. The primary functions of IFNγ - i.e. enhancing antigen presentation, recruiting effector cells, promoting direct tumour cell killing, and facilitating cytotoxic granule trafficking - appear to dominate in most immunocompetent settings, particularly during early response phases. This explains the loss of therapeutic efficacy observed when T cells cannot produce IFNγ; reducing adaptive resistance via IFNGKO simultaneously blunts the early anti-tumour response, neutralizing any potential benefit of the KO. This is exacerbated at low E:Ts as we observed, as the early response is inherently weaker in such settings. In the presence of IFNγ, the potency of the early response is conserved resulting in initial tumour control. It is only when tumours persist long enough under continuous immune pressure that the balance of IFNγ signalling shifts toward immune evasion. This dual nature of IFNγ biology can explain why complete ablation of IFNγ production by T cells compromises anti-tumour immunity severely despite alleviating adaptive resistance. The differential requirements between hematological malignancies and solid tumours further highlight the tissue-specific nature of these effects, where factors such as tumour microenvironment, vascularity, and inherent immunogenicity may determine the relative importance of IFNγ-supported cytotoxicity. Thus, rather than viewing tumour-directed paracrine IFNγ signalling as simply pro or anti-tumour, a more nuanced perspective should recognize its essential role in initiating effective anti-tumour immunity, with its pro-tumourigenic effects representing evolutionary adaptations that emerge when initial elimination fails.

While the above discussion focuses on paracrine IFNγ signaling in tumour cells, an equally important consideration is the autocrine response of T cells to IFNγ, and whether signaling to T cells impacts their function differently from the effects of IFNγ on tumours. Evidence from Mazet et al. describes an inhibitory role of autocrine function of T cell derived IFNγ[112]. In the context of a B16F10-OVA murine model, IFNGR1 KO in CD8+ OT1 T cells enhanced tumour control and survival, primarily as a function of enhanced T cell expansion and persistence, indicating chronic exposure to IFNγ restricted the efficacy of the TCR driven platform. To confirm whether this observation held true for our KIR-CAR platform, we generated IFNGR1KO T cells and functionally evaluated them. We found that IFNGR1KO T cells maintained full cytotoxic capacity against tumour targets in-vitro, unlike IFNGKO T cells which showed reduced killing efficiency, suggesting autocrine IFNγ signalling to be dispensable in this setting. Additionally, supplementation with rhIFNγ in our IFNGKO cultures rescued cytotoxicity to Mock levels, while neutralizing IFNγ in both Mock and IFNGR1KO cultures significantly blunted anti-tumour efficacy. Perhaps the most striking evidence supporting our argument that autocrine but not paracrine IFNγ feedback is dispensable for anti-tumour function is the *in-vivo* tumour control data, where IFNGR1KO T cells exhibited equivalent therapeutic efficacy compared to Mock T cells, despite being unable to receive autocrine IFNγ signaling. This stands in stark contrast to the loss of function observed with IFNGKO T cells, highlighting that the critical role of IFNγ in this system is primarily through its paracrine effects on the tumour microenvironment rather than through autocrine conditioning of the T cells themselves.

Our findings regarding the effects of IFNGR1KO on T cell dynamics diverge from previously published results. Unlike the work by Mazet et al, KIR-CAR T cell expansion both *in-vitro* and *in-vivo* was no different between Mock and IFNGR1KO. One explanation for the observed functional differences may be the cell population on which knockout was conducted. Notably, Matzet et al. conducted conditional knockout in mature CD8+ T cells using a transgenic murine cre-lox system, while we performed it in CD3/CD28/CD2 tetramer activated Human PBMCs via CRISPR/Cas9. Canonically, IFNγ plays a crucial role in CD8+ T cell responses, promoting effective differentiation into memory and effector populations, only functioning to restrict T cell function when cells are exposed to persistent, elevated levels of IFNγ. Indeed, in a typical anti-viral response, IFNγR is rapidly downregulated upon receptor binding with IFNγ, and remains low. Upon resolution of the infection, IFNGR1 is re-expressed, aiding in contraction of the immune response[73,138]. While this regulatory mechanism works effectively under normal settings, it may prove overly restrictive in the TME with the continued exposure of T cells to IFNγ, which can drive negative feedback mechanisms and terminal differentiation causing T cells to become prematurely exhausted and allowing for tumour outgrowth. This suggests that while IFNγR signalling in the TME is indeed restrictive due to its chronic nature, initial activation of anti-tumour T cells may still rely on it for optimal performance, requiring a delicate balance between the two. Thus, it is possible by knocking IFNGR1 out early during manufacturing, our IFNGR1KO CD8 T cell population was inherently

more different from the Mock product than we considered, leading to fundamental differences in differentiation and effector function. Alternatively, it is possible that the dose of T cells used in our studies was too high to elucidate more subtle differences in function. Other differences including the use of xenograft models over syngeneic ones, and the different methods of IFNGR1 disruption may also contribute to the observed results.

IFNs simultaneously act as both pro and anti-inflammatory, where initial IFNγ signalling is stimulatory, but chronic exposure drives exhaustion. To best optimize T cell function without knocking out IFNGR entirely, one possible strategy includes promoting expression of key IFN-regulated transcription factors like IRF1, which drives expression of genes essential for effector function, while inhibiting expression of regulatory factors that limit T cell responses. IRF2 functions as a competitive antagonist to IRF1, binding to the same promoter sequence in IFN inducible genes but instead functioning as a transcriptional repressor, not activator. Recently, IRF2 signalling has been shown to promote exhaustion in CD8+ T cells in an IFN dependent manner[113]. As a transcriptional repressor, IRF2 can inhibit the expression of genes that maintain T cell effector function, thereby accelerating the exhaustion program when chronically exposed to IFN signals in the tumour microenvironment. Like IRF1, IRF2 expression is also potently induced by IFN signalling[106]. Thus, we hypothesized knockout of IRF2 would result in increased effector function (cytotoxicity, expansion) in our KIR-CAR T cells by allowing unfettered access for IRF1 to its target sequence. Our evaluations found no benefit to expansion or survival as a result of IRF2KO, which contradicts the results of Lukhele et al. IRF2 expression across multiple rounds of stimulation in our IFNGR1KO T cells was no different than Mock. This indicates that other factors – likely type I IFNs - play a significant role in regulating IRF2 expression. Nevertheless, IRF2KO was not sufficient to prevent T cell exhaustion, as we observed no difference from Mock T cells after its deletion. Differences between our observations and the published report could be a result of numerous elements. We generated our ACT product via CRISPR/Cas9 deletion of IRF2 in activated Human PBMCs, whereas Lukhele et al. utilized a the cre-lox system to remove IRF2 from T cells during development. As IRF2 is involved in development and activation, it is likely that the observed differences are a result of fundamentally different T cell products. We also utilized the KIR-CAR platform, while Lukhele et al. employed a TCR transgenes. To our knowledge, this is the first study to evaluate IRF2KO in the context of human T cells. Further work should focus on understanding the connection between IRF2 and IFNGR signalling. As transcriptional profiling tools like RNAseq become more widely accessible, a deeper investigation of the connections between IFNγ signalling and T cell exhaustion may reveal other avenues to mitigate exhaustion in the TME.

Beyond its dual role in the TME, augmenting IFNγ signalling continues to be an important avenue for improving engineered T cell therapies due to its propensity to exert on-target/off-tumour toxicities through interactions with other immune cell subsets. Clinically, elevated IFNG signature has been associated with greater severity of CRS[121]. Knockout of IFNG in CAR T cells reduced CRS in an *in-vivo/in-vitro* hybrid model of

ALL. Deletion of IFNG in CAR T cells led to reduction in macrophage activation and thus decrease in CRS indicated cytokines, without compromising CAR T cell efficacy[139]. This suggests IFNγ drives CRS through on-target/off-tumour interactions with macrophages. More recently, IFNγ was shown to drive hematotoxicity of healthy hematopoietic stem/progenitor cells (HSPCs) in a model of AML. Due to the similarities between leukemic stem cells and HSPCs, CAR T cell treatments for AML result in significant depletion of the endogenous HSPC pool. However, genetic knockout of IFNG in AML directed CAR T cells allowed for effective tumour clearance without any negative impact on the endogenous HSPC population[140]. Therefore, a deeper understanding of IFNγ signalling will not only enable enhanced therapeutic efficacy for engineered T cell products, but also has the potential to improve the safety and tolerability of these treatments.

This study identifies a role for IFNγ in promoting both optimal cytotoxicity and T cell exhaustion simultaneously in the context of solid tumours. One key limitation to this work is the use of xenograft models for all *in-vivo* analysis. In a physiological setting, T cells are not the only immune cell present in the TME, nor are they the only ones capable of producing and responding to IFNγ. As evidenced in the literature, endogenous immune cells like macrophages, NK cells, and MDSCs all play significant roles in the TME[94]. Through our use of xenograft models, we regressed these factors out, leaving only T cell and tumour cell interactions. While this simplifies the analysis of T cell responses in the presence and absence of IFNγ, it does not accurately capture the complexities of a physiological TME. This difference remains a possible explanation for the divergence of our results and the published literature. Future work should seek to include other accessory and immune cells in *in-vitro* cultures, and utilize syngeneic models where possible.

5.0 Concluding Remarks

Taken together, this study demonstrates that IFNγ is a double-edged sword in the context of KIR-CAR T cell therapies, augmenting cytotoxic efficacy while simultaneously driving adaptive resistance in solid tumours. While our IFNGKO product successfully reduced tumour adaptive resistance, it also displayed decreased efficacy both in vitro and in vivo, indicating that the beneficial effects of IFNγ outweigh the potential negatives ones in the context of adoptive T cell therapies in solid tumours.

While IFNGKO in our KIR-CAR system was detrimental to anti-tumour efficacy despite reducing, enhancing anti-tumour efficacy through modulation of the IFNGR pathway in T cells remains an attractive prospect. The apparent contradictions in the role of IFNγ in the TME may point to opportunities for more nuanced approaches to IFNγ signaling manipulation. Rather than complete ablation of signaling components, targeted modifications of specific downstream pathways, like IRF family members, might selectively preserve beneficial effects while mitigating inhibitory ones. Alternatively, temporal control of IFNγ signaling—allowing initial beneficial signaling while preventing chronic exposure—via inducible gene expression systems, could potentially emerge as a method to optimize T cell function. Strategies such as knockouts, pathway-

specific inhibitors, or engineered receptors with modified signaling domains like an IFNGR-based switch receptor may enable more precise tuning of the IFNγ response. Furthermore, combining IFNGR pathway modulation with additional engineering approaches, such as checkpoint blockade resistance or metabolic enhancements, could potentially overcome the limitations observed in our system and leverage aspects of IFNγ biology to improve therapeutic outcomes.

Ultimately, the insights gained from this study provide a foundation for the development of more effective and refined T cell therapies that harness the full potential of IFNγ while mitigating its undesirable consequences. By unraveling the intricate interplay between IFNγ and engineered T cells, we can pave the way for the next generation of cancer immunotherapies that offer improved efficacy and safety for patients.

Materials and Methods

Lentivirus production

Lentivirus encoding the desired transgene was produced as using a third generation packaging system. Briefly, 12 x 10^6 HEK293T cells plated on a 15-cm dish (NUNC (Thermo Fisher Scientific)) were transfected with plasmids pRSV-REV (6.25 μg), pMD2.G (9 μg), pMDLg-pRRE (12.5 μg), and pCCL (or pCDH for later experiments) HER2 KIR-CAR (or other receptor on a per experiment basis) (32 μg) encapsulated with Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM media (Gibco). 12-16 hours post-transfection, media was exchanged with HEK293 media supplemented with 1 mM sodium butyrate (Sigma-Aldrich). 24-36 hours later, supernatant was harvested, filtered through a 0.45 μm asymmetric polyethersulfone filter (Thermo Fisher Scientific) to remove cellular debris. Viral particles were then concentrated using an Amicon Ultra 15 100 kDa centrifugal filter (Millipore Sigma) and stored at -80°C. Viral titre (TU/mL) was determined post-thaw by serial dilution and transduction of HEK293T cells, and enumeration of percent $NGFR^+$ cells by flow cytometry.

Generation of engineered human T cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy patient donors who provided informed written consent in accordance with the Hamilton Integrated Research Ethics Board, or were collected from commercial leukapheresis products (HemaCare, Northridge, CA and STEMCELL Technologies, Vancouver, BC, Canada). In the case of leukapheresis products, samples were transported at room temperature and processed within 24 hrs of collection. Whole blood was collected from donors using BD CPT sodium heparin collection tubes (BD Biosciences). PBMCs were isolated from blood or leukapheresis by Ficoll-Paque Plus gradient centrifugation (Cytiva, Vancouver, BC, Canada) and cryopreserved in inactivated human AB serum (Corning, Corning, NY) containing 10% DMSO (Sigma-Aldrich), or in CryoStor10 (STEMCELL Technologies) for healthy donors, and RPMI (Gibco) containing 12.5% human serum albumin (Sigma-Aldrich) and 10% DMSO (Sigma-Aldrich) for myeloma patient donors. Samples were cryopreserved in an isopropanol controlled-rate freezer (Thermo Fisher Scientific) at -80°C for 24-72 hrs prior to long term storage in liquid nitrogen. Post-thaw, PBMCs were activated with 25 μL ImmunoCult soluble anti-CD3/28/2 tetrameric complexes (STEMCELL Technologies) per mL PBMC, and cultured in RPMI 1640 (Gibco) containing 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche), 1 mM sodium pyruvate (Sigma-Aldrich), 1 mM non-essential amino acids (Gibco), 55 μM β-mercaptoethanol (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 1.5 ng/mL rhL-2 and 10 ng/mL rhIL-7 (PeproTech, Cranbury, NJ). 16-24 hours later, activated T cells were transduced with applicable lentivirus at an MOI of 1-2. T cells edited by CRISPR/Cas9 were washed with 1x PBS on day 3 to remove soluble activator, otherwise T cells were washed on day 4. Culture yields were enumerated every 2-3d and supplemented with cytokine-containing media to dilute cultures to ~ 1.0×10^6 cells/mL. Engineered T cell products were expanded for a total culture period of 14-15 days prior to use. In all cases, engineered T cell products were cryopreserved prior to use.

In short, after culture cells were cryopreserved in CryoStor10 according to manufacturer's directions at 20×10^6 cells/mL (for downstream *in vitro* assays). Prior to use of cryopreserved engineered T cell products in any *in vitro* assay, cells were thawed according to manufacturer's directions and rested for 24 hrs in cytokine-containing media (as above).

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

Cell lines

Human colorectal cancer cell line HCT116 and glioblastoma line U251 were both kindly provided by Dr. Karen Mossman, McMaster University. SKOV-3 and A549 cell lines were acquired from ATCC. All cell lines were cultured in RPMI 1640 (Gibco (Thermo Fisher Scientific), Waltham, MA), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop Canada Inc., Burlington, ON, Canada), 10 mM HEPES (Roche Diagnostics, Laval, QC, Canada), 1 mM sodium pyruvate (Sigma-Aldrich Canada Co. (Millipore Sigma), Oakville, ON, Canada), 1 mM nonessential amino acids (Gibco), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). To generate luciferase-expressing or NLRed cell lines, parental cell lines were transduced with lentivirus encoding enhanced firefly luciferase (effLuc) or Sartorius proprietary NLRed lentiviral vector(42) as well as puromycin N-acetyltransferase at an MOI 10 and selected in culture media supplemented with 8 µg/mL puromycin (InvivoGen, San Diego, CA). HEK293T cells (kindly provided by Dr. Megan Levings, University of British Columbia, Canada) were cultured in DMEM (Gibco), supplemented with 10% FBS (Gibco), 2 mM L-glutamine (BioShop), 10 mM HEPES (Roche Diagnostics), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco), or 0.1 mg/mL normocin (InvivoGen). All cells were cultured at 37°C, 95% ambient air, 5% CO2, and routinely tested for mycoplasma contamination using the PlasmoTest detection kit (InvivoGen, San Diego, CA, USA).

CRISPR/Cas9 editing of T cells

T cell gene-editing was accomplished by electroporation of complexed gRNA/Cas9 ribonucleoprotein (RNP). To generate RNP, a triple sgRNA pool (Synthego, Redwood City, CA, USA) or negative control gRNA (IDT, Newark, NJ, USA) was complexed with 20 pmol Alt-R HiFi Cas9 V3 (IDT) at a 3:1 sgRNA , Cas9 molar ratio for 10-15 min at room temperature. Prior to electroporation activated T cells were pooled and washed with 1x PBS. $5x10^5$ T cells per electroporation were resuspended in buffer T (Thermo Fisher Scientific) and mixed with complexed RNP (20 pmol on a Cas9 basis) and shocked using a Neon or Neon NxT electroporation system (Thermo Fisher Scientific) set to 1600 V, 10 ms pulse width, and 3 pulses. Immediately after electroporation, T cells were dispensed into antibiotic-free T cell medium containing rhIL-2 and rhIL-7. Genomic DNA was collected from T cells on d14/15 via the Monarch Genomic DNA Extraction Kit, following manufacturer's instructions, and targeted exons were amplified by PCR and sequenced by sanger sequencing. Indel analysis was performed by the Synthego ICE

tool. A table containing all primer and sgRNA sequences used in this thesis can be found below.

Gene	sgRNA sequence	Exon
IFNG sgRNA1	TTTTTAAtAGtACttGtttG	2
IFNG sgRNA2	agctgactaattattcggtg	3
IFNG sgRNA3	cgaaaagctgactaattatt	3
IFNG sgRNA4	gacattcatgtcttccttga	3
IFNG sgRNA5	tgcatcgttttgggttctct	1
IFNG Pool (Synthego)	GAAAUAUACAAGUUAUAUCU	1
	UGCAUCGUUUUGGGUUCUCU	1
	CUUCUUUUACAUAUGGGUCC	1
IFNGR1	UACUCCCAAUAUACGAU	2
	ACUCCCAAUAUACGAUA	2
	GUAACAUUAGUUGGUGU	2

Table 1.1 *sgRNA sequences*

Cytokine production analysis

A total of 5×10^5 engineered T cells were stimulated with were stimulated with 0.05ug/ml PMA (Sigma) and 1uM Ionomycin (Sigma) for 4 hours at 37C and 5% CO2in a 96 well U bottom plate. 1X GolgiPlug (cat. no. 51-2301Kz, BD Biosciences) was added at the start of stimulation. Cells were stained for surface expression of CD4 (BD Pharmingen) and CD8 (eBioscience), fixed, and permeabilized in Cytofix/Cytoperm buffer (cat. no. 51-2090KZ, BD Biosciences), and stained with APC-conjugated mouse anti-human IFNγ (cat. no. 554702, BD Pharminge and FITC-conjugated mouse anti-human TNF-α (cat. no. 554512, BD Pharmingen). Flow cytometry data were acquired and analyzed as indicated above.

RNA Expression Analysis

Tumours were collected from mice on the day of sacrifice and subsequently flash frozen. Tumours were homogenized using the Kinematica Polytron System PT2011 Homogenizer, and RNA was extracted using the QIAGEN AllPrep DNA/RNA/miRNA Universal Kit (cat no. 80224, Qiagen). Reverse transcription was conducted using the Superscript IV First-Strand Synthesis System (cat no. 18091050, ThermoFisher Scientific). The following TaqMan Assay probes from ThermoFisher Scientific were used for qPCR: IRF1 (Assay ID Hs00971965_m), IDO1 (Assay ID Hs00984148_m1), PD-L1 (Assay ID Hs01125296_m1), GAPDH (Assay ID Hs02786624_g1) and ICAM1 (Assay ID Hs00164932_m1). All qPCR data was collected using a StepOnePlus (ThermoFisher Scientific)

In vitro cytotoxicity assay

HCT116 colorectal cancer cells were engineered with Nuclight Red lentivirus (Sartorius, cat. no. 476). SKOV3 and A549 cells were engineered with eGFP lentivirus made in

house. In these next day KIR-CAR αβ T cells were added to the tumour cells at various effector-to-target ratios. When indicated, 50ng/ml rhIFNγ (Stemcell Technologies) or 4ug Anti-IFNγ (BioXCell) were also included. Cells were co-cultured for 4–5 days at 37°C and 5% CO2 in the Sartorius Incucyte S3 Live cell imaging system with nine images per well taken every 8 h. For the HCT116 Nuclight Red cells, the red image mean for each image was used to determine tumour cell groMockh. The area under the groMockh curve (AUC) was analyzed using PRISM GraphPad and used as a metric for tumour cell groMockh for these data. The larger the area, the greater the tumour cell groMockh that occurred over the incubation period. The area under the curve for the tumour alone control control and each condition were used to calculate the % cytotoxicity.

Percent cytotoxicity was calculated as follows:

%Cytotoxicity=((AUCTumourAlone−AUCSample)/AUCTumourAlone)×100%

SKOV3 and A549 analyses were conducted in the same manner, but calculated using green image mean instead of red.

Proliferation assay

HER2 KIR-CAR engineered T cells (5E5 cells) labeled with CTV dye (cat. no. C34557, Invitrogen) were incubated with HCT116 cells, at an effector:target ratio of 1:1, or left unstimulated in media. All proliferation assay samples were incubated for 3 days at 37°C and 5% CO2. Cells were then stained with live/dead fixable near-IR stain (cat. no. L10119, Invitrogen), PerCP-Cy5.5-conjugated mouse anti-human CD8α (cat. no. 45-0088-42, eBioscience), Alexa Fluor 700-conjugated mouse anti-human CD4 (cat. no. 56-0048-82, eBioscience), VioBright FITC-conjugated mouse anti-human NGFR (cat. no. 130-113-423, Miltenyi Biotec), and. Flow cytometry data were acquired as indicated above. Results were analyzed with FCS Express (De Novo Software) by determining the starting generation peak based on the unstimulated sample and using the software proliferation package for fitting a proliferation model and collecting corresponding statistics, such as percent divided.

Mouse Studies

All animal studies were approved by the McMaster Animal

Research Ethics Board. To summarize, 6- to 8-week-old NOD.Cg-Rag1 tm1Mom Il2rg tm1Wjl/SzJ (NRG) mice were bred in-house and injected subcutaneously with $2.5x10^6$ HCT116 or U251 tumour cells. Mice bearing tumours approximately $200mm^3$ (MM1.S) were treated intravenously with $6x10^6$ engineered KIR-CAR T cells. Tumour burden was monitored by caliper measurement. For *in-vivo* T cell tracking experiments, T cell expansion was quantified via bioluminescent imag ing using intraperitoneally injected D luciferin and an IVIS Spectrum. Images were analyzed with Living Image Software v4.2 (PerkinElmer, Waltham, MA, USA). Tumour burden was quantified as the sum of whole-body dorsal and ventral average radiance (p/s/cm 2 /sr). Endpoint was defined as tumour burden greater than $2000mm^3$ or loss of >20% body weight.

Phenotypic characterization of T cell products

Surface expression of HER2 KIR-CAR constructs was determined by staining with recombinant human Her2-Fc protein (R&D Systems, Minneapolis, MN), followed by goat anti-human IgG (to detect Her2-Fc) PE (Cat No. 109-115-098, Jackson ImmunoResearch, West Grove, PA). Other phenotypic markers were detected with Pacific Blue (Cat No. 558116, BD Biosciences, Mississauga, ON, Canada) or AlexaFluor700 (Cat No. 56-0048-82, eBioscience (Thermo Fisher Scientific))-conjugated mouse anti-human CD4, AlexaFluor700 (Cat No. 56-0086-82, eBioscience) or PerCP-Cyanine5.5 (Cat No. 45-0088-42, eBioscience)-conjugated mouse anti-human CD8α, and PE – conjugated mouse anti human IFNGR1 (Cat No. FAB673P, RnD Systems). Staining was assessed by flow cytometry.

Flow cytometry

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

Phospho-STAT signaling characterization of T cell products

Cryopreserved engineered T cells were thawed into T cell media supplemented with rhIL-2 and rhIL-7 and let to rest overnight. Cells were washed and placed into base T cell media without cytokines and rested again overnight. $5x10^5$ HER2 KIR-CAR TAC T cells were stimulated with 0 or 50 ng/mL rhIFNG (Cat No. 200-38, PeproTech) for 1 hour at 37°C, or indicated length for time course experiments. Cells were then immediately fixed with an equal volume Cytofix (BD Biosciences), washed, then permeabilized with Phosflow Perm III (BD Biosciences) for 30 minutes on ice as per manufacturer's instructions. Cells were washed twice with FACS buffer then resuspended in 100 µL FACS per 1x106 cells and stained with PE-conjugated anti-pSTAT1 pY701 (Cat No. 612564, BD Biosciences) as per manufacturer's instructions. Cells were then analyzed by flow cytometry.

In vitro cytokine quantification by enzyme-linked immunosorbent assay

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

Western blotting

T cell IRF1 and IRF2 analysis was accomplished by first stimulating 5x105 Mock, IFNGKO, or IFNGR1KO T cells on 0.05 µg/mL plate-bound HER2-Fc antigen or 0.03ug/ml plate-bound anti-CD3 antibody in a 24-well plate for 4, 8, 12, 16, 20 and 24

hours. After stimulation, T cells were collected, washed twice in 1x PBS, then frozen as cell pellets at -20°C. Protein lysates were generated from cell pellets by lysis with RIPA lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (Roche, Basel, Switzerland) for 15 min on ice, followed by 15 min centrifugation.. Proteins were then transferred to a nitrocellulose membrane, and stained for total protein (Li-COR Biosciences, Lincoln, NE, USA). Immunoblotting was performed overnight at 4°C with the primary antibodies, IRF1 (Cell Signaling Technologies) or IRF2 (AbCam). The next day, bound primary antibodies were probed with secondary antibodies conjugated to either Alexa Fluor680 (Thermo Fisher Scientific) or IRDye 800CW (Li-COR Biosciences) at room temperature for 1 hour, then blots were scanned on the Odyssey Infrared Imaging System (Li-COR Biosciences).and then blotted for IRF1 (Cell Signaling Technologies) or IRF2 (AbCam). All imaging was accomplished using an Odyssey Lx Imaging System (Li-COR Biosciences) set to auto mode. Blots were analyzed using Empiria Software (Li-COR Biosciences) where IRF1 or IRF2, signals were normalized to total protein signal of their respective wells.

Recursive Stimulation Assay

$5x10^5$ Mock or IFNGR1KO HER2 KIR-CAR T cells were cocultured with indicated tumour cell lines at a 1:1 E:T for 3 days. After 3 days, cultures were collected and stained with Pacific Blue CD4 (BD Pharmingen) and AF700 CD8 (eBioscience), Live/DeadFixable NearIR (ThermoFisher Scientific), and BV605 CD3 (BioLegend). Cell count was enumerated using 1 2 3 Count eBeads Counting Beads. Cells were then assessed via flow cytometry, prior to replating T cells and targets at 1:1 E:T based on the FACS enumerated counts for subsequent stimulations.

Statistical analyses. A Student's t-test was used to compare the means of two groups, while a one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test was employed to compare three or more groups within an experiment. The Log-rank (Mantel-Cox) test was used to compare survival curves unless otherwise noted. Statistical significance and p-values were calculated using GraphPad Prism 10.2.3 for macOS. The significance levels were as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = not significant.

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