

Enhancing CAR T cell therapy

By

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ABSTRACT

Adoptive cell therapy using chimeric antigen receptor (CAR) T cells has shown remarkable efficacy in the treatment of haematological malignancies, with complete remission rates of 90% reported in early clinical trials. Following this success, two CD19-targeting CAR T cell products have been approved in Australia for the treatment of Acute Lymphoblastic Leukaemia (ALL) and non-Hodgkin lymphoma. However, acquired resistance to this therapy through CD19 antigen loss is an emerging problem. Moreover, such promising results have not been recapitulated against solid tumours. This is thought to be due in part to poor CAR T cell trafficking and infiltration, the immunosuppressive tumour microenvironment and antigen heterogeneity in solid tumours, as well as intrinsic and acquired tumour resistance. Therefore, it is necessary to uncover mechanisms of resistance to CAR T cell therapy in both the haematological and solid tumour settings, in order to improve patient outcomes.

In this thesis, we sought to uncover the tumour-intrinsic mechanisms of resistance to CAR T cell therapy. Whole-genome loss-of-function CRISPR-Cas9 screening has recently emerged as a powerful tool to screen all protein-coding genes in the genome for conferring resistance to various immune and drug pressures. Herein, we utilised a genome-wide mouse pooled-sgRNA library to investigate genes that protect MC38 mouse adenocarcinoma cells from CAR T cell killing. Here, we identified that loss of cytokine response pathways, in particular, TNF and IFN- γ signalling were critical for the effector function of CAR T cells. Additionally, we report for the first time that loss of the transcriptional co-binding partner *Cbfb* protects tumour cells against T cell killing. Investigation into the mechanisms of *Cbfb*-mediated resistance uncovered that loss of tumour cell *Cbfb* protected tumour cells against T cell-derived TNF. Importantly, we report that mice bearing *Cbfb*-deficient MC38 tumours did not respond to α -PD-1 in vivo, and analysis of clinical trial data suggests that in melanoma cohorts, low expression of CBFB or the transcriptional co-binding partner RUNX1 was correlated with a poorer prognosis following immune checkpoint blockade or adoptive cell therapy.

Next, we sought to design a rational combination therapy to overcome some of the challenges associated with CAR T cell therapy against solid tumours and enhance CAR T cell therapy in this context. Based on data from the CRISPR screen which highlighted the role of TNF in CAR T cell-mediated cytotoxicity, we sought to enhance TNF-mediated killing using a small-molecule smac-mimetic. We demonstrated that antagonism of the Inhibitor of Apoptosis Proteins with the smac-mimetic birinapant, significantly enhanced CAR T cell killing in a TNF-dependent manner. Using a syngeneic HER2⁺ self-antigen model, we report that birinapant significantly enhanced CAR T cell-mediated tumour clearance in a combination therapy approach, and we demonstrated enhanced human CAR T cell killing in patient biopsy-derived tumouroids. Critically, we report that birinapant significantly enhanced TNF-mediated “bystander killing”, which occurred in the absence of target antigen expression, which may be a strategy to overcome the challenge of tumour antigen heterogeneity associated with solid tumours.

Finally, we sought to uncover mechanisms by which B cells lose CD19 antigen expression following CAR T cell attack. By utilising a second genome-wide CRISPR screening approach, we applied a human pooled sgRNA library to the CD19⁺ B-ALL cell line MHH-CALL4, and sorted 3 times the cells with the lowest 20% CD19 surface expression by flow cytometry, in order to uncover genes or pathways of interest that are implicated in down-regulation of CD19 surface expression. Along with several known factors, such as CD81 trafficking and N-linked glycosylation, we also identified several novel processes including histone methylation, aryl hydrocarbon receptor processing and E3 ubiquitin ligase activity, which will be the focus of future investigations.

In summary, insights gained from the work in this thesis elucidate some of the resistance pathways utilised by tumour cells in order to evade immune pressure from CAR T cells, and highlight the cytotoxic potential of TNF in CAR T cell therapy. In addition, we herein present a novel strategy to enhance CAR T cell efficacy in solid tumours, and demonstrate the potential

for combination therapy approaches to overcome the challenges associated with CAR T cell therapy against solid tumours. Taken together, this work significantly addresses several of the existing limitation of CAR T cell therapy in both B cell leukaemias and against solid malignancies, and the novel combination therapy we describe is poised to be rapidly translated in to the clinic.

DECLARATION

This declaration is to certify that:

- i. This thesis comprises only my original work towards the PhD except where indicated in the Preface section.
- ii. Due acknowledgement has been made in the text to all other material used
- iii. The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliography and appendices.

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PREFACE

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PUBLICATIONS

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LIST OF ABBREVIATIONS

A2AR	Adenonsine A2A Receptor
ACK	Ammonium-chloride-potassium
ACT	Adoptive cell therapy
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCR	B cell receptor
CAR	Chimeric antigen receptor
CBA	Cytometric bead array
CCL	Chemokine C-C motif ligand
CCR	Chemokine CC motif receptor
CD	Cluster of differentiation
Ch-IP	Chromatin Immunoprecipitation
CR	Complete response
CRC	Colorectal cancer
CRISPR	Clustered regularly interspaced short palindromic repeats
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FDA	Food and Drug Administration
FDR	False-discovery rate
Foxp3	Forkhead box P3
GFP	Green fluorescent protein
GO	Gene ontology

GSEA	Gene Set Enrichment Analysis
GVHD	Graft versus host disease
Gy	Gray
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
IAP	Inhibitor of Apoptosis Protein
ICAM1	Intercellular Adhesion Molecule 1
ICOS	Inducible T cell co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
IU	International units
IV	Intravenous
KIR	Killer cell immunoglobulin-like receptor
KLR	Killer cell lectin-like receptor
LAG-3	Lymphocyte activation gene 3
mAb	Monoclonal antibody
MDSC	Myeloid derived suppressor cell
MFI	Mean fluorescent intensity
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
NCR	NK cytotoxicity receptor
NFκB	Nuclear factor kappa B
ng	Nanogram
NGS	Next generation sequencing
NK	Natural killer
nm	Nanometre
Ova	Ovalbumin
PAMP	Pathogen associated molecular pattern

PBS	Phosphate-buffered saline
PD-1	Programmed death receptor 1
PD-L1/2	Programmed death ligand 1/2
pg	Picogram
PR	Partial response
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cells expressed and secreted
RNA	Ribonucleic Acid
RPMI	Roswell park memorial institute
RT	Room temperature
ROR γ t	Retinoic acid receptor-related orphan receptor gamma-T
scFv	Single chain variable fragment
SD	Stable disease
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
sgRNA	Single guide RNA
Smac	Second mitochondrial activator of caspases
STAT	Signal transducer and activator of transcription
TAA	Tumour associated antigen
Tbet	T box transcription factor
TCGA	The Cancer Genome Atlas
Tcm	Central Memory T cell
TCR	T cell receptor
Tem	Effector Memory T cell
TGF β	Transforming growth factor beta
Th	T helper
TIGIT	T cell immunoglobulin and ITIM domain
TIL	Tumour-infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin domain containing molecule 3
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF2	TNF receptor-associated protein 2

TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
WT	Wildtype
μCi	Microcurie
μg	Microgram
μL	Microlitre

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CHAPTER 1: Literature Review

1. Introduction to the immune system

The immune system is a complex interplay of host cells, lymphoid organs and effector molecules that has evolved in order to protect the host from a range of pathogens and disease. The immune system can be broadly divided into two arms; the innate and the adaptive immune system. The innate immune system is considered to be the non-specific line of defence, and the adaptive immune system consists of antigen-specific immune responses [1]. While the two are considered to be distinct arms, they are both essential for the health of the host, and a complex interplay exists between them with various shared molecules.

1.1 Innate immunity

The innate immune system is the first line of host defence against invading pathogens and foreign material, and consists of physical barriers, various inflammatory and signalling molecules and cellular immune mediators. Whilst the innate immune system is not responsible for eliciting antigen-specific responses, it is able to distinguish between proteins that are associated with the host, known as “self” and foreign material, known as “non-self” [2]. The innate immune system is broadly protective against a wide range of non-self threats, but does not result in the formation of immunological memory and subsequent or repeated threats result in an immune response of similar orders of magnitude, even when the invading pathogen has been encountered before.

The cellular component of the innate immune system consists of natural killer (NK) cells, neutrophils, macrophages, dendritic cells (DCs), basophils and eosinophils. In order to be protective against pathogens in a non-antigen specific manner, the cellular components of the innate immune system possess pattern recognition receptors (PRRs), which have evolved in order to recognise pathogen associated molecular patterns (PAMPs), which are associated with, and broadly conserved across a wide array of infectious pathogens [3]. These receptors also recognise danger-associated molecular patterns (DAMPs), which are host molecules that are released from damaged

or dying cells in order to signal danger and tissue damage [4]. Activation of the innate immune system releases a variety of pro-inflammatory molecules in order to initiate an inflammatory response.

NK cells are the predominant cytotoxic lymphocyte component of the innate immune system, and are primarily involved in immunological surveillance [5, 6]. NK cell effector function relies on the balance between the binding of germline-encoded activating and inhibitory receptors [7]; killer cell immunoglobulin-like receptor (KIR), killer cell lectin-like receptor (KLR) and NK cytotoxicity receptors (NCR), which respond to ligands on the target cell surface [8]. Inhibitory receptors on NK cells recognise and bind to the constitutively expressed human leukocyte antigen (HLA) surface proteins such as the major histocompatibility complex class I (MHC I) [9], which is expressed on the surface of professional antigen-presenting cells (APCs) and tumour cells. Altered or loss of expression of these proteins, commonly referred to as the 'missing' or 'altered-self hypothesis' [10], enables NK cells to determine if a target cell is "self" or "non-self", if it has been virally infected or has undergone malignant transformation, and allows the destruction of these cells. Following recognition of target cells; NK cells form an immunological synapse that results in killing mediated by the cytotoxic molecules perforin and granzyme B, which will later be discussed in section 2.2.6. In addition, target cell recognition results in NK cell release of a multitude of cytokines to promote the immune response.

1.2 Adaptive immunity

The other arm of the immune system which functions to complement the broad defence offered by the innate immune system, is the antigen-specific adaptive immune system. This can be broadly divided into two central arms; humoral immunity mediated by B cells, so called because they mature in the bone marrow, and cell-mediated immunity which is mediated by T cells, which mature in the thymus. The antigen-specific activation of lymphocytes results in clonal expansion of antigen-specific cells and the subsequent differentiation into effector, helper or memory subtypes. Unlike innate immunity, the adaptive immune system is able to form long-lasting immunological memory towards

specific previously encountered antigens, mediated by memory subtypes.

1.2.1 B cells

Activation of B cells by the cognate antigen via binding to the B cell receptor (BCR) results in the secretion of antibodies that are specific for that antigen, which have an array of protective immunological functions. Antibodies are responsible for the neutralisation of toxins and the opsonisation of pathogens and foreign material [11]. This serves to aid recognition and engulfment by innate immune cells, to activate and propagate the complement cascade, and contribute to cell-mediated immune responses via T cell interactions.

Each BCR has one of the five BCR constant regions that is associated with one of the five antibody isotypes it is able to generate: IgG, IgD, IgM, IgE and IgA. Antibodies contain a hypervariable region, which is extremely diverse and allows for the recognition of a wide range of antigens [12]. Antibodies consist of two distinct regions, the Fab region that binds to the cognate antigen, and the Fc region, which binds to Fc receptors, expressed on an array of innate immune cells that trigger effector function.

Following clonal expansion of antigen-specific B cells due to interactions with their cognate antigen, and the subsequent resolution of the threat, memory B cells also known as plasma cells remain circulating long-term. This forms the basis of immunological memory [13], in a mechanism that has been utilised in the development of vaccination. There are a variety of surface markers that are highly expressed on B cells that differentiate them from other adaptive immune cells. Specific B cell markers include CD (cluster of differentiation) 19, CD20 and CD22, which will later be discussed in Chapter 5. B cells are also known as professional APCs, and express MHC class II, whereby their interaction with T lymphocytes aids the immune response.

1.2.2 T cells

The other major class of immune cells that make up the adaptive immune system, are the T lymphocytes. Whilst B cells elicit their effector function through the generation of antibodies, T cells elicit a cell-mediated effector

function. All T cells rely on the interaction between the T cell receptor (TCR) and MHC class I or class II. The TCR is a membrane bound receptor that comprises of α and β chains, which contain a constant and a variable region [14]. During thymic maturation, T cells experience rearrangement of their germline TCR genes, known as V(D)J recombination, which results in the generation of a vast number of single cells each specific to a particular antigen [15]. During this process, a number of self-reactive T cells will develop by chance, which in the majority of cases will be eliminated in order to prevent auto-reactive T cells and subsequent autoimmunity.

Full activation and effector function of T cells requires three complementary signals. First, recognition of a cognate antigenic peptide displayed on MHC by the specific T cell. Second, a co-stimulatory activation signal such as CD28, binding to its ligands CD80 and CD86 on APCs [16], and adequate inflammatory cytokine stimulation, such as interleukin (IL)-2 or IL-7 [17], which serve to amplify the immune response and to aid T cell survival. TCR recognition of an antigen presented by APCs in the lymph nodes results in extensive antigen-specific proliferation, and subsequent migration to the inflamed tissue.

There are two main subsets of T lymphocytes, distinguished by the presence of CD4 or CD8 cell surface molecules. During their development, T cells begin to express both CD4 and CD8 surface molecules, but following the completion of thymic development, T cells can be broadly classified into $CD4^+$ and $CD8^+$ subsets, which have complementary functions.

1.2.3 $CD4^+$ T helper cells

$CD4^+$ T cells, commonly known as “T helper cells”, highly express the CD4 surface marker, which functions as a co-receptor for TCR-MHC class II ligation. Whilst $CD4^+$ T cells are able to elicit direct cellular cytotoxic functions [18, 19], they are prolific cytokine producers, and therefore their main role is to assist the inflammatory response and other immune cell types through the production and release of inflammatory cytokines and chemokines. The $CD4^+$ T cell subset can be broadly further subdivided into distinct Th lineages

including Th1, Th2, Th17 and regulatory T cells (Tregs), which will briefly be discussed.

The differentiation of CD4⁺ T cells into the Th1 lineage occurs following transcription by T-bet [20], as a result of Signal Transducer And Activator of Transcription (STAT) 1 and 4 activation by IL-12. Th1 cells potentiate cellular immune responses through the production of the cytokines interferon- γ (IFN- γ) and tumour necrosis factor (TNF). Th1 cells are also characterised by their ability to secrete chemokines such as macrophage chemotactic protein-1 (MCP-1) and Regulation on Activation, Normal T cells Expressed and Secreted (RANTES/ CCL5) [21], which act to promote the recruitment of macrophages and other innate immune cells. Th1 T cells are considered a highly inflammatory subtype, and therefore are often associated with a favorable outcome in response to infection and cancer.

Th2 cells differentiate via GATA-3 transcription and activation of STAT6 as a result of stimulation with IL-4 [22]. Th2 cells are classically associated with clearance of infectious pathogens and parasites, the production of IL-4, IL-5, IL-13 and allergic responses through eosinophil recruitment.

CD4⁺ T cells differentiate into Th17 cells due to the transcription of ROR γ t, which is associated with IL-6 stimulation, often in the context of exposure to transforming growth factor beta (TGF β) [23]. Th17 cells are so named because their primary function is the production of IL-17, a cytokine that is associated with inflammation and the recruitment of innate immune subsets such as neutrophils.

Regulatory T cells (Tregs) play a critical role in restraining the immune system in order to limit inappropriate immune responses. Treg differentiation is characterised by the expression of the Forkhead box P3 (Foxp3) transcription factor. Tregs can be further characterised in to two subtypes, natural or induced. Natural Tregs are thymus-derived cells that have a critical role in the prevention of autoimmunity through peripheral tolerance mechanisms. Induced Tregs differentiate from CD4⁺ T cells in the periphery due to

exposure to TGFβ, resulting in the development of FoxP3⁺ Treg cells, or IL-10, resulting in the FoxP3⁻ subtype [24].

1.2.4 CD8⁺ cytotoxic T cells

The main mediators of cellular cytotoxicity are the cytotoxic CD8⁺ T lymphocytes. CD8⁺ T cells are able to specifically recognise antigens presented on MHC I through the TCR and eliminate transformed cells, through the release of granule-associated cytotoxic molecules perforin and granzyme B, which will be discussed in section 2.2.6. CD8⁺ T cells differentiate in to either effector or the two memory subsets; effector memory (T_{em}) and central memory (T_{cm}) [25]. In mice, these populations are associated with differential expression of the surface markers CD62L and CD44 (Figure 1) amongst others, where the memory subsets remain circulating to mediate long-term immunity to previously encountered antigens [26].

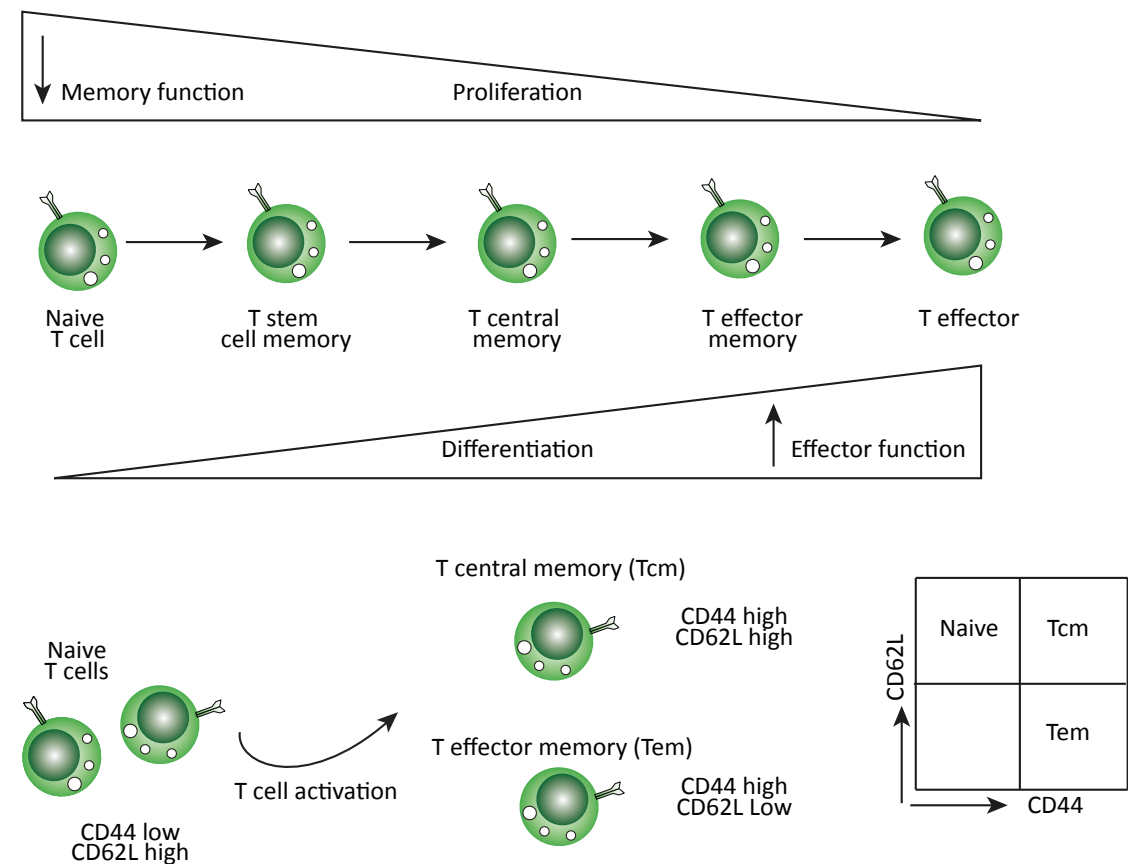


Figure 1. T cell activation results in the formation of memory and effector T cell populations.

Upon T cell activation, receptors that are up-regulated include CD28, OX40, CD137 and inducible T cell co-stimulator (ICOS) [27]. Conversely, a number of inhibitory receptors act to dampen T cell-immune responses in order to prevent autoimmunity as a result of T cell over-activation. Inhibitory receptors include Cytotoxic T lymphocyte associated protein 4 (CTLA-4), Programmed death receptor-1 (PD-1), T cell immunoglobulin and mucin domain containing molecule-3 (TIM-3) and Lymphocyte-activation gene 3 (LAG-3) [28]. These inhibitory receptors are the subject of much research in the context of cancer immunotherapy, and these therapeutic approaches will be discussed in Chapter 1, section 3.

2. The immune system in the context of cancer

Cancer is a broad collection of diseases that can affect nearly every tissue, which is characterised by the malignant transformation and subsequent evasion of apoptosis of cells, that allows for unchecked cell division, neoplastic development and ultimately tumour migration and metastasis. Since the initial observation that immunocompromised mice were more susceptible to developing cancer [29], the role of the immune system in this context has been extensively studied over the last 50 years. A number of innate and adaptive immune cells are involved in the recognition and destruction of malignantly transformed cells, and therefore the immune system plays a critical role in the protection against the development of cancer. The seminal cancer immunosurveillance theory, proposed in 1957 that immune cells continually survey the body in order to recognise and destroy malignantly transformed cells, and therefore the development of cancer represents a fundamental failing of immune surveillance and detection of malignancy [30]. Although not immediately accepted, more recent evidence that tumours from immunodeficient mice are more immunogenic than tumours from immunocompetent mice supports this hypothesis [31]. Immune surveillance by NK cells is the first line of defence against malignantly transformed cells, through the detection of lost or altered MHC class I. Where malignantly transformed cells do not lose MHC expression, often peptide

neoantigens that distinguish them from “self” are displayed on MHC class I where they can be detected by T lymphocytes.

2.1 Cancer immunoediting

Our understanding of the role of the immune system in the development of cancer is concisely described by the principle of immunoediting, where the failure of the immune system to eliminate malignantly transformed cells results in cancer progression [32]. In the first stage of cancer immunoediting, immune cells recognise malignantly transformed cells and efficiently destroy them in order to prevent tumour development, which known as elimination. T cells recognise tumour-associated antigens (TAAs) presented in the context of MHC, and NK cells recognise altered or missing MHC and kill cancerous cells through cell-mediated cytotoxicity.

In optimal conditions, anti-cancer immunity would result in tumour elimination and therefore no further disease progression. When the immune response has been unable to successfully eliminate the tumour, this leads to the equilibrium stage, where malignant tissue remains in a dormant state whilst being controlled by the immune system. Finally, tumour immune escape occurs when the immune system is no longer able to control the developing tumour, and is characterised by tumour growth and the development of the tumour microenvironment (TME).

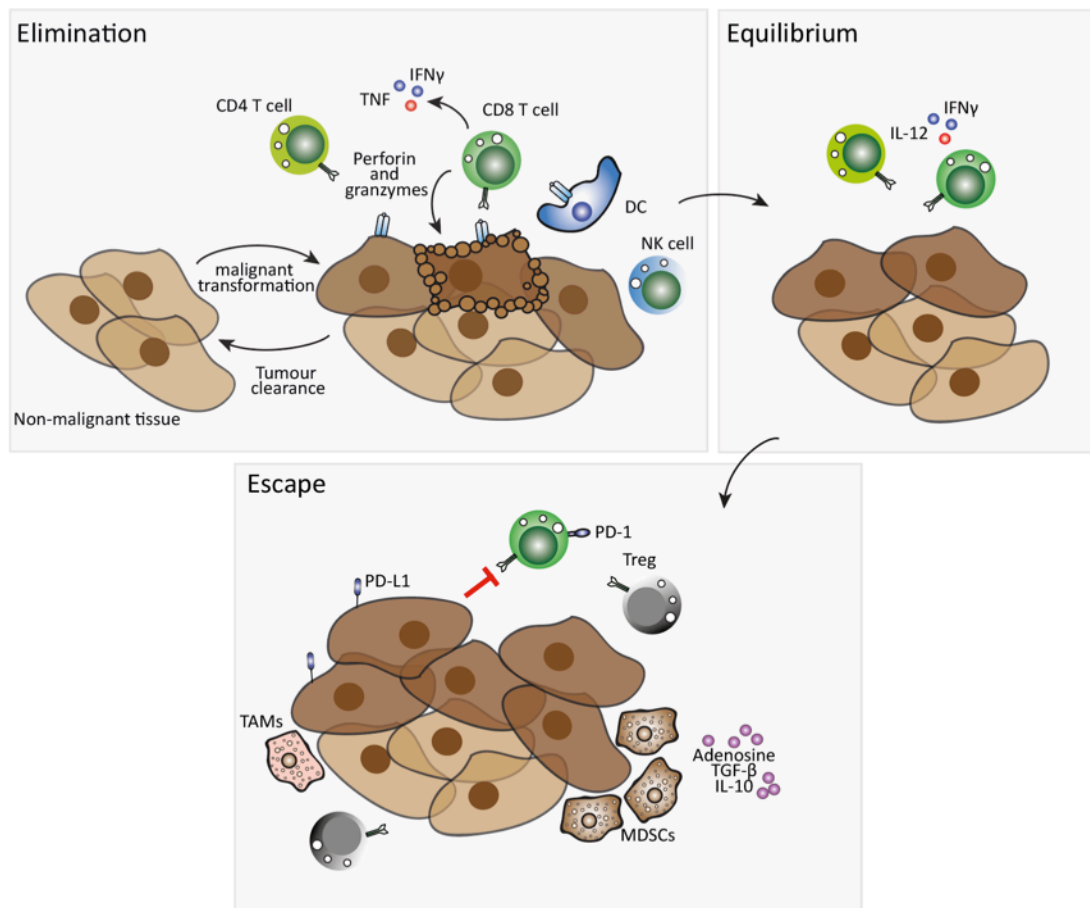


Figure 2. Principles of cancer immunoediting. Three distant phases occur; elimination, where the immune response is adequate to eliminate malignantly transformed cells, equilibrium, where the tumour remains in a dormant state whilst being controlled by the immune response, and escape, where the tumour evades immune control and is able to develop. Adapted from O'Donnell et al, (2019) [33].

2.2 The Cancer immunity cycle

The “cancer immunity cycle”, outlined in Figure 3, explains how T cells are able to recognise malignantly transformed cells, and elicit their cytotoxic anti-cancer effector function, leading to tumour clearance under optimal conditions. The stages of the cancer immunity cycle and challenges associated with them will be discussed in the following sections.

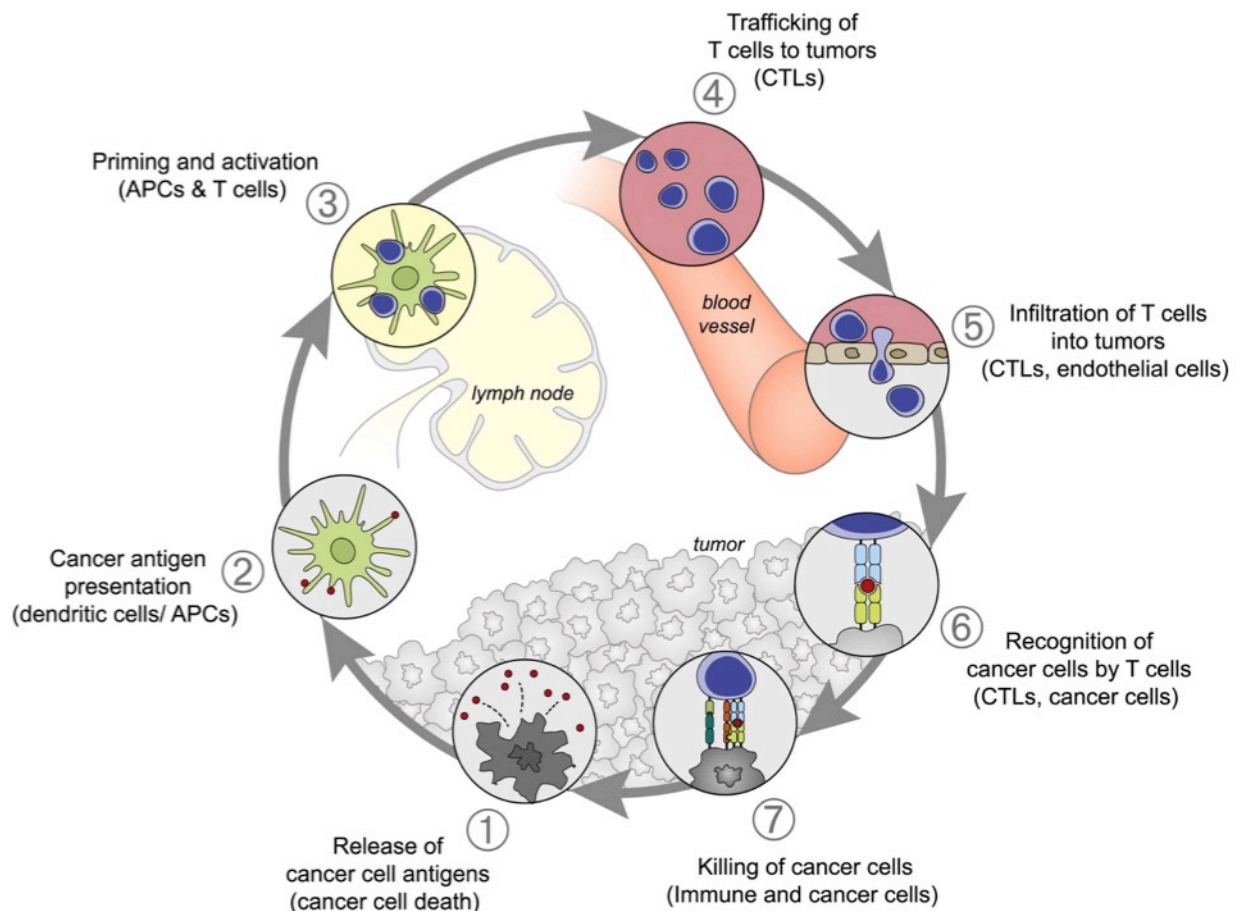


Figure 3. The cancer-immunity cycle outlines the role of T cells in anti-cancer immunity. T cells are primed by neo-antigens presented on APCs, traffic to the tumours and infiltrate the tumour microenvironment and following antigen recognition, kill the malignantly transformed cells. Taken from Chen and Mellman (2013) [34].

2.2.1 Release of cancer cell antigens

The cancer immunity cycle begins when the process of malignant transformation results in the production and release of peptides that are absent from the normal genome, known as neoantigens. In cancers that arise following viral infection, these may include viral epitopes, however in the absence of viral etiology, such antigens are generated as a result of genetic alterations that generate new protein epitopes [35]. As a result, cancers that arise following a number of mutations, known as the mutational burden, are associated with the production of neoantigens and tumour immunogenicity,

which has been implicated in patient prognosis following immunotherapy [36, 37].

2.2.2 Neoantigen presentation

Tumour neoantigens are taken up by dendritic cells (DCs), a type of innate immune cell that primarily function to present antigenic material to T cells. DCs are therefore considered professional APCs and essential mediators of immunity [38]. DCs express a number of receptors for antigen uptake, such as intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [39], which enables antigen recognition, uptake, processing and ultimately the presentation of peptide antigens in the context of MHC I and II.

2.2.3 Priming and activation

DCs enable the priming of CD4⁺ and CD8⁺ T cells through TCR recognition of peptide antigens presented by MHC I and II, respectively, and by providing critical signals for the co-stimulation of T cells, where the expression of co-stimulatory molecules enables effective T cell activation. DCs are also able to produce a range of inflammatory cytokines, which aids the orchestration of the immune response, and effective cell-cell communications. As a result, high DC infiltration in primary tumours is associated with reduced incidence of metastasis [40], while low DC infiltration in tumours is associated with poorer patient outcomes [41], highlighting the importance of DCs in mediating an effective immune response against cancers. Following DC-mediated T cell priming, which typically occurs in the lymph node, a cancer-antigen specific T cell response is mounted, leading to T cell trafficking to the site of the tumour.

2.2.4. Trafficking of T cells to tumours

Following T cell priming and activation, T cells scan for MHC-antigen complexes that are recognised by their specific TCRs. T cells are recruited from the circulation to a lesion through a complex interplay of distinct processes, including adhesion, tethering, chemotaxis and extravasation [42]. Following T cell activation, T cells lose expression of surface molecules such as CD62L, which facilitate lymph node access, and gain expression of homing molecules to aid their trafficking, including chemokine receptors such as

CXCR3 that receive signals from the inflammatory chemokines CXCL9 and CXCL10 [43]. In solid tumour mouse models, the secretion of CXCL9 and CXCL10 by the tumour is associated with enhanced immune recruitment and anti-tumour response [44, 45]. In addition to the tumour cells themselves, macrophages are key producers of chemokines within the tumour [46].

Trafficking of T cells into the tumour is often hampered by dysregulation of tumour blood flow and aberrant angiogenesis. Absent pericytes associated with the tumour result in “leakier” vasculature and inefficient T cell trafficking [47]. This effect is further potentiated by the rapid and uncontrolled proliferation of transformed cells, which results in insufficient blood supply and limited oxygen availability. As a result, hypoxia is a feature of the TME in most solid tumour types [48], which is predictive of a poor outcome [49]. In addition, T cell extravasation is mediated through T cell adhesion events, controlled in part by the expression of trafficking molecules such as intracellular adhesion molecule 1 (ICAM-1) on endothelial cells. Receptors highly expressed by tumour-associated endothelium such as endothelin B receptor (ET_BR), inhibit ICAM-1 expression and suppress T cell adhesion to blood vessels. Tumours with high expression of ET_BR, such as ovarian cancer are associated with poorer T cell infiltration and lower TIL numbers [50].

2.2.5 Infiltration of T cells in to tumours

In addition, during tumour development a myriad of factors result in the development of a metabolically hostile and highly immunosuppressive TME, which facilitates immune exclusion and an immunologically “cold” tumour. The TME is characterised by elevated numbers of immunosuppressive cells, including Tregs, tumour-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC). In addition, the hypoxic environment drives the recruitment of immature myeloid cells, and fosters their differentiation into MDSCs and TAMs, which exacerbates TME hostility [51]. Elevated numbers of Tregs in the TME or circulation have been reported in numerous cancer types, including lung [52], breast [53], liver [54], pancreatic [55] and ovarian [56]. Furthermore, Treg tumour infiltration is associated with a decrease in CD8⁺ T cell to Treg ratio, which correlates with patient prognosis in breast

[57], ovarian [58] and gastric [59] cancers. Tregs highly express several co-inhibitory molecules, which are critically important for their suppressive function, and produce cytokines that favor tumour progression, such as IL-10 and TGF- β .

Soluble mediators that favor tumour progression, such as TGF- β often accumulate in the TME. Produced by both tumour cells and Tregs, TGF- β inhibits the effector function of innate and adaptive immune cells, and TGF- β signalling predicts adverse patient outcomes [60]. The accumulation of TGF- β is associated with decreased NK cell mediated anti-tumour activity [61], and promotes down-regulation of MHC II [62, 63] which may contribute to reduced tumour cell immunogenicity, contributing to the escape of tumour cells from immune surveillance [64]. TGF- β has been implicated in reduced T cell activation, and suppression of the cytotoxic T cell response that promotes tumour development [65]. As a result, TGF- β signalling in the tumour microenvironment is considered a determinant of tumour-mediated T cell exclusion and is associated with poor immunotherapy response [66, 67].

Adenosine is a purine nucleoside that forms from extracellular ATP breakdown by ectoenzymes CD39 and CD73 [68]. In T cells, adenosine primarily signals through the A_{2A}R, a G protein-coupled receptor, which leads to potent inhibition of T cell function through accumulation of intracellular cAMP. CD73 is expressed on tumour cells and immunosuppressive immune cells such as Tregs and MDSCs, and therefore extracellular adenosine accumulates at immunosuppressive concentrations within the TME [69], where CD73 expression negatively correlates with patient prognosis in several tumour types [70-72]. T cells and other immune cells are sensitive to adenosine accumulation, and therefore become excluded from the TME at high concentrations.

2.2.6 T cell killing of cancer cells

Activated CD8⁺ cytotoxic lymphocytes that have successfully trafficked to and infiltrated the solid tumour, can now unleash their potent anti-tumour cytotoxic function, via the recognition of the TCR and the cognate antigen which is

presented by MHC I. Cytotoxic lymphocytes are capable of mediating cellular cytotoxicity through the formation of an immunological synapse between the effector and target cell (Figure 4). Killing of the tumour cells results in the release of additional neo-antigens, from dead and dying cells, which propagates the cancer immunity cycle.

In migrating T cells, as with other migratory cells, actin accumulates at the leading edge, while the microtubule organising centre (MTOC) is organised in a distinctive uropod [73]. Upon target cell recognition, TCR ligation induces robust actin polymerization responses, where actin accumulates at the edge of the T cell-target cell interface [74], which correlates with the translocation of the MTOC and the polarisation of cytotoxic secretory granules to the leading edge [75]. In addition to TCR-MHC interactions, the formation of an immunological synapse is dependent on several adhesion molecules, such as LFA-1, a member of the integrin family, which recognises the ligand ICAM-1 that is expressed on all nucleated cells [76]. Adhesion molecules facilitate sustained cell-cell interactions, antigen recognition and execution of effector functions [77].

During the formation of a synapse, fusion of the cytotoxic granules with the presynaptic membrane results in release of the granule contents, including perforin and granzymes, into the synaptic cleft. Perforin is a monomeric protein that upon exposure to extracellular Ca^{2+} binds to lipid moieties in the target cell membrane and polymerises to form 10-20 nm diameter pores comprising of several subunits [78] in the membrane of target cells. Immune effector cells are resistant to the cytotoxic effects of perforin, due to perforin monomers being more readily able to bind to disordered membranes on target cells, and less able to bind to highly ordered membranes. Therefore, cytotoxic lymphocytes are able to protect themselves from the cytotoxic effects of perforin by incorporating lipid rafts into their membranes in order to readily change the ordered state of the membrane [79].

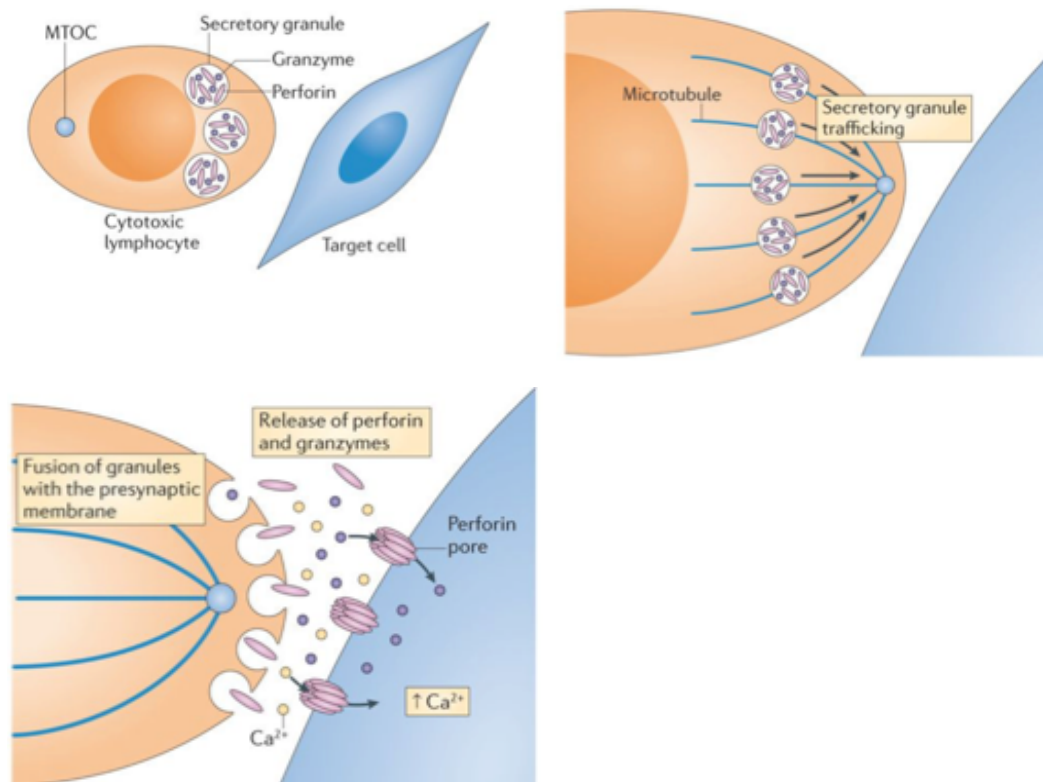


Figure 4. Schematic of the immunological synapse. Upon recognition of a “non-self” or altered cell, cytotoxic lymphocytes cells form an immunological synapse with the target cell, leading to target cell killing. Figure taken from Voskoboinik and Trapani (2015) [80].

The formation of perforin pores allow for the delivery of cytotoxic granzymes into the target cell in order to elicit their cytotoxic functions. 5 distinct granzymes have been identified in humans (A, B, H, K, and M) however, granzyme B is among the most well-characterised and considered most important for mediating cytotoxicity against malignantly transformed or virally infected cells. Granzyme B is a caspase-like serine protease that cleaves acidic residues, in particular Aspartic acid, and induces apoptosis by activating the intrinsic apoptotic pathway in target cells. Granzyme B cleaves the pro-apoptotic protein BH3-interacting-domain death agonist (BID), and induces mitochondrial outer membrane permeabilisation, and subsequently the release of the mitochondrial proteins cytochrome c and second mitochondrial activator of caspases (smac) [81].

Direct cell-contact mediated target cell lysis can also be facilitated through the Fas/Fas ligand pathway [82], or through TNF-related apoptosis-inducing ligand (TRAIL) [83]. T cell-tumour recognition also results in the release of inflammatory cytokines, such as TNF and IFN- γ , which are critically important for the anti-tumour immune response.

2.2.7 The role of IFN- γ in anti-tumour immunity

IFN- γ is a type II interferon that plays a critical role in modulating both innate and adaptive immunity, and is associated with an array of biological functions including apoptosis, inflammation and the cell cycle [84]. In addition, IFN- γ regulates the activation and differentiation of Th1 and Treg cells, restricts Th2 differentiation [85], and enhances NK cell activity. The canonical IFN- γ signalling pathway is activated following IFN- γ binding to its heterodimeric receptor consisting of alpha (IFNGR1) and beta (IFNGR2) subunits. Each subunit promotes activation of the janus-activated kinases JAK1 and JAK2 [86], which function to phosphorylate intracellular binding domains of STAT1 [87]. Activation of the JAK/STAT pathway leads to downstream interferon regulatory factor 1 (Irf1) transcription, which upregulates the expression of MHC I and β -2 microglobulin (B2M) [88], a structural component of MHC, and induces expression of transporter-associated antigen processing (TAP) [89], which is critical for peptide antigen processing. Overall, this facilitates the upregulation of MHC I on the target cell surface (Figure 5), and enhances the immunogenicity of tumours by aiding T cell recognition [90].

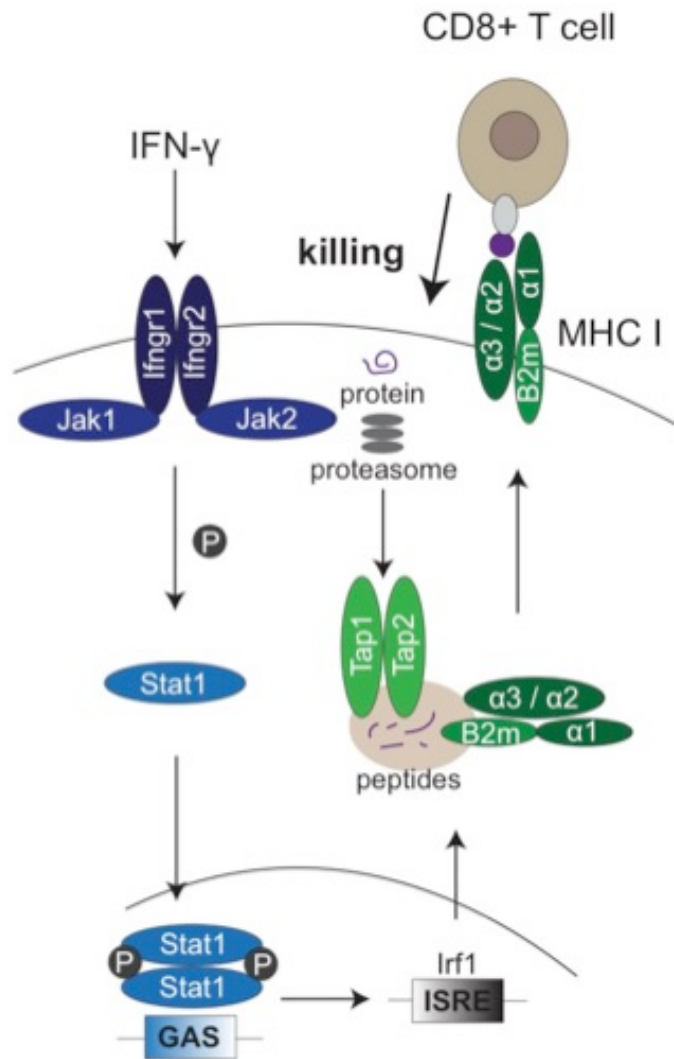


Figure 5. Release of the inflammatory cytokine IFN- γ by immune cells results in the activation of the IFN- γ signalling pathway in target cells. Signalling is mediated through the JAK/ STAT pathway, which results in Irf1 transcription and the upregulation of MHC I, which aids cytotoxic lymphocyte recognition. Figure taken from Freeman et al, (2019) [91].

In addition to the upregulation of antigen presentation, IFN- γ signalling has other roles in facilitating anti-tumour immunity. IFN- γ signalling has cytostatic activity in some cell types [92], and induces chemokine expression, such as RANTES and MIP-1 α [93] which enhances chemotaxis and facilitates a more inflammatory TME. IFN- γ stimulation can also lead to the upregulation of other cell surface molecules including TNF receptors [94], promoting the tumour cell signalling of another major inflammatory cytokine secreted by activated

cytotoxic lymphocytes. TNF has direct cytotoxic activity against some malignantly transformed cells, via the extrinsic apoptosis pathway, and has profound implications for anti-cancer immunity, which will be discussed in greater detail in section 4.

2.2.8 Unconventional T cells in cancer

In addition to the conventional cytotoxic lymphocytes, innate-like unconventional T cells compromise a significant proportion of the human leukocyte population, and many have been implicated in anti-tumour immunity. Mucosal associated invariant T (MAIT) cells, constitute up to 10% of blood CD8 T cells and up to 40% of liver T cells. MAIT cells recognise bacteria-derived vitamin B metabolites, which are presented in the context of the major histocompatibility class I-related gene protein (MR1) [95], and activated MAIT cells retain expression of cytotoxic effector molecules [96] and anti-tumour responses [97]. Another major unconventional T cell subset, the $\gamma\delta$ T cells, so named because their TCR complex is comprised of both TCR γ and δ chains, constitute up to 5% of blood T cells. $\gamma\delta$ T cells recognise phosphorylated non-peptide antigens, known as phosphoantigens, which are typically generated by microbes and malignant cells, which results in an increase in $\gamma\delta$ T cell abundance in infection and cancer. $\gamma\delta$ T cells require the combination of two immunoglobulin superfamily members butyrophilin 2A1 and BTN3A1, in order to recognise these phosphoantigens [98]. Genomic analysis from The Cancer Genome Atlas (TCGA) has identified $\gamma\delta$ T cells as the most prognostically important cell type in cancer, paving the way for anti-cancer properties of unconventional T cells to be elucidated and potentially utilised in immunotherapy approaches [99].

2.3 Resistance to T cell killing

In the context of cancer progression, the cancer-immunity cycle is considered to be performing sub-optimally, and one key area that has been identified is tumour cell resistance to T cell killing. In Chapter 3 of this thesis, we sought to uncover tumour-intrinsic mechanisms of resistance to T cell killing, however known mechanisms of resistance include expression of inhibitory ligands on tumour cells, APCs and immunosuppressive immune cells within the TME.

2.3.1 Inhibitory ligands and immune checkpoint blockade

Inhibitory ligands are part of T cell immunosuppressive pathways utilised by the immune system in order to restrain immune responses and enable the development of immunological tolerance and limit immune-pathologies such as auto-immunity. However, tumour cells hijack such immune checkpoints in order to dampen the immune response mounted against them and enable immune evasion [100]. A number of immune checkpoint molecules have been characterised, leading to the development of therapeutic approaches to overcome the inhibitory interactions. Targeting of these immune checkpoints is the basis of many cancer immunotherapy strategies, and immune checkpoint blockade is a relatively new treatment modality demonstrating robust anti-cancer efficacy in the clinic.

Cytotoxic lymphocytes are efficient killers of cancer cells and unlike conventional cancer treatments, do so with unparalleled specificity for malignant cells whilst leaving healthy cells unaffected. Immune checkpoint blockade aims to boost immune responses to specific cancer-related antigens and enhance T cell activation, proliferation, persistence and killing in an antigen-dependent manner. This is in contrast to traditional treatment strategies, such as chemotherapy, which have broad physiological effects and limited specificity and which results in collateral damage to healthy cells.

2.3.2 Programmed death ligand 1 (PD-1)

The most characterised inhibitory ligand is programmed death-ligand 1 (PD-L1, CD274), which is overexpressed in up to 50% of breast [101] or bladder cancers [102]. PD-L1 binds to Programmed cell death protein 1 (PD-1) receptors on T cells, the ligation of which results in an inhibitory signal being delivered to the cytotoxic lymphocyte, resulting in impaired T cell activation through inhibited kinase signalling downstream of the TCR, and subsequently impaired anti-tumour cytotoxic function. PD-L1 expression on both normal and malignantly transformed cells is upregulated following IFN- γ exposure, as a response to immune pressure. High PD-1 expression on T cells is associated with T cell exhaustion due to chronic antigen stimulation, which may occur in the context of prolonged viral infections, immune tolerance or cancer, and

ultimately can lead to T cell anergy. PDL-1/PD-1 interactions may also promote T cell apoptosis [103], and high PD-L1 expression on tumour cells and upregulation of PD-1 on TILs in the TME is associated with loss of cytotoxic function, which has resulted in considerable research efforts in this area. Subsequently, neutralising antibodies directed against both PD-L1 and PD-1 have been developed in order to inhibit this interaction and therefore block the inhibitory signal being delivered to the T cell by the tumour cells.

In 2014, two monoclonal α -PD-1 antibody products known as Pembrolizumab and Nivolumab, which were designed to neutralise this inhibitory interaction, received FDA approval for the treatment of melanoma. Subsequently, α -PD-1 has been approved for a host of other malignancies including lung cancer, gastric cancer, cervical cancer and renal cell carcinoma [104]. In melanoma, α -PD1 therapy has a clinical partial or complete response rate of around 20% [105], and a 5 year survival rate of 44% [106]. In many cases, it is used both as a first line treatment and as an adjuvant therapy alongside surgery.

In addition to PD-1 blockade, neutralising antibodies have been developed in order to inhibit the other arm of the axis, PD-L1. PD-L1 inhibitors are approved for the treatment of high PD-L1 expressing tumours, such as Merkel cell carcinoma [107] or urothelial carcinoma [108] where clinical response rates are typically between 17 and 30%. A second Programmed death receptor inhibitory ligand, known as PD-L2, has also been identified, and its expression on APCs, including macrophages and DCs has been characterised [109]. PD-L2 presents a less attractive target for cancer immunotherapy due to its more restricted expression. However, its role in the modulation of anti-cancer immunity and in immunotherapy is being investigated, as α -PD-1 therapy has demonstrated enhanced efficacy in both PD-L1 and PDL-2 positive tumours [110].

2.3.3 Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)

Another inhibitory receptor, known as cytotoxic T lymphocyte-associated protein 4 (CTLA-4, CD152) is a CD28 homologue expressed on CD8 T cells upon activation, which binds to co-stimulatory molecules CD80/CD86 on

APCs. CTLA-4 binds to CD80 and CD86 with a higher affinity than CD28, therefore outcompeting CD28 and diminishing the co-stimulatory signal. By limiting CD28 signalling, CTLA-4 increases the activation threshold of T cells, which restrains immune responses against weaker antigens, such as tumour antigens [111]. CTLA-4 is constitutively expressed on Tregs, as an immunosuppressive mechanism of these cells [112]. Along with other inhibitory receptors, exhausted T cells are often characterised by high CTLA-4 expression.

Neutralisation of the inhibitory signal via CTLA-4 blockade with a monoclonal antibody results in enhanced T cell activation, IFN- γ response and enhanced tumour cell clearance [113, 114]. In clinical trials, CTLA-4 blockade demonstrated dose-dependent responses in melanoma patients [115], and improved survival in patients with metastatic disease [116]. Ipilimumab was FDA approved for the treatment of melanoma in 2011, where response rates are typically around 28% [117]. Meta-analyses of metastatic cutaneous melanoma patient outcomes treated with α -CTLA-4 or α -PD-1 compared to conventional chemotherapy demonstrated superior outcomes following immunotherapy, with overall survival at 1 year of 51.2% vs 38.8% [118].

Given the complementary functions of the immune checkpoint molecules, checkpoint blockade is considered more effective when used in combination. Monotherapy with checkpoint inhibitors is associated with the up-regulation of compensatory checkpoints [100], and pre-clinical data suggests enhanced efficacy with the blockade of multiple immune checkpoints. In B16F10 melanoma mouse models, α -CTLA-4 and α -PD-1 combination therapy demonstrated improved anti-tumour efficacy than either monotherapy, an effect that was attributed to increased effector T cell infiltration and an increase in IFN- γ in the tumour, promoting a more inflammatory TME [119].

The mechanism by which dual checkpoint blockade can overcome tumour immune suppression is outlined in Figure 6. In the first clinical trial investigating the use of dual checkpoint blockade, combination therapy was associated with an improved overall response rate, 40% compared to 20% in

the CTLA-4 monotherapy group [120], although adverse events were more common following combination therapy. In larger trials, for the treatment of metastatic melanoma, dual checkpoint blockade of α -PD-1 and α -CTLA-4 results in improved overall survival (52%), compared with either α -PD-1 (44%) or α -CTLA-4 (26%) as a monotherapy [106]. In addition to metastatic melanoma, dual checkpoint blockade efficacy continues to be assessed in clinical trials for a variety of solid tumours including breast, head and neck, cervical and prostate cancer.

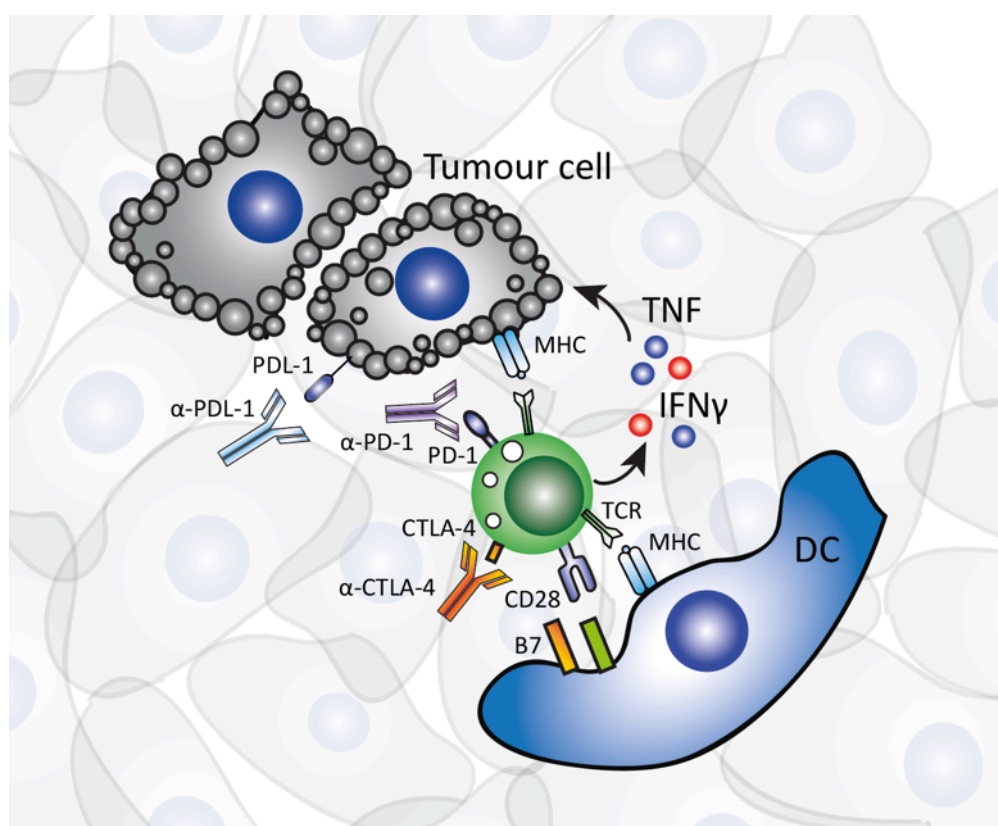


Figure 6. Mechanisms of overcoming tumour-associated immune suppression by utilising α -PD-1 and α -CTLA-4 checkpoint inhibitors.

Following the clinical success of immune checkpoint blockade, immunotherapy has rapidly emerged as the fourth pillar of cancer treatment, where PD-1 inhibitors are part of routine clinical treatment for more than 20 indications. Based on this success, the Nobel Prize for Physiology or Medicine in 2018 was jointly awarded for the discovery of immune checkpoints CTLA-4 and PD-1.

2.3.4 *Acquired resistance*

Despite the success of checkpoint blockade, clinical benefit is somewhat restricted to specific disease types, such as melanoma and metastatic lung cancer, and is associated with high mutational and neoantigen load [121-125]. Therefore, a significant proportion of patients fail to respond to therapy [126, 127], and only a minority of patients experience long-term and durable responses. Resistance to immune checkpoint blockade is an emerging problem and can be broadly classified into primary resistance, referring to patients that do not respond and acquired resistance, regarding patients that respond but later experience disease progression. To overcome primary resistance, checkpoint blockade is typically more efficacious when administered in combination with another cancer therapy, such as conventional chemotherapy [128]. Overall, there remains an unmet clinical need for predictive biomarkers for responsiveness to immune checkpoint blockade.

On the other hand, acquired resistance is an emerging problem, the scale of which appears to differ between cancer types. In a melanoma cohort treated with nivolumab, 39% of responders experienced progressive disease after 5 years [106], however in a non-small cell lung cancer (NSCLC) cohort, 65% were reported to have progressed after 4 years [129]. While many cases have uncertain mechanisms of resistance, several distinct mechanisms have thus far been identified, including defects in antigen presentation, loss of IFN- γ signalling, neoantigen depletion and additional inhibitory checkpoints [130].

Acquired mutations that impair antigen presentation on MHC I have been identified as a reoccurring mechanism of resistance to T cell-mediated immunotherapies. Loss of function mutations in B2M leading to loss of MHC I surface expression, and consequently tumour immune escape following checkpoint blockade have been reported by several groups in a number of cancer types [131-134]. In addition, several clinical reports have demonstrated the acquisition of inactivating mutations in JAK1 and JAK2 [131], or STAT1 [135] resulting in loss of IFN- γ signalling [136]. Given the

importance of IFN- γ signalling in antigen presentation, it is likely that these resistance mechanisms function similarly.

It is evident that checkpoint blockade is most efficacious where strong T cell responses to neoantigens are present, and therefore loss of neoantigens either through clonal selection or epigenetic silencing has been implicated as a resistance mechanism [137]. In NSCLC patients who acquired resistance to checkpoint blockade, analysis of the pre- and post-treatment exome data identified loss of several mutations that were predicted to produce neoantigens [138], suggesting that loss of tumour immunogenicity may be a resistance mechanism. Finally, there are several reports of upregulation of other immune checkpoints, including TIM-3 [139] and LAG-3 [134], at the time of acquired resistance. While the association has not been shown to be causal, these findings suggest that multiple checkpoint blockade combination therapies might demonstrate enhanced efficacy and overcome acquired resistance in some cases.

Identification of mechanisms of acquired resistance to checkpoint blockade paves the way for the development of therapies that can overcome this resistance. Although few successful strategies have been reported to date, therapeutic approaches that are currently being investigated include inhibitors of the RNA-editing enzyme ADAR1 [140], or pharmacological ablation of the histone demethylase, LSD1 [141, 142], which profoundly sensitise resistant tumours checkpoint blockade *in vivo*.

3. Cellular immunotherapy

In addition to the development of antibodies that block the inhibitory arms of the immune system, other forms of immunotherapy, known as cellular immunotherapy, have been developed. Cellular immunotherapies, such as adoptive cell therapy (ACT) and chimeric antigen receptor (CAR) T cell therapy, which are based on the modification of patient-derived immune cells in order to enhance their anti-tumour immunity, will be discussed in the following section.

3.1 Adoptive cell therapy

Adoptive cell therapy (ACT) was one of the first clinically investigated cancer immunotherapies and in some instances has demonstrated robust anti-tumour efficacy. The technique involves isolating tumour-infiltrating lymphocytes (TILs) from within the patient's tumour, and following selection for the appropriate tumour-reactive CD8⁺ T cells and subsequent *ex-vivo* expansion of these cells, they are re-administered in large quantities back into the patient [143]. ACT has shown remarkable promise in the clinic, where tumour regression typically occurs in 20-25% of treated patients [144-147], with up to 50% response rates in refractory metastatic melanoma [148]. The observed clinical efficacy of ACT in metastatic melanoma is likely due to the immunogenicity of this cancer type, which typically has a high mutational burden and neo-antigen expression [149].

Whilst striking clinical results have been observed in some melanoma patients, up to 50% of patients have no clinical benefit following ACT [149]. Factors that are associated with clinical response include the number, differentiation and persistence of the infused TILs [145, 147, 150] and the neoantigen load of the tumour [149].

Whilst ACT has demonstrated positive clinical results in the treatment of metastatic melanoma, these responses have not been observed across the broad range of cancers. This is largely due to the inability to obtain sufficient numbers of tumour-reactive TILs, as the trafficking of the T cells into tumours, and the *in vivo* persistence of these cells is limiting [42, 151]. Furthermore, as ACT is an MHC I-dependent immunotherapy, acquired mutations that cause loss of tumour antigen presentation, is a clinical problem that limits the effectiveness of this therapy.

3.2 Chimeric antigen receptor T cell therapy

Given the clinical promise of ACT, solutions were sought in order to overcome the associated limitations, such as the insufficient numbers of tumour-reactive TILs and the potential for acquired resistance. The main limitation of conventional T cell-mediated immunotherapy is that their application is

restricted to antigens presented by MHC I [152], given their dependency on signalling through the TCR. Therefore, a major advance in ACT within the last decade has been the clinical development of chimeric antigen receptor (CAR) T cells. CAR T cells are based on the idea of combining the antigen-binding properties of an antibody with the cytotoxic function of T cells, in order to redirect the T cells towards any target antigen [153].

Considerable research efforts have enabled the clinical development of CAR T cell therapy, based upon this first conceptual idea. Two CAR T cell products, Kymriah and Yescarta, are now approved for the treatment of relapsed and refractory B cell acute lymphoblastic leukaemia (ALL) in patients under the age of 25. This treatment modality involves the isolation of T cells by apheresis from the peripheral blood of individual patients, and then the genetic engineering of these cells to express a synthetic chimeric antigen receptor that targets a surface antigen expressed on the patient's tumour. Genomic incorporation of the CAR is conventionally achieved through viral transduction, however non-viral genetic manipulation strategies, including CRISPR-Cas9 gene editing are being optimised [154], and are likely to be utilised in the future as part of an optimised CAR T cell generation protocol.

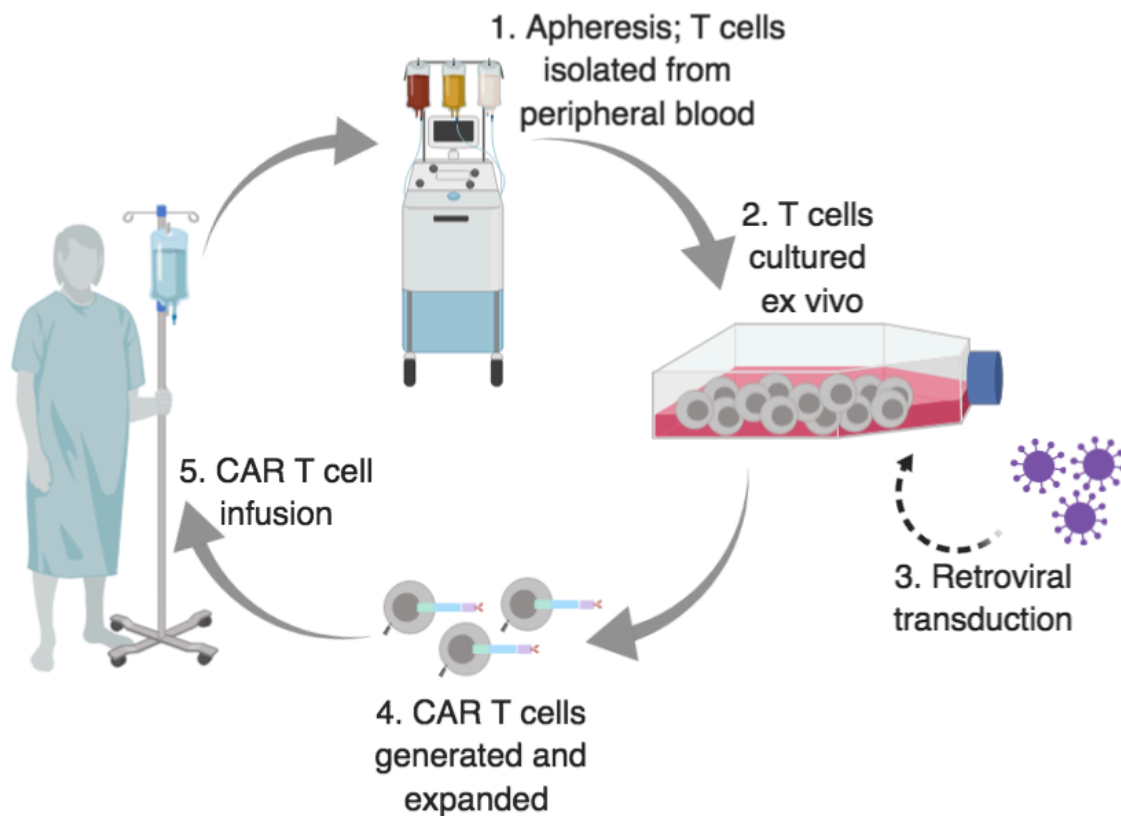


Figure 7. CAR T cell generation for clinical use. T cells are isolated from the peripheral blood of a patient, virally transduced to express a synthetic chimeric antigen receptor and then re-administered into the patient following an ex-vivo expansion protocol.

Critically, a major advantage of CAR T cell therapy is their ability to recognise target surface antigens that are not presented in the context of MHC, and CAR T cell killing occurs independently of the TCR. This is in contrast to other T cell-mediated immunotherapies, which can only recognise the MHC-peptide complex [155]. Furthermore, unlike endogenous conventional CD8⁺ T cells, CAR T cells are also able to recognise lipid and carbohydrate targets [156].

3.2.1 Chimeric antigen receptor construct and design

First-generation chimeric antigen receptors constitute synthetic receptors composed of 3 distinct domains:

1. An extracellular antigen-specific target-binding domain, derived from the single chain variable fragment (ScFv) of an antibody against a target surface antigen.
2. A transmembrane hinge fragment derived from CD8 [157] or IgG-based [158].
3. An intracellular domain, consisting of CD3 ζ T cell signalling molecule or Fc receptor γ chain [159].

Due to limited activation of the first generation CAR T cells [153, 160], attempts were made to further enhance their activation through the incorporation of the CD28 co-stimulatory signalling domain into the CAR construct, adjacent to the ζ domain [161]. These were termed second-generation CAR T cells, which showed superior activation and enhanced anti-tumour efficacy [162]. The structure of the second-generation CAR T cells that were used throughout this thesis is outlined in Figure 8.

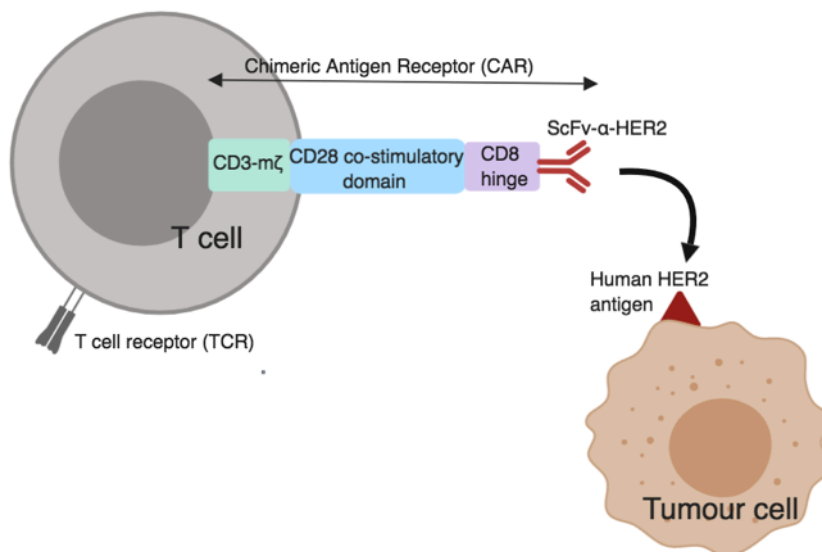


Figure 8. Schematic of HER2-targeting CAR T cell construct used throughout this thesis. Second-generation CAR T cell contains distinct domains; CD3 ζ activating domain, transmembrane CD28 co-stimulatory domain, CD8 hinge and HER2 targeting ScFv. Figure adapted from Michie et al, (2019) [163].

Other co-stimulatory domains have been incorporated in to the CAR, in place of CD28, such as 4-1BB. Both CD28 and 4-1BB CAR T cells are approved for clinical use, however 4-1BB CAR T cells are considered to have enhanced *in vivo* persistence in the clinic [164, 165]. Third-generation CAR T cells have been developed by incorporating a third co-signalling molecule, such as both CD28 and 4-1BB or OX40, however their safety and advantages in a clinical setting over second generation CAR T cells continue to be investigated [166, 167]. Potential pitfalls to this approach might include increased toxicity, or CAR T cell tonic activation leading to T cell anergy or exhaustion [168].

3.2.2 Clinical success in haematological malignancies

Clinically, the adoptive transfer of CAR T cells has proven hugely successful in the treatment of haematological cancers, where complete remission rates of up to 90% have been reported in patients with relapsed acute lymphoblastic leukemia (ALL), following infusion of CAR T cells targeting the B cell-specific antigen CD19 [157, 169]. CD19 is an excellent CAR T cell target, as it is exclusively expressed on B cells during all stages of differentiation and maintained on B cells that have undergone malignant transformation [170]. Subsequently, the first CD19-directed CAR T cell product was FDA approved in 2017, paving the way for CAR T cell therapy development for the treatment of other malignancies.

3.2.3 CAR T cell relapse

Although CAR T cells do not require MHC I-mediated antigen presentation, resistance has emerged as a problem in a subset of patients. In approximately 25% of patients [171], immune evasion of CD19-CAR T cell therapy often occurs due to the emergence of antigen negative clones, which in some cases are present at treatment onset [172], or are subsequently acquired. Clinically, resistance has emerged through acquired CD19 mutations or alternative splicing events [173] or through acquisition of a CD19-negative myeloid phenotype, known as lineage switching [174]. The more durable the clinical response to CD19-CAR T cell therapy, the greater the selection pressure for the development of CD19⁻ clones, which in turn makes antigen negative relapse more likely [175]. There is currently an unmet

need to uncover the specific mechanisms of CD19 antigen loss in order to prevent antigen-negative relapse. This question forms the basis of Aim 3, and will be discussed in further detail in Chapter 5.

3.2.4 CAR T cell limitations in solid tumours

Whilst the success of CAR T cell therapy in haematological cancers has been celebrated, CAR T cell therapy against solid tumour has shown less promising results to date. A number of clinical trials have investigated the efficacy of CAR T cells designed to target a variety of antigens including human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), prostate-specific membrane antigen (PSMA) and disialoganglioside (GD2) for the treatment of solid tumours. However, meta-analyses of CAR T cell therapy in solid tumours show complete response rates between 4.1% [176] and 9% [177], compared to 54.4% complete response rate for CD19-CAR T cell therapy [176]. Several factors limit the efficacy of CAR T cell therapy against solid tumours, including availability of an appropriate antigen and antigen heterogeneity, T cell homing and penetration and the hostile tumour microenvironment.

As previously discussed, CD19 constitutes a suitable CAR T cell target antigen, due to its near ubiquitous expression on B cells at all maturation stages. However CD19-directed CAR T cell therapy results in long-term B cell aplasia, as the antigen is not specific for malignantly transformed cells, which results in cancer remission but also chronic hypogammaglobulinemia and susceptibility to infection. However, CD19 is an ideal target because immune function can be restored following regular treatment with maintenance IgG replacement therapy, and therefore the patient does not suffer long-term health implications due to CAR T cell on-target but off-tumour “collateral damage”. In contrast, such off-tumour cytotoxicity arising from CAR T cells in the solid tumour context would be clinically unacceptable and potentially life threatening.

Unlike B cell malignancies, solid tumours rarely express one specific tumour antigen, which has limited CAR T cell usage against solid tumours thus far. In

an attempt to recapitulate the success of CD19-directed CAR T cell therapy, tumour associated antigens that are enriched on the tumour cell but expressed at low levels in normal tissue have been identified for solid tumours [178]. CAR T cells targeting a wide range of target antigens including HER2 [179], EGFR [180] and NY-ESO-1 [154, 181] have been developed and investigated in clinical trials, albeit to limited clinical success. Tumour heterogeneity often results in poor antigen distribution through the solid tumour, and subsequent escape of antigen-negative variants [182, 183]. Therefore, target antigen selection for solid tumours requires careful consideration.

As previously discussed as part of the cancer immunity cycle, several factors limit T cell trafficking and penetration in to the solid TME, which also present considerable challenges for CAR T cell therapy. One reason for excellent CAR T cell efficacy against haematological malignancies is that the T cells encounter tumour cell targets within the circulation [184]. In stark contrast, in solid tumours CAR T cells are required to traffic to the tumour site, and subsequently penetrate the hostile TME to elicit anti-tumour cytotoxicity. The number of infiltrating T cells positively correlates with disease outcome and response to immunotherapy in a number of cancer types [185], including breast [186, 187] and colon [188]. Therefore, trafficking of T cells into the tumour microenvironment is critical for the success of immunotherapies, and poor trafficking into the tumour is believed to be one of the reasons for poor CAR T cell efficacy *in vivo* [42].

Additionally, the site of the tumour and the microenvironment in which it forms can alter response to immunotherapies [189]. The hostile TME hinders CAR T cell migration and is one of the reasons that CAR T cell therapy against solid tumours results in significantly poorer clinical responses compared to haematological malignancies [190]. As previously discussed, the TME often includes the presence of immunosuppressive molecules such as PD-L1, and both endogenous T cells and adoptively transferred CAR T cells are susceptible to exhaustion and elevated expression of PD-1 [191] under these conditions.

3.2.5 CAR T cell combination therapies

In order to overcome these limiting factors associated with the treatment of solid tumours, there have been considerable research efforts to design rational CAR T cell combination therapies. As with endogenous T cells, neutralising the PDL-1/ PD-1 inhibitory interaction with checkpoint blockade has shown to enhance CAR T cell efficacy pre-clinically [192], and in clinical trials [193]. Another approach has been to combat CAR T cell exhaustion induced by tumour PD-L1 expression by engineering CAR T cells that secrete α -PD-1 [194] or α -PD-L1 [195] monoclonal antibodies, which has demonstrated enhanced efficacy *in vivo*. Dual checkpoint blockade of α -PD-1 and α -CTLA-4 enhances adoptively transferred tumour-reactive T cells [196] and EGFR-targeting CAR T cells secreting both α -PD-1 and α -CTLA-4 for the treatment of advanced solid tumours are in clinical trials (NCT03182816).

Combination therapies designed to overcome the inhibitory properties of soluble mediators have also been reported. Blockade of T cell adenosine signalling with an A2AR antagonist enhances CAR T cell efficacy *in vivo*, which was potentiated by additional PD-1 blockade [197]. Blockade of TGF β enhances anti-tumour activity of TCR modified T cells [198], and deletion of TGF- β receptor II (TGFB2) in CAR T cells enhances their efficacy against solid tumours *in vivo* [199].

In addition to blockade of inhibitory ligands and soluble immune mediators, there are a number of activating ligands expressed by T cells, including 4-1BB and OX40, and there may be a role for the use of T cell activating agonistic antibodies to enhance CAR T cell killing. Second generation CAR T cells incorporating a CD28 co-stimulatory domain can be enhanced by the concurrent administration of α -4-1BB agonist antibodies, which activates the 4-1BB activation pathway, resulting in increased CAR T cell proliferation, IFN- γ expression, and reduced host immunosuppressive cells at the tumour site [200]. In addition to 4-1BB, other T cell activation receptors such as OX-40 have been incorporated in to the chimeric antigen receptor as a co-stimulatory domain, however the potential to enhance CAR T cell killing via combination therapy with agonist antibodies is still being investigated. Taken together,

these studies highlight the potential for CAR T cell therapy in the context of a combination therapy approach, which might overcome some of the challenges associated with the treatment of solid tumours.

4. Augmenting TNF to enhance immunotherapy

T cells rely, in part, on their ability to produce cytokines in order to elicit their cytotoxic function. In particular, TNF can induce tumour cell apoptosis directly in a number of tumour types [201] and disabling the TNF-signalling pathway has been identified as a mechanism of tumour immune evasion [202, 203]. Furthermore, TNF secretion by CAR T cells predicts enhanced efficacy in patients and is associated with a better response to treatment [175]. Given the potent cytotoxic function of CTL-derived TNF in cancer immunotherapy, considerable research efforts are directed to enhancing the tumour immune response as a mechanism to augment and enhance immunotherapy [202-204]. One such strategy that might be employed to enhance the tumour TNF response in the context of immunotherapy is by targeting endogenous proteins within the TNF-signalling pathways, such as the Inhibitor of Apoptosis Proteins (IAPs) [163, 204, 205], or TNF receptor-associated protein 2 (TRAF2) [203].

4.1 Publication in Cells

There is increasing evidence of the role of TNF in determining response to immunotherapies, and research in to strategies that augment the tumour responsiveness to TNF is on-going. One such treatment strategy is the antagonism of IAPs. In Chapter 4 of this thesis and in the context of Aim 2, we identify for the first time that antagonism of the IAPs, with a clinical smac-mimetic, enhances CAR T cell efficacy. The rest of this section in Chapter 1 is a published review in the journal *Cells*, where the role of TNF and the immuno-modulatory properties of IAP antagonists in cancer immunotherapy is extensively discussed, as per the following reference:

Michie, J, Kearney, C.J, Silke, J, Hawkins, E.D, and J Oliaro. 2020 'The Immuno-modulatory effects of IAP antagonists in cancer immunotherapy', *Cells* 9(1), 207.

Review

The Immuno-Modulatory Effects of Inhibitor of Apoptosis Protein Antagonists in Cancer Immunotherapy

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Abstract: One of the hallmarks of cancer cells is their ability to evade cell death via apoptosis. The inhibitor of apoptosis proteins (IAPs) are a family of proteins that act to promote cell survival. For this reason, upregulation of IAPs is associated with a number of cancer types as a mechanism of resistance to cell death and chemotherapy. As such, IAPs are considered a promising therapeutic target for cancer treatment, based on the role of IAPs in resistance to apoptosis, tumour progression and poor patient prognosis. The mitochondrial protein smac (second mitochondrial activator of caspases), is an endogenous inhibitor of IAPs, and several small molecule mimetics of smac (smac-mimetics) have been developed in order to antagonise IAPs in cancer cells and restore sensitivity to apoptotic stimuli. However, recent studies have revealed that smac-mimetics have broader effects than was first attributed. It is now understood that they are key regulators of innate immune signalling and have wide reaching immuno-modulatory properties. As such, they are ideal candidates for immunotherapy combinations. Pre-clinically, successful combination therapies incorporating smac-mimetics and oncolytic viruses, as with chimeric antigen receptor (CAR) T cell therapy, have been reported, and clinical trials incorporating smac-mimetics and immune checkpoint blockade are ongoing. Here, the potential of IAP antagonism to enhance immunotherapy strategies for the treatment of cancer will be discussed.

Keywords: smac-mimetics; TNF; cancer immunotherapy; checkpoint blockade; CAR T cells

1. Inhibitor of Apoptosis Proteins

The capacity to evade apoptosis, a form of physiological cell death that relies on the activation of a family of cysteine proteases known as caspases [1], is a common trait of malignantly transformed cells [2]. During apoptotic cell death, endogenous second mitochondrial activator of caspases/Direct IAP-Binding Protein With Low PI (smac/DIABLO), is released from the mitochondrial inter-membrane space where it binds to, and inhibits, the three major inhibitor of apoptosis proteins; cellular IAP 1 (cIAP1, *BIRC2*) and 2 (cIAP2, *BIRC3*) and X-linked IAP (XIAP, *BIRC4*) [3,4]. The inhibitor of apoptosis (IAP) proteins are a family of endogenous proteins that function as key regulators of caspase activity, and are defined by the presence of at least one Baculoviral IAP Repeat (BIR) domain. These approximately 70-residue zinc-binding domains enable their interaction with, and suppression of, caspases, and therefore facilitate the inhibition of apoptosis [5]. Only XIAP is a potent direct inhibitor of caspases, however, the physiological significance of this activity is unclear, because cells from patients with XIAP mutations [6] and murine XIAP knockout mice, are not more

sensitive to apoptosis than wild type cells [7]. Importantly, IAPs also contain a RING finger E3 ligase domain at the C-terminus [8,9], enabling these proteins to participate in diverse cellular processes, including signal transduction events that promote inflammation, cell cycle progression and migration. Notably, IAPs are critical regulators of both canonical and alternative (non-canonical) nuclear factor kappa light-chain enhancer of activated B cells (NF- κ B) signalling, downstream of various members of the Tumour Necrosis Factor Receptors Superfamily (TNFRSF).

1.1. Inhibitor of Apoptosis Proteins in NF- κ B Signalling

IAPs are required for the activation of the canonical NF- κ B pathway downstream of several receptors [10,11]. One of the best studied is downstream of TNF Receptor 1 (TNFR1) (Figure 1). In this pathway, TNFR1 ligation by TNF results in the formation of a complex comprising RIPK1, TRADD, and TRAF2 (Complex I), where TRAF2 is the primary factor required for the recruitment of IAPs [12–14]. IAPs ubiquitylate several components within this complex, although the best studied is RIPK1 [15–18]. The downstream signalling pathway consists of the trimeric canonical I κ B kinase (IKK) complex, composed of IKK α and IKK β subunits, as well as the regulatory subunit IKK γ (also known as NF- κ B essential modulator (NEMO)). IAP-mediated ubiquitylation of Complex I mediates the recruitment of the linear ubiquitin chain assembly complex (LUBAC) [19], which is comprised of HOIL-1L, HOIP and Sharpin [20]. LUBAC generates M1 linked ubiquitin chains on Complex I components such as RIPK1 and IKK γ [21], which stabilizes Complex I and allows full activation of the IKK complex (consisting of IKK1, IKK2 and IKK γ /NEMO) and a TAK1 containing complex. IKK2 phosphorylates I κ B α , resulting in its proteasomal degradation and the release of the p50 and p65/RelA NF- κ B heterodimer, which allows their translocation to the nucleus [22,23], while TAK1 activation leads to activation of the MAPK pathway. This results in the induction of pro-survival and inflammatory transcriptional programs [24].

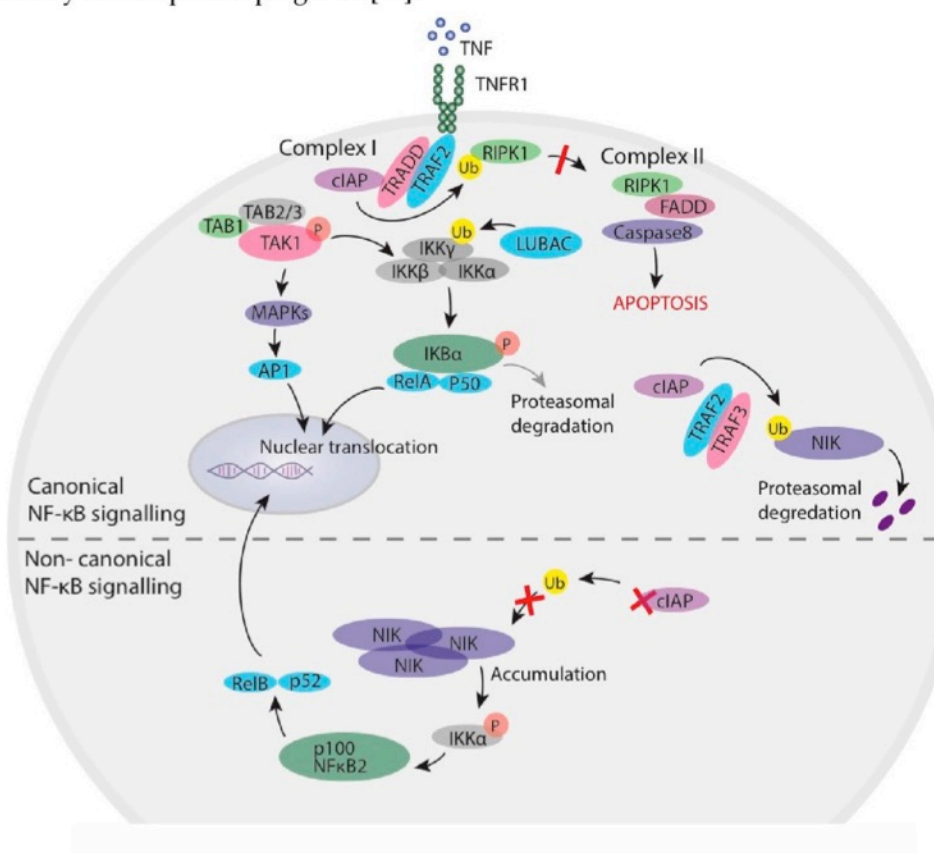


Figure 1. The Inhibitor of Apoptosis Proteins (IAPs) are critical regulators of both canonical and non-canonical NF- κ B signalling. During canonical NF- κ B signalling, the ubiquitylation of Complex I

components by cIAPs results in the nuclear translocation and activation of pro-survival canonical NF- κ B and limits the formation of pro-apoptotic Complex II. cIAPs also target NIK for proteasomal degradation preventing the activation of non-canonical NF- κ B. Loss of IAPs results in the formation of Complex II and activates caspase-mediated apoptosis, and results in the accumulation of NIK, which causes downstream non-canonical NF- κ B activation.

IAP-mediated ubiquitylation of RIPK1 in Complex I also limits RIPK1 association with FADD and caspase 8 to form the ripoptosome (Complex II) [25]. Together MAPK, IKK activation and IAP ubiquitylation therefore suppress TNF induced apoptosis. As a result, antagonism, or the absence of, IAPs results in signalling through TNFR1 that activates caspase-mediated apoptosis, rather than the induction of NF- κ B pro-survival signalling [26–28]. IAPs also inhibit cell death induced by the TNFSF death ligands, FasL and TRAIL, as well as chemotherapy agents [28–31]. In these cases, it is likely that preventing RIPK1 from generating a FADD caspase-8 activating complex is the primary mechanism of inhibition, while NF- κ B activation plays a less significant role.

The cellular IAPs (cIAPs) also have a critical role in the regulation of NF- κ B-inducing kinase (NIK) through K-48 ubiquitylation; a process that leads to its continuous degradation and therefore low-level expression [26,27]. Therefore, the loss or inhibition of IAPs leads to NIK stabilization and accumulation, phosphorylation and activation of IKK α , phosphorylation and processing of p100 (NF κ B2) and nuclear translocation of the p52 and RelB NF- κ B heterodimer; resulting in activation of the so-called non-canonical NF- κ B pathway.

1.2. IAPs and Smac-Mimetics in Cancer

IAPs are amplified in approximately 3% of cancers [32], and several IAPs can act as proto-oncogenes as a result of genetic alterations in various cancer types [33]. As IAPs promote cell survival in response to TNFSF ligands and chemotherapy, their upregulation renders cancer cells resistant to the cytotoxic effects of these agents, and is associated with failure of treatment and poor prognosis in multiple cancer types [24,25,34]. For example, XIAP expression has been implicated in cisplatin resistance in ovarian cancer [35], correlated with disease severity in AML [36], and is a prognostic indicator in renal cell carcinoma [37]. Following the discovery of a potent antagonist of XIAP [38], small molecule mimetics of smac/DIABLO (smac-mimetics; SMs) were developed to overcome the survival benefits of IAP upregulation in cancer cells, through their antagonism of IAP activity and promotion of tumour cell death [39–42]. SMs promote IAP degradation by binding to the BIR3 domain of cIAPs, which allows the RING to dimerize, and in turn, activate the E3 ubiquitin ligase function of the cIAPs [26,27,43]. As they are not bound to a natural substrate such as NIK, this results in their own ubiquitylation and degradation [44,45]. Although SMs also bind to the BIR3 of XIAP, very few of them promote degradation of XIAP, although they nevertheless can antagonise normal XIAP function [11,46,47].

A number of SMs have been developed over the past decade with differing chemistries, including monovalent and bivalent varieties, which both mimic the N-terminus of SMAC and subsequently promote RIPK1-caspase8 complex formation and apoptosis. However, bivalent SMs are typically more cytotoxic than the structurally related monovalent IAP antagonists. Biochemical analyses of various monovalent and bivalent SMs have revealed that bivalency promotes cIAP1 degradation more effectively than monovalent IAP antagonists, and monovalent SMs are also less effective at depleting TRAF2-associated cIAP1, and thus inhibiting canonical NF- κ B signalling [48]. SMs not only degrade IAPs to promote tumor cell death, but also induce the production of TNF in a subset of tumor cells, which can act in an autocrine or paracrine manner to cause tumor cell death [24,26,27,49,50]. In this manner, single agent efficacy of SMs has been reported, particularly for the treatment of certain leukaemias [51–53]. However, these monotherapy studies have shown that, for the most part, antagonism of IAPs alone does not result in immediate cell death; rather, SM-induced assembly of the ripoptosome can prime cells for death—a process which requires an additional RIPK1 activating signal [1,30,31], which lowers the threshold for TNF-dependent apoptotic cell death. The importance of the ripoptosome in SM-induced cell death was highlighted by a recent study

demonstrating resistance to SM therapy by chronic lymphocytic leukaemia cells as the result of an inability to form the ripoptosome complex [54]. SMs do not usually sensitize non-transformed cells to TNF-induced death, as is evident from the good safety profile of these drugs, but how healthy cells are protected from this effect is not well understood.

Several SMs have now entered clinical trials, including monovalent LCL161 and Debio 1143 (AT-406) and one of the most clinically advanced SMs—the bivalent and pan-IAP antagonist birinapant [39]. Despite promising pre-clinical efficacy, this has largely failed to translate into positive outcomes in clinical trials, with several reports of the poor efficacy of SMs when used as single agents [32,55]. However, due to their favourable safety profile, attention is now being directed to the incorporation of SMs in combination therapies and as immuno-modulatory agents for the treatment of cancer.

1.3. *The Immuno-Modulatory Properties of Smac-Mimetics in Cancer*

Our understanding of the physiological roles of IAPs has undergone a transformation since they were originally identified as a potential target for cancer therapy. Their functions in regulating activation of canonical NF- κ B, inhibiting cell death and inhibiting activation of the non-canonical NF- κ B pathway all play a role in regulating inflammatory and immune responses in normal development and disease [56–60]. Both the IAPs and NF- κ B signalling play a critical role in the normal functioning of the immune system, including in B cell development [57,61], in the regulation of myeloid cells [62] and in cytotoxic lymphocyte activity (see later sections). For this reason, SMs have been reported to exhibit broad immuno-modulatory effects on both the innate and adaptive immune systems.

The immuno-modulatory effects observed following SM therapy, along with the ubiquitous inflammatory properties of TNF, have led to a number of studies reporting the effects of SMs on the immune system in cancer. TNF-dependent anti-tumour activity mediated by bacillus Calmette-Guérin (BCG)-stimulated neutrophils following SM treatment has been reported [63]. In murine models of human ovarian carcinoma or murine sarcoma, SM administration was associated with a rapid inflammatory burst of TNF and interferon-gamma (IFN γ), due to the reversion of tumour-associated macrophages from a pro-tumoural M2 phenotype to a pro-inflammatory M1-like phenotype [64]. The SM compound BV6, can sensitize resistant tumour cells to killing by cytokine-induced killer cells [65] and synergise with interferon-alpha (IFN α) to induce apoptosis in acute myeloid leukaemia cells [66]. Synergy between IFN γ and various SMs, including BV6 and the clinical compound birinapant, has been reported to induce apoptosis in lung cancer cells [67,68]. The pre-clinical efficacy of LCL161, a SM which potently induces apoptosis in some multiple myeloma cell lines with over-expression of XIAP has been reported [69], and can also sensitise osteosarcoma cells to TNF-mediated apoptosis [70].

The administration of SMs also stimulates myeloma cells to secrete soluble factors that induce tumour cell phagocytosis by macrophages; demonstrating the immuno-modulatory properties of SMs [32]. SM-mediated activation mimicking CD40 engagement was directly responsible for inducing an inflammatory response in macrophages and dendritic cells in vitro, which was further potentiated in vivo by the presence of SM-sensitive tumour cells. Two weeks of LCL161 treatment was curative in 15% of tumour bearing mice, an anti-myeloma response that was attributed to activation of tumour cell type I interferon signalling, resulting in myeloid activation and tumour cell phagocytosis, rather than direct cell killing [32].

As SMs become more clinically advanced, there is increasing data on the immunomodulatory effects of SMs in patients. In some instances, clinical data corroborates the pre-clinical findings, suggesting that SMs have wide-reaching effects on immunity. A phase 1 study of LCL161 against solid tumours of lung, skin, colon, pancreas and other origins (NCT01098838) determined pharmacodynamic activity with a favourable safety profile up to 1800 mg weekly, where cytokine release syndrome was the dose-limiting toxicity in 60% of patients [71]. Phase 2 evaluation of LCL161 against relapsed refractory multiple myeloma (NCT01955434) demonstrated some efficacy, with a partial response achieved in a number of patients. Immuno-modulatory properties of LCL161 were observed in patients following treatment, where a type I interferon response was required for activity, induction of inflammatory cytokines in the bone marrow plasma of patients after three weeks of

LCL161 treatment was observed, and there was evidence of increased macrophage-mediated phagocytosis [32]. However, other SM clinical studies show less profound immunomodulation. In Phase 1 (NCT00993239) and 2 (NCT01098838) clinical trials of birinapant, no significant differences in serum levels of TNF, interleukin-6 (IL-6) or IL-8 were observed regardless of dose [55,72], and only modest effects on immune cell populations were observed [55].

While SMs are likely to be effective as a monotherapy only where tumours release autocrine TNF following the degradation of IAPs, innate immune stimuli capable of inducing a clinically safe cytokine storm in the context of the tumour microenvironment may result in significant bystander killing in the presence of SMs [73]. As a result, there are now significant research efforts being directed towards the use of IAP antagonists as part of combination strategies that incorporate various immunotherapy approaches.

2. IAP Antagonists and Immunotherapy Approaches

Immunotherapy has had some remarkable successes in the clinic, however the benefit is limited to only a subset of patients. For example, checkpoint blockade therapy, such as α -PD-1 treatment, results in a partial or complete clinical response rate in only ~20% of patients with solid tumours [74]. The application of chimeric antigen receptor (CAR) T cell therapy has also been limited to haematological malignancies, largely due to poor CAR T cell infiltration and the hostile tumour microenvironment associated with solid tumours. Several properties of SMs, including their immuno-modulatory effects on a range of immune subsets, may overcome these challenges and enhance the efficacy of immunotherapies when used in combination. SM therapy can also synergise with innate immune stimuli such as oncolytic viruses, poly(I:C) or CpG, to promote anti-tumour immunity in mouse models [50]. The potential of SMs to promote the anti-tumour activity of CD8+ cytotoxic T lymphocytes (CTLs) is particularly promising, as these are the key immune cells required for the clinical efficacy of both adoptive cell therapies and checkpoint blockade immunotherapies.

2.1. Role of Smac-Mimetics in T Cell-Specific Immunotherapies

CTLs possess remarkable anti-tumour cytolytic capacity, and therefore are key targets for enhancing anti-tumour immunity. Activation of naïve T cells occurs following the interaction between a peptide antigen presented in the context of major histocompatibility complex and the T cell receptor (TCR). T cell activation also requires co-stimulation through the interaction of the T cell receptor, CD28, and the membrane bound glycoproteins, CD80 or CD86, expressed on professional antigen presenting cells, such as dendritic cells [75]. In CD4+ T cells, ligation of CD28 promotes T cell survival through the induction of NF- κ B-dependent expression of anti-apoptotic BCL-XL [76]. Appropriate T cell activation occurs following both activating signals, which results in T cell proliferation, cytokine production and antigen-specific killing of target cells.

Upon activation, T cells express several co-stimulatory TNFRSF members, including TNFR2, OX40 and 4-1BB, which all activate NF- κ B2 in a cIAP dependent manner [77]. In this way, cIAPs can function as negative regulators of T cell co-stimulation [58,78]. Activating non-canonical NF- κ B signalling following SM treatment is therefore likely to affect all T cell subsets. Indeed, inhibiting cIAPs in human and murine CD4+ and CD8+ T cells delivers a co-stimulatory signal that enhances activation in the presence of TCR stimulation [79]. Overall, this results in an enhancement of CD8+ and CD4+ T cell proliferation and functions such as cytokine production [78], and may therefore augment immunity by stimulating the adaptive immune response *in vivo*.

Moreover, it has been shown that the bivalent SM, APG-1387, positively regulates T cells *in vitro*, reducing regulatory T (Treg) cell differentiation and downregulating PD-1 expression on CD4+ T cells [80]. More recently, the molecular and functional imprinting capacity of SM treatment on T cells at the transcriptional and proteomic level was demonstrated, where non-canonical NF- κ B signalling was shown to skew non-differentiated T cells into a more Th2-like, and less Th17-like, differentiation state [81]. While the direct effects of SMs on T cells are still being uncovered, it is clear that they can modulate T cell activation and differentiation, which will have important consequences when combined with immunotherapy approaches that target and promote the anti-tumour activity of T

cells. It is not yet known if SMs will elicit co-stimulatory effects on T cells that are activated through a synthetic chimeric antigen receptor, which might have clinical applications in the context of adoptive CAR T cell therapies for cancer.

The pro-inflammatory properties of smac-induced cell death play an important role in T cell mediated anti-tumour immunity; highlighted in a study where delivery of the cytosolic form of smac resulted in immunogenic tumour cell death and triggered an anti-tumour immune response leading to decreased tumour growth in two melanoma models, and total tumour regression in a mastocytoma model [82]. In this instance, dendritic cells were activated upon uptake of dying tumour cells, resulting in increased functional CTL numbers and decreased Treg numbers. Administration of SMs also improved the outcome of both prophylactic and therapeutic anti-tumour vaccines in vivo, with the SM, LBW-242, reported to synergise with a vaccine against B16 melanoma, delaying tumour growth and prolonging survival [79].

The TNF-mediated apoptotic pathway is a potent mechanism of inducing tumour cell death [83], and IAP antagonists rapidly sensitise tumour cells to killing by CTL-derived TNF. More recently, the potential of agents, such as birinapant, that lower the tumour TNF cytotoxicity threshold and augment responses to immunotherapy has been demonstrated [84]. Indeed, T cell killing in vitro is enhanced following the addition of birinapant—an effect that was TNF-dependent [85]. In support of these findings, a synergistic enhancement of T cell effector function due to birinapant and α -PD-1 co-administration was reported; an effect attributed to enhanced TNF secretion [32]. Intracranial glioblastoma cells are also sensitive to the effects of IAP antagonists (either LCL161 or birinapant) in combination with exogenous TNF or TRAIL [86]. SMs synergised with immune checkpoint inhibitors to promote tumour immunity against glioblastoma, an effect that was dependent on both CD8+ T cells and TNF. SM and immune checkpoint blockade combination therapy was associated with increased expansion of CD8+ T cells, a decrease in Treg numbers and increased sensitivity of the tumours to TNF-induced cell death mediated through the RIPK1-Fadd-Caspase8 complex. Based on the pre-clinical success of SM and checkpoint blockade combination strategies, phase I/2 clinical trials are on-going to assess the toxicity and efficacy of the combination of these treatment modalities, including birinapant, APG-1387, LCL161 and Debio1143 (Table 1).

Table 1. Summary of smac-mimetics in clinical trials incorporating an immunotherapy approach.

Identifier	Smac-mimetic	Combination Therapy	Malignancy	Phase
NCT03270176	Debio 1143 (AT-406)	Avelumab (α -PDL-1)	Advances solid tumours and advanced/metastatic non-small cell lung carcinoma	1
NCT02587962	Birinapant (TL32711)	Pembrolizumab (α -PD-1)	Various solid tumours	1/2
NCT031111992	LCL161	PDR001 (α -PD-1) CJM112 (anti-IL-17a) PDR001 (α -PD-1)	Multiple myeloma	1
NCT02890069	LCL161	Everolimus (mTOR inhibitor) Panobinostat (HDAC inhibitor)	Colorectal cancer, non-small cell lung carcinoma, triple negative breast cancer, renal cell carcinoma	1
NCT03166631	BI891065	B I754091 (α -PD-1)	Advanced and/or metastatic malignancies	1
NCT03697304	BI891065	BI 754111 (anti-LAG-3) BI 754091 (α -PD-1)	Advanced and/or metastatic solid tumours (pre-treated)	2
NCT03871959	Debio 1143	Pembrolizumab (α -PD-1)	Non-MSI-high advanced or metastatic pancreatic ductal adenocarcinoma or colorectal cancer	1
NCT03386526	APG-1387	Pembrolizumab (α -PD-1)	Advanced solid tumours or haematological malignancies	1/2

Birinapant also significantly enhanced CAR T cell therapy, resulting in prolonged survival and tumour regression in tumour bearing mice, and significantly enhanced tumour cell death in a patient-derived tumour model, compared to CAR T cell or birinapant monotherapy [87]. Importantly, the ability of SMs to sensitize antigen negative cells to TNF-mediated apoptosis, an effect known as bystander killing (Figure 2), may be an effective strategy to avoid antigen negative relapse following CAR T cell therapy [88]. Harnessing tumour-antigen specific CAR T cells as a vehicle to deliver TNF specifically to the tumour in combination with birinapant may have a significant therapeutic advantage. CAR T cells secrete inflammatory cytokines only following tumour antigen recognition through the CAR, which avoids the potential detrimental effect of systemic TNF secretion. α -PD-1 therapy increases CAR T cell-derived TNF [89], suggesting that the combination of CAR T cell therapy, SM treatment and checkpoint blockade may be a potent treatment strategy that could be rapidly translated into the clinic.

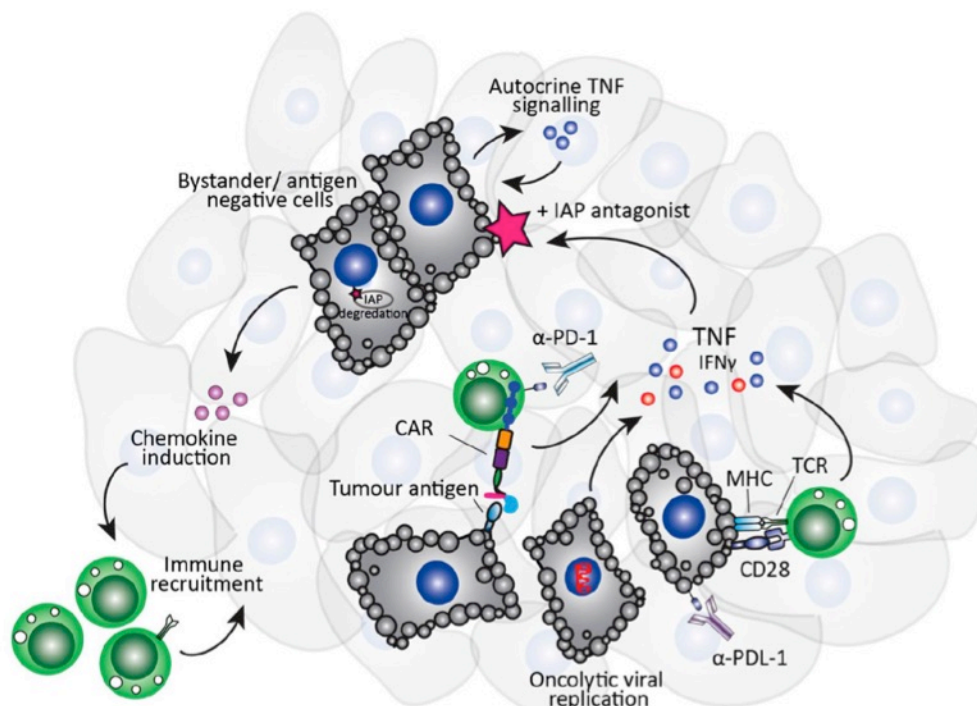


Figure 2. Bystander killing mediated by tumour cell IAP antagonism. TNF released following checkpoint blockade, CAR T cells, or from tumour cells following oncolytic virus-induced death can cause death of surrounding “bystander” tumour cells, due to IAP degradation.

SMs can also mediate the induction of systemic cytokines such as TNF, and chemokines such as RANTES and CXCL1 [90], which results in enhanced immune cell recruitment *in vivo*. In combination with adoptive cell therapy, SMs may therefore enhance the trafficking of immune cells into the tumour microenvironment. TNF produced by immune cells contributes to SM efficacy, particularly against tumours that do not secrete TNF following degradation of IAPs. As a result of this, SMs are more efficacious *in vivo* when combined with inflammatory adjuvants that promote TNF production [50,90,91]. Overall, SMs can elicit a pro-inflammatory cell death in cancer cells that engages an adaptive anti-tumour immune response, and may result in an increase in tumour-infiltrating lymphocytes, cytokine secretion and enhance inflammation in an otherwise immunologically “cold” tumour.

The SM, Debio 1143, also has radiosensitization properties in non-small cell lung cancer [92] and head and neck squamous cell carcinoma; an effect that has been attributed to enhanced TNF sensitivity [93]. However, the immune-modulatory properties of SMs in this context have only recently been explored. Synergistic anti-tumour effects between Debio 1143 and ablative

radiotherapy have been reported, where addition of the SM not only induced a tumour-specific CD8⁺ T cell and cytokine response, but was able to overcome the recruitment of immunosuppressive host cells into the tumour microenvironment [94]—which typically occurs following radiotherapy. As a result of this pre-clinical efficacy, a phase I clinical trial combining re-irradiation therapy and birinapant for the treatment of head and neck squamous cell carcinoma is ongoing (NCT03803774).

2.2. Role of Smac-Mimetics in NK Cell-Specific Immunotherapies

While the success of CAR T cell therapy and checkpoint blockade has highlighted the use of T cell-centric immunotherapies for the treatment of cancer, there is potential for natural killer (NK) cell-specific immunotherapies to provide an alternative or overcome relapse or resistance to these therapies [95,96]. Like T cells, IAPs also regulate aspects of NK cell biology, however their role has not been extensively studied.

There have been several reports that IAP antagonists potentiate Hodgkin lymphoma susceptibility towards NK cell-mediated killing [97,98], and a TNF-dependent increase in the effector function of NK cells in vitro following the administration of birinapant has been reported [85]. Similarly, BV6 improves NK cell-mediated killing of rhabdomyosarcoma cells by sensitising them to NK cell-mediated killing through TNF, whilst simultaneously enhancing the cytotoxic potential of the NK cells through increasing NK cell activation [99]. Furthermore, the administration of AG-1387 resulted in a five-fold increase in NK cell tumour infiltration, as well as inducing tumour cell apoptosis directly in a hepatocellular carcinoma xenograph model [80]. More recently, the cytotoxic activity of NK cells against hepatocellular carcinoma was shown to be enhanced following AG-1387 administration [100]. Based on this favourable pre-clinical efficacy, and mounting evidence for the role of the PD-1/PDL-1 inhibitory axis on NK cell cytotoxic activity [101–103], a Phase 1/2 clinical trial with AG-1387 in combination with pembrolizumab (α -PD-1), is ongoing (Table 1). IAP activity, particularly XIAP, is also implicated in NKT cell development [104], and SM treatment has been reported to enhance cytokine production from mouse and human NKT cells and promote the anti-tumour activity of NKT cells in a B16F10 lung metastasis model [105], providing excellent rationale to combine SMs with immunotherapy approaches.

Whether NK cell-based immunotherapies will become commonplace in clinical practice is still unknown, but some clinical success has been reported following the adoptive transfer of the NK92 cell line or NK cells engineered to express a chimeric antigen receptor against a tumour associated antigen [106,107]. The role of NK cells in anti-tumour immunity should not be underestimated, and given the potent cytotoxic capacity of NK cells to kill tumour cells, the potential to enhance their effector function with SMs is a promising approach. Indeed, emerging literature suggests that tumour cells may be sensitised to NK cell-mediated and TNF-dependent killing through administration of a SM, and potentially overcome resistance to T cell-directed immunotherapies [96].

2.3. Role of Smac-Mimetics in Oncolytic Virus Immunotherapy

Oncolytic virus immunotherapy is a treatment modality that uses genetically modified viruses that selectively replicate within, and directly lyse, tumour cells. This treatment has emerged as a promising tool to treat solid tumours, due to their remarkable capacity to selectively replicate in cancer cells without affecting non-malignant cells [108]. Indeed, clinical trials have reported clinical benefit in patients following oncolytic viral immunotherapy [109,110].

Following oncolytic cell death, tumour cells release tumour-associated neo-antigens, viral pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs), which serve to induce an innate and adaptive immune response against cancer cells. Combined with the release of cytokines from tumour cells, this promotes the activation of CD4⁺ and CD8⁺ T cells, which traffic to sites of established tumour cell growth and mediate anti-tumour immunity, releasing more inflammatory cytokines into the tumour microenvironment [111]. The use of oncolytic viral immunotherapy to modulate the tumour microenvironment and release inflammatory cytokines provides a strong rationale for combination with other immunotherapies, such as checkpoint blockade [112,113] and/or SMs, to improve therapeutic responses.

Indeed, SMs have shown impressive and broad activity when combined with oncolytic viruses [91], likely due to the increase in local TNF production as a result of oncolytic cell death. The combination of SMs and oncolytic virus therapy, both of which have significant immunomodulatory properties, can synergise to promote anti-tumour T cell responses due to apoptosis of SM-treated cells triggered by TNF secreted during viral infection [114]. Treatment with the oncolytic virus, M1, through the release of associated PAMPs, results in an increase in inflammatory cytokine secretion via the host innate immune system. SMs also sensitise tumour cells to inflammatory cytokines induced by M1, enhancing a bystander killing effect mediated by IL-8, IL1A and TRAIL [115].

The generation of oncolytic viruses that express TNF resulted in an enhanced anti-tumour effect when combined with LCL161, due to an increase in TNF-mediated bystander killing and collapse of tumour-vasculature [116]. It has also been reported that SMs themselves may have tumour-vasculature targeting properties, through the sensitisation of endothelial cells to TNF-mediated death [117]. Taken together, these studies highlight the multi-purpose role of SMs in promoting anti-tumour immunity and the potential for combination therapies that harness the favourable effects of SMs on the immune system.

3. Conclusions

While IAPs were originally characterised as modulators of cell death through their interaction with caspases, it is now clear that they have broad immuno-modulatory properties, affecting both the innate and adaptive immune system. As such, IAP antagonists are poised to enter the clinic as a promising approach to overcome some of the challenges associated with conventional immunotherapies. One of the key immuno-modulatory effects of SMs is their ability to potentiate TNF-mediated bystander killing of tumour cells.

Combination therapies that enhance the efficacy of SMs are those that typically increase the local concentration of inflammatory cytokines, which can then be harnessed and potentiated by SMs via their capacity to sensitise tumour cells to TNF-mediated apoptosis. Therefore, therapies specifically designed to enhance the local secretion of inflammatory cytokines in the tumour microenvironment, such as CAR T cell or oncolytic virus therapy, have resulted in a synergistic effect with SMs and significantly enhanced tumour cell death. It may be possible to create triple combination therapies based on this approach, by incorporating an immune checkpoint blockade—a therapy which has been shown to enhance T cell secretion of inflammatory cytokines—to further enhance this synergistic effect. As many of these agents with potential for synergy with SMs are already in clinical trials, we can expect a rapid translation of such combination therapies in to the clinic.

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5. Hypotheses and aims

As discussed in this chapter, CAR T cell therapy has demonstrated remarkable efficacy in the clinic, however the development of resistance is an emerging problem. In haematological malignancies, immune pressure resulting in antigen loss results in relapse due to the emergence of antigen negative clones. In solid tumours, CAR T cell efficacy is limited due to several factors including poor CAR T cell trafficking and penetration of the microenvironment and potentially tumour-specific resistance mechanisms.

In this thesis, the mechanisms of tumour immune evasion in the context of immunotherapy will be discussed, using a series of genome-wide loss-of-function CRISPR-Cas9 screening approaches. Based on this data, we designed a rational combination therapy in order to overcome immune evasion and enhance CAR T cell therapy in the solid tumour context, and

subsequently we demonstrated the efficacy of this combination in various *in vitro* and *in vivo* models. Finally, the potential mechanisms of CD19 antigen loss following CAR T cell therapy in B cell ALL will be discussed.

Aim 1: To conduct genome-wide CRISPR-Cas9 screening in order to uncover mechanisms of CAR T cell resistance

Hypothesis: Loss of genes associated with TNF-dependent apoptosis will confer resistance to CAR T cell therapy

Aim 2: To investigate the potential for smac-mimetic combination therapy to enhance CAR T cell efficacy

Hypothesis: The clinical smac-mimetic birinapant will synergise with CAR T cell-derived TNF to enhance efficacy.

Aim 3: To conduct genome-wide CRISPR screening in order to uncover mechanisms of CD19 antigen loss in a B cell acute lymphoblastic leukaemia cell line.

Hypothesis: Expression of the CD19 antigen on B cells is dependent on epigenetic regulation

CHAPTER 2: Materials and Methods

1. Mice

C57BL/6 wildtype (WT), C57BL/6 human-HER2 (hHER2) transgenic mice, C57BL/6 OT-I and C57BL/6 perforin^{-/-} (pfp^{-/-}) mice were bred at the Peter MacCallum Cancer Centre (Melbourne, Victoria, Australia). C57BL/6 TNF^{-/-} were gifted from Professor John Silke at the Walter and Eliza Hall Institute (WEHI, Parkville, Victoria, Australia). Mice were used from age of 6 weeks, and were housed in PC2 specific pathogen-free conditions. All animal studies were conducted under a protocol approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee.

2. Cell lines

Murine cell lines (MC38 and E0771) were cultured in DMEM medium and human cell lines (HeLa and AU565) were cultured in RPMI medium (Gibco, Melbourne, Victoria, Australia; Invitrogen, Scoresby, Victoria, Australia) both supplemented with 10% FCS (Thermo Scientific, Scoresby, Australia), penicillin/streptomycin (Gibco), and incubated at 37 °C in 10% CO₂.

The parental tumour lines MC38 and E0771 were transduced with a retroviral vector murine stem cell virus (MSCV)-GFP to express a truncated form of human HER2, which lacks the intracellular signalling components [206-208]. MHH-CALL-4 cells were obtained from DSMZ-German Collection of Microorganisms and cell cultures GmbH (Liebniz Institute, Germany) and cultured in B cell media [50% RPMI medium (Gibco, Melbourne, Victoria, Australia; Invitrogen, Scoresby, Victoria, Australia), 50% Minimum Essential Medium Eagle (Sigma-Aldrich) supplemented with 20% FCS, L-glutamine, non-essential amino acids, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and penicillin/streptomycin. Tumour lines were verified to be Mycoplasma-negative by the Victorian Infectious Diseases Reference Laboratory by PCR analysis.

3. Antibodies, cytokines and drugs

Birinapant was acquired from Medivir (Sweden). Neutralising antibodies used: Mouse anti-TNF (Biolegend, San Diego, USA, clone MP6-XT22), Human anti-TNF (BioLegend, San Diego, USA, clone MAb11), anti-PD-1 (clone RMP1-14, IgG2a, Bio X Cell) and Ig2a istoype (clone 2A3; Bio X Cell). Enbrel® (etanercept) was obtained from the Walter and Eliza Hall Institute of Medical Research (Parkville, Victoria). Western blotting: anti-cbfb (Ab33516) and anti-runx1 (Ab35962) were purchased from Abcam, Cambridge, United Kingdom). Secondary antibody was polyclonal swine anti-rabbit, purchased from DAKO (Jena, Germany). Antibodies used for immunofluorescence microscopy: anti-TNF (Biolegend, clone MP6-XT22), IFN- γ (eBiosciences, Scoresby, Australia, clone XMG1.2), Tubulin (Rockland, USA). ProLong antifade with DAPI were purchased from Molecular Probes (Scoresby, Victoria, Australia). Antibodies for T cell stimulation and CAR detection: anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) antibodies were purchased from BD Biosciences (USA). SIINFEKL peptide (OVA257-264) was purchased from Auspep Tullamarine (Victoria, Australia). Recombinant cytokines: IL-2 and IL-7 were obtained from the NIH and PeproTech (Rocky Hill, NJ), respectively. Recombinant mouse TNF and IFN- γ were purchased from PeproTech (Rocky Hill, New Jersey, USA). Antibodies used for flow cytometry are listed in Table 1.

Table 1. List of antibodies and dilutions used for flow cytometry in these studies

Specificity	Species	Fluorochrome	Clone	Company	Dilution
c-Myc Tag (CAR	Mouse	-	9B11	Cell Signalling Technology	1:50
c-Myc Tag (CAR)	Mouse	APC	9B11	Cell Signalling Technology	2 µL neat
CD19	Human	FITC	340364	BD Biosciences	1:200
CD19	Human	PE	SJ25C1	BioLegend	1:200
CD19	Human	APC	H1B19	BD Biosciences	1:200
CD4	Mouse	APCcy7	GK1.5	BD Biosciences	1:200
CD44	Mouse	BV785	IM7	BioLegend	1:400
CD62L	Mouse	BV421	MEL-14	BD Biosciences	1:400
CD8	Mouse	FITC	53-6.7	BioLegend	1:100
H-2Kb	Mouse	PE	AF6-885	BioLegend	1:200
HER2	Human	APC	24D2	BD Biosciences	1:200
IgG1 Isotype	Mouse	APC	MOPC-21	BD Biosciences	1:200
PD-L1	Mouse	BV421	10F.9G2	BioLegend	1:200
Viability	-	Fixable Yellow BV510	-	Thermo Fisher	1:1000
Viability	-	PI	-	Sigma-Alrich	1:1000

4. T cell activation and transduction

Retrovirus was generated from the viral packaging GP+E86 cell line containing LXS^N-anti-HER2 CAR, acquired from Dr Paul Beavis. The CAR construct used in these studies was composed of an extracellular scFv–anti–human HER2 fused to an extracellular CD8 hinge region, a transmembrane CD28 domain and an intracellular CD3 ζ domain. The CAR T cell generation protocol is outlined in Figure 9.

Spleens from C57BL/6 WT, Tnf^{-/-} or perforin^{-/-} mice were harvested and processed into a single cell suspension through a 70 μ m filter (BD Falcon, San Jose, California, USA). Cells were centrifuged at 1250 RPM for 5 minutes and the supernatant was aspirated prior to removal of red blood cell with Ammonium-chloride-Potassium (ACK) lysis buffer for 3-5 minutes. Cells were cultured overnight in 6 well plates (Greiner Bio One, Frickenhausen, Germany) in 5 mL (density 5x10⁶ T cells/ mL) enriched T cell media [RPMI supplemented with 10% FCS, penicillin/streptomycin, L-glutamine, non-essential amino acids, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate (Calbiochem, Macquarie Park, New South Wales, Australia)] containing α -CD3 and α -CD28 (both 0.5 μ g/mL) activating antibodies and in the presence of IL-2 (100 IU/mL) and IL-7 (2 ng/mL).

The following day, live T cells were isolated from the cultures by the addition of a Ficoll Paque layer (Sigma Aldrich, Castle Hill, NSW, Australia) followed by centrifugation (protocol: 1400 RPM, 10 minutes, acceleration/ deceleration: 4). 2.5 mL virus-containing supernatant from overnight culture of GP+E86 packaging cells was centrifuged (1200 g, 30 minutes) on to retronectin-coated (10 μ L) 6 well plates (non-treated, Corning Costar, New York, USA). Live isolated T cells were re-suspended in additional virus-containing supernatant supplemented with IL-2 (100IU/mL) and IL-7 (2 ng/mL), and added to retronectin-coated plates, resulting in a T cell density of 5x10⁶/ well for transduction. Cells were centrifuged again at 1200g for 90 minutes prior to overnight incubation at 37°C 5% CO₂.

The following day, T cells were harvested from 6 well plates and the transduction protocol was repeated excluding Ficoll separation. After final centrifugation, T cells were incubated at 37°C 5% CO₂ for 3-5 hours, and then re-suspended in fresh supplemented RPMI (5 mL/ well) with IL-2 (100 IU/mL) and IL-7 (2 ng/mL). CAR T cells were topped up with additional RPMI and cytokines when deemed necessary, and used on day 5-7 for adoptive transfer or *in vitro* assays.

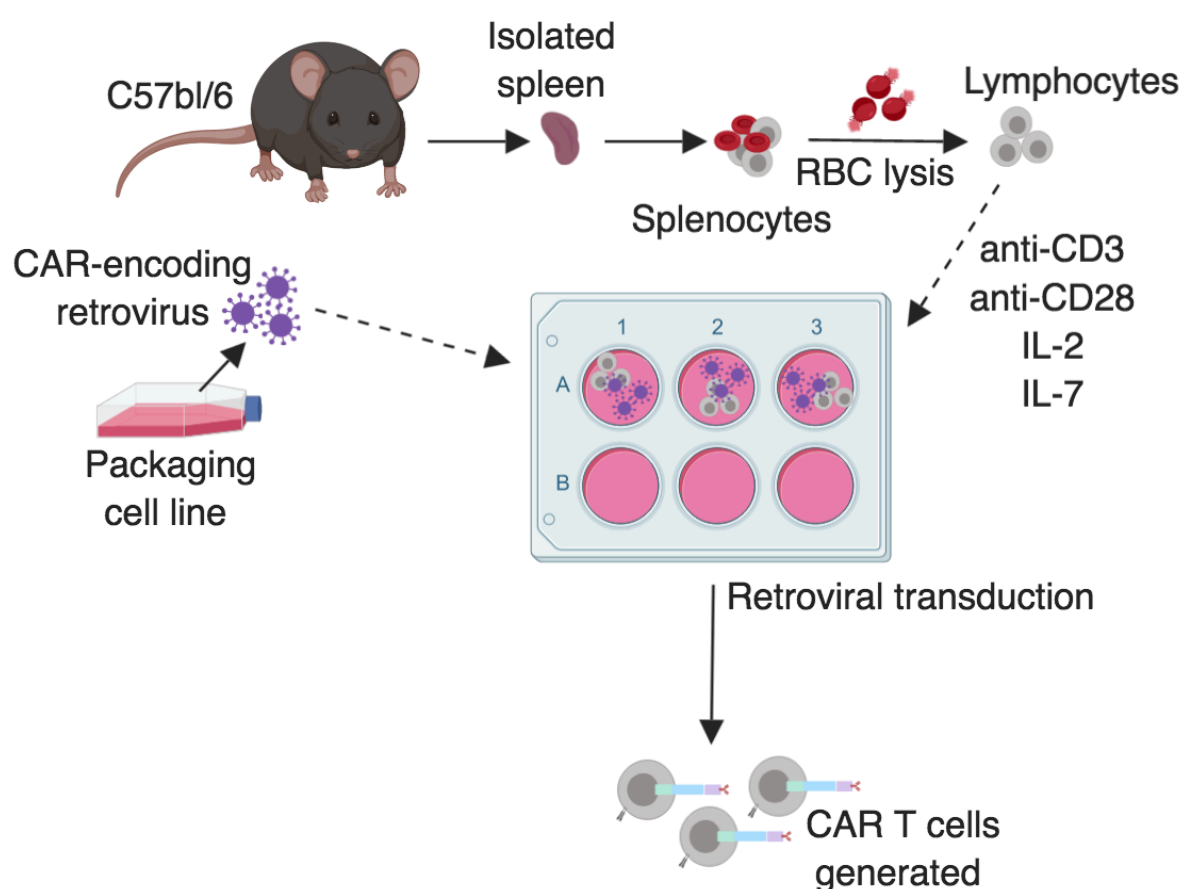


Figure 9. Generation of murine CAR T cells from splenocytes. Splenocytes were isolated from the C57BL/6 mice, and activated with anti-CD3 and anti-CD8 activating antibodies and cultured in IL-2 and IL-7. T cells were transduced twice with a CAR-encoding retrovirus and expanded in culture for 3-5 days.

Human CAR T cells were generated from donor whole blood as follows. 15 mL donor blood was mixed in equal parts with sterile PBS (in house). T cells were isolated by Ficoll Paque separation (15 mL, centrifugation protocol 10 mins 1000g acceleration 6, deceleration 0). T cells were cultured for 2 days at 1×10^6 / mL in enriched T cell media supplemented IL-2 (600 IU/mL) and soluble α -CD3 (OKT3, BioLegend, 30 ng/mL). Viral supernatant was harvested from PG13-anti-HER2 CD28/CD3 ζ packaging cells and 5 mL was added to retronectin-coated untreated 6 well plates, as above and incubated at 37°C 5% CO₂ for 4 hours. T cells were added to plates in 5 mL fresh viral supernatant supplemented with IL-2 (600 UI/mL) at a total density of 2.5×10^6 T cells/ well and cultured overnight. The following day 5 mL fresh viral supernatant was harvested and centrifuged on to retronectin-coated plates (1000g, 1 hour, acceleration/ deceleration 5) and incubated at 37°C with 5% CO₂ for 3 hours. T cells from previous day were harvested, re-suspended in fresh viral supernatant, centrifuged (1000g, 1 hour, acceleration/ deceleration 5) and incubated at 37°C 5% CO₂ for 3 hours. T cells were re-suspended in fresh enriched T cell media supplemented with IL-2 (600 UI/mL) for 2-3 days. T cells were then re-suspended at a total density 1.5×10^6 in IL-2 containing media in large culture flask (T75).

Primary OT-I T cells were isolated from the spleens of C57BL/6 OT-I transgenic mice. Spleens were harvested and processed into a single cell suspension through a 70 μ m filter (BD Falcon, San Jose, California, USA). T cells were cultured in enriched T cell media, in the presence of IL-2 (100 IU/mL) and activated with OVA₂₅₇ peptide (Auspep, Tullamarine, Victoria, Australia). Cells were incubated at 37°C, 5% CO₂ and used on day 4-8 post-activation.

5. In vitro assays

5.1 Cytotoxicity assays

CAR T cell, OT-I T cell or TNF cytotoxic activity was measured using a standard chromium release assay. 1×10^6 tumour cell targets were labelled with 100 μ Ci ⁵¹Cr for 1 hour at 37°C with 5% CO₂, and subsequently washed

three times with DMEM media by centrifugation (1800 rpm, 2 minutes). Cells were re-suspended in DMEM and plated at a density of 1×10^4 cells/ well and co-cultured with CAR T cells/ OT-I T cells/ recombinant cytokines at the indicated ratio. Wells containing only labelled targets were lysed with 20% solution of Triton X-100 detergent (Sigma Aldrich) to determine total ^{51}Cr release. Targets were incubated with T cells/ recombinant cytokines for 4, 6 or 18 hours and then centrifuged (1400 rpm, 4 minutes) and supernatant harvested. Chromium release was measured from 100 μL of supernatant from each well using an automatic gamma counter (Wallac Wizard 1470, General Electricity Healthcare, Amersham, Western Australia).

The percentage specific killing was determined using the formula: $(\text{Sample } ^{51}\text{Cr release} - \text{Spontaneous } ^{51}\text{Cr release}) / (\text{Total } ^{51}\text{Cr release} - \text{Spontaneous } ^{51}\text{Cr release}) \times 100$, and represented as a Michaelis–Menten kinetic trend. All assays were performed using triplicate wells. To generate relative killing bar graphs, tumour cell killing at the E:T ratio that resulted in 50% maximal killing of the least cytotoxic condition was compared, using Michaelis-Menten trends, as performed previously [163, 204, 209].

Relative killing from flow cytometry-based PI killing assays was calculated as follows: Cell death was determined by PI uptake and morphology. Background cell death was determined by an untreated control for each cell type, and background cell death was subtracted from all other conditions. Percentage cell death was determined at each TNF concentration, and represented as a Michaelis-Menten kinetics trend. Relative killing based on this trend was calculated as outlined above.

5.2 Cytokine and chemokine assays

Cytokine release assay co-cultures were set up as with the chromium release assays. Briefly, tumour cell targets were added to V-bottom plates with recombinant cytokines or CAR T cells added at indicated E:T ratios, and incubated at 37°C , 5% CO_2 for 4-6 hours. Supernatants were harvested and cytokines/chemokines were detected using a mouse inflammation CBA kit (BD Biosciences, 552364) as per manufacturer's instructions and analysed on

a FACS Verse (BD Biosciences, North Ryde, Australia). All assays were analysed using triplicate determinations.

5.3 Flow cytometry

Cells assessed via flow cytometry were plated out in V-bottom plates in 100 μ l media, pelleted by centrifugation at 1400 RPM for 4 minutes and resuspended in 100 μ l FACS buffer (PBS + 2% FCS). Cells were then stained with the appropriate antibodies for 30 minutes on ice in the dark. Following this, cells were washed twice in FACS buffer, resuspended in 100 μ l FACS buffer, containing a viability marker where applicable. Analysis of samples was performed on a FBD LSR II (BD Biosciences), and data were analysed with FlowJo (Tree Star, Ashland, Oregon, USA).

5.4 Western blotting

Western blotting using NaDodSO₄ PAGE (SDS-PAGE) was conducted as previously described [210]. Briefly, samples were prepared in 2% SDS, 50 mM Tris-HCL (pH 6.8), 10% glycerol, 2.5% 2-ME, then boiled for 10 minutes. Lysates were loaded into 8-12% polyacrylamide gels and electrophoresed at 100 V and resolved proteins were transferred onto nitrocellulose membranes at 40 mA for 12-18 hours. Membranes were blocked for 1h (5% BSA, 0.05% NaN₃ in PBS, Tween 20), and then incubated with primary Ab overnight. Proteins were detected using HRP-conjugated secondary Ab, using the ECL system (Amersham Bioscience) according to the manufacturer's protocols using an iBright FL1500 imaging system (Thermo Fisher Scientific).

5.5 Fixed and time-lapse microscopy

Microscopy of T cell-tumour cell conjugates was done as previously described [204]. The slides were imaged using a FV1000 confocal microscope (Olympus, NY, USA). CAR T cells selected for quantitation had a single contact site with one tumour cell, indicating a single synapse event. The percentage of T cells that were positive for the indicated cytokine in a synapse or not, was quantitated manually by confocal microscopy. A minimum of 100 cells was counted in each condition.

For time-lapse imaging, CAR T cells were labelled with Cell Trace Violet (Molecular Probes), and added to adherent targets, in media containing propidium iodide. Imaging was conducted as previously described [91]. Chamber slides were mounted within a temperature-controlled chamber maintained at 37°C, and constant CO₂ concentration of 5% was maintained ('The Brick'; Ibidi). Optical sections were acquired through sequential scans or Brightfield/DIC using a TCS SP5 confocal microscope (Leica Microsystems, Deerfield, IL, USA) using a 40x (NA 0.85) air objective and Leica LAS AF software. Image analysis was performed using Meta-Morph Imaging Series 7 software (Universal Imaging, Downingtown, PA, USA).

6. In vivo mouse experiments

6.1 CAR T cell experiments

C57BL/6 human HER2 transgenic mice were injected subcutaneously with 2.5×10^5 MC38-HER2 cells. At day 7 after tumour injection, mice were preconditioned with total-body irradiation (4 Gy) prior to the administration of 1×10^7 CAR T cells on days 7 and 8. Mice were also treated with 50,000 IU IL-2 on days 0–4 after T cell transfer. Mice were treated with either vehicle or birinapant (12 mg/kg per mouse) on days 0, 4, and 8 after T cell transfer. Tumour growth was monitored approximately every second day using a caliper square to determine the product of two perpendicular tumour diameters. The mice were culled when the tumour size reached the ethical limit (150mm²) and recorded for the survival plot.

6.2 Checkpoint blockade experiments

C57BL/6 mice were inoculated subcutaneously with 1×10^6 MC38 cells. When tumours reached an average size of 20 mm², mice were treated with 2 doses of α-PD-1 (200 µg/mouse, Bio X Cell) or isotype control 4 days apart. Tumours were measured every 2 days until mice began reaching ethical endpoint (150 mm²), after which the mice were recorded for survival.

7. Sequencing

7.1 3' RNA Sequencing

Tumour cells were left untreated or co-cultured with CAR T cells at a 2:1 ratio at 37°C for 6 hours. Cells were washed thoroughly with PBS to remove contaminating CAR T cells and cells were lysed and total RNA isolated as per manufacturer's instructions (Nucleospin® RNA extraction kit, Macherey-Nagel, Bethlehem, PA). The quality of the extracted RNA was analysed on the TapeStation (Agilent TapeStation 2200), and RNA with integrity number values greater than 9 were used in subsequent library preparation. The QuantSeq 3' mRNA Library Prep Kit (Lexogen) was used to prepare libraries. Single-end 75-bp RNA sequencing (RNA-Seq) was performed in-house at the Peter MacCallum Cancer Centre Molecular Genomics Core on the NextSEquation 500 (Illumina). Demultiplexing of the reads was performed using CASAVA v1.8.2, and sample quality control was performed using FastQC (Babraham Bioinformatics, Babraham Institute, low-quality reads $Q < 30$ were removed. Cutadapt (v1.9) was used to trim polyA-derived sequences and biased 3' reads resulting from random hexamer priming. HISAT2 (v2.1.0) was used to align the resulting reads to the human or mouse reference genome. Read counting was performed using FeatureCounts; part of the Subread package (v1.5.0). Voom-LIMMA [211] workflow was used to normalize data for differential gene expression. Gene set enrichment analysis was performed using GSEA2 (v3.0) for identification of enriched signatures obtained from the MSigDB Hallmarks datasets.

7.2 Generation of boutique transcriptional signatures

Published transcriptional MC38 data [202] treated with recombinant TNF and IFN- γ was utilised. Genes that were significantly upregulated ($p = < 0.005$) following TNF or IFN- γ treatment was used to generate a stringent boutique signature, which was subsequently applied by GSEA to the RNA seq generated as described above.

7.3 Genome-wide CRISPR-Cas9 screens

Genome-wide CRISPR-Cas9 screen was performed using MC38 HER2 cells and MHH-CALL4 cells transduced with mCherry-Cas9 (FUCas9Cherry, Addgene plasmid: 70182). MC38 HER2-Cas9 cells were transduced with a mouse CRISPR knockout pooled library (Brie) (Addgene Cat: 73633), and MHH-CALL-4 cells were transduced with a human CRISPR knockout pooled library (Brunello) (Addgene Cat: 73178). Transductions were performed at a multiplicity of infection of 0.3 to ensure integration of single sgRNA constructs per cell. After transduction, the cells were selected with puromycin (MC38: 6 µg/mL, MHH-CALL-4 1 µg/ml) for 5 days. The MC38 HER2-Cas9 cells were co-cultured with HER2-targeting mouse CAR T cells at effector to target (E:T) ratios of 2:1. The tumour cells were exposed to a total of three rounds of CAR T cell killing for resistance screen, or 1 overnight co-culture for dropout screen, after which the pellets were snap-frozen. MHH-CALL-4 cells were subjected to three successive rounds of cell sorting by flow cytometry on the lowest 20% CD19 expression.

Genomic DNA extraction, sequencing and analysis has been described previously [91, 202]. DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen), as per the manufacturer's instructions. The libraries were multiplexed and run on the NextSeq 500 (Illumina) generating 75-bp single-end reads. After demultiplexing with CASAVA (v1.8), the vector-derived sequence reads were removed, and reads of 20 bp were extracted using cutadapt (v1.7). Subsequently, MAGeCK (v0.5.6) [212] was used to count the reads and perform gene/sgRNA enrichment and statistical analysis. Graphs were generated in R Studio, using the ggplots2 package.

8. Tumouroid assays

All human biopsy studies were conducted under a protocol approved by the Peter MacCallum Cancer Centre Human Ethics Committee and according to their guidelines. Methods for tumouroid derivation and cytotoxicity assays are published as supplementary information associated with the publication [163] in Chapter 4 of this thesis, and outlined below.

Biopsy-derived tumour cells were plated on μ -Plate 96 Well (iBidi), by adding 20 μ L of Matrigel/cell suspension per well, and placed to set in 37°C incubator for 30-60 minutes. Following incubation, 350 μ L of OB Media supplemented with: 500 nM A8301 (Torcis, Bioscience), 2X B27™ (Gibco, ThermoFisher), 50 ng/mL Epidermal Growth Factor (Sigma-Aldrich), 1 mg/mL Gastrin (Sigma-Aldrich), 1 mM N acetyl Cyst (Sigma-Aldrich), 5 μ M SB202190 (Sigma-Aldrich), 10 μ M SB431542 (Sigma-Aldrich), 10mM Y27632 (Sigma Aldrich) was added to each well, and tumouroids grown in hypoxic conditions (37°C, 5% O₂, 5% CO₂) for 5-7 days. Tumouroids were grown to >50 μ m and checked for viability using a light microscope on the day of assay, before proceeding. Tumouroids were deemed suitable for co-culture after 10-14 days post initial biopsy.

A total volume of 350 μ L supplemented OB media was applied to the Matrigel-embedded tumouroids. Propidium Iodide (PI) (Sigma-Aldrich) was added, for a final concentration of 2 mg/mL, 2.5 x10⁵ CAR T cells/well were then resuspended in this media. Wells were set up with the following conditions: tumouroids + vehicle control, tumouroids + CAR T cells/ TNF, tumouroids + birinapant, tumouroids + CAR T cells/ TNF + birinapant, CAR T cells + birinapant + TNF neutralising antibody (80 μ g/mL). Tumouroids were incubated at 37°C, 5% CO₂ for 24 hours, and then end-point images were acquired.

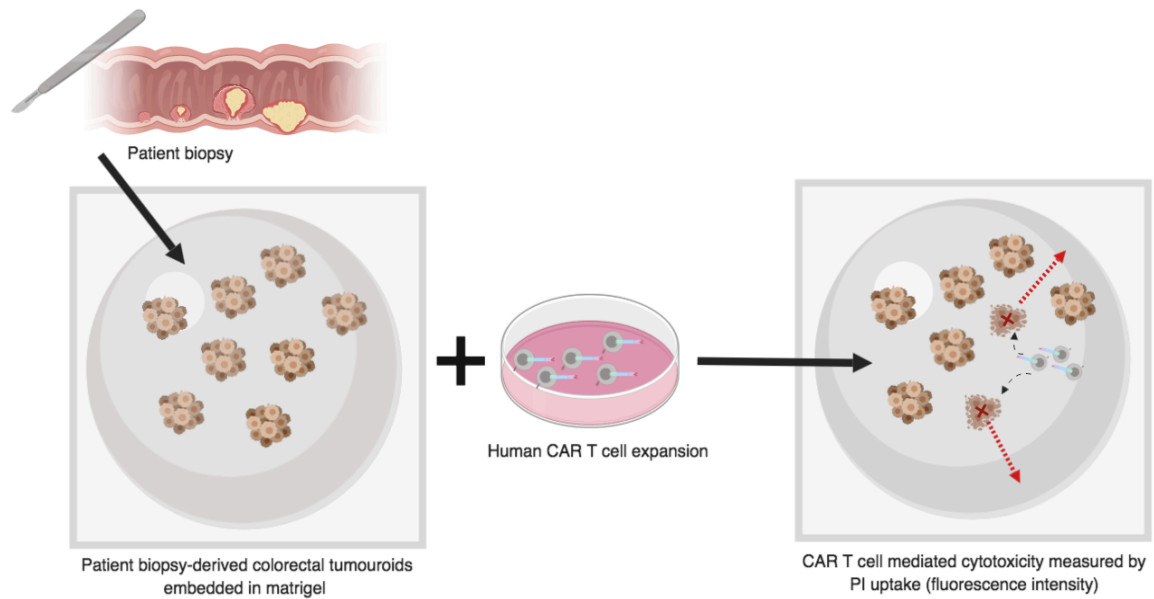


Figure 10. Schematic of tumouroid assay. Upon colorectal tumours resection, tumouroids were established and passaged in matrigel. Donor HER2-CAR T cells were expanded *in vitro* and applied in to tumouroids embedded in matrigel in PI containing media to measure tumouroid cell death.

U-Plate 96 well plate was mounted on a heated stage in a temperature controlled chamber, maintained at 37°C, 5% CO₂ ("The Brick"; iBidi). Using the cellSens software (Olympus), imaging of the plate was obtained on an UPLSAPO 4X (NA 0.16) air objective using a Hamamatsu ORCA-Flash 4.0 camera. Z-stack images were acquired by taking 25 sequential images (Z spacing 50 nm) of PI (emission 656 nm, exposure 368.644 ms) through the Matrigel, and Z stack images converted to one image in EFI format. Image quantitation was conducted using Meta-Morph Imaging Series 7 Software. An identical region of interest was identified within the matrigel and integrated morphometry analysis was used to filter out individual T cells based on size. A threshold was applied to delineate PI positive pixels and integrated intensity was measured. Identical conditions were applied to compared images.

9. Generation of gene-edited tumour cell lines

Tumour cells were transiently transfected using the Amaxa Nucleofector 4D System (Lonza) using Cas9 Nuclease V3 (IDT), and two synthetic guides simultaneously (Synthego) as listed below:

Table 2. Oligonucleotides used to stably deplete genes of interest in these studies.

Gene of interest	Oligonucleotide (5')
B2m	ACTCACTCTGGATAGCATAC
B2m	TCGGCTTCCCATTCTCCGGT
Cbfb	AACCCATACCATCCAATCTG
Cbfb	GCCTTGCAGATTAAGTACAC
Jak1	TCCGAACCGAATCATCACTG
Jak1	TGAATAAATCCATCAGACAG
KMT2D	CCCCTATCCTAGTCCTACGG
KMT2D	CCTTCGAAGCGAAAGTACTG
Negative control (non-targeting)	GCACUACCAGAGCUAACUCA
Runx1	CGGTCCCTACACTAGGACAT
Runx1	TGCGCACTAGCTCGCCAGGG

10. Viral transductions

10.1 Lentivirus

Lentiviral transductions were performed as previously described [91]. MC38 cells were transduced with lentivirus containing lentiGuide-Crimson (Addgene Plasmid: 70683), MHH-CALL-4 cells were transduced with KMT2D guide cloned in to FgH1tUTG lentiviral plasmid (Addgene Plasmid: 70183) in the presence of sequabrene (5 µg/mL; Sigma-Aldrich). Virus was generated from overnight transfection of HEK293T cells in the presence of polyethylenimine (PEI; Sigma-Aldrich) using the following lentiviral envelope and packaging plasmids: pMD2.G, pMDL & pRSV-Rev. Forty eight hours after transfection, the supernatant was harvested and was filtered (0.45 µm) and subsequently

added to adhered MC38 cells. After at least 2 days post-transduction, cells were sorted by flow cytometry at the Peter MacCallum Flow Cytometry Core Facility using a FACS Aria Fusion flow cytometer (BD Biosciences).

10.2 Retrovirus

For generation of *Cbfb* re-expression or over-expression cells, *Cbfb* was cloned in to murine stem-cell virus-internal ribosomal entry site-blue fluorescence protein (MSCV-IRES-BFP). Virus was generated from HEK293GP cells on Poly-D-lysine (PDL) coated plates, in the presence of lipofectamine 2000 (Invitrogen), MSCV-construct and VSV.G (Addgene plasmid: 14888). 48 hours after transfection, virus was filtered and added to MC38 cells, and subsequently BFP⁺ cells were FACS sorted as described above.

11. TCGA and clinical data analyses

The following publicly available human datasets were analysed. TCGA Melanoma: The TCGA melanoma dataset consisted of 300 cases with whole exome sequencing and RNA Seq data available as well as survival follow up [213]. Data were downloaded from cBio cancer genomics portal [214]. Cases were classified as *CBFB* deleted if heterozygous or homozygous copy number deletions were present. The whole cohort was stratified into high-*CD8A* and low-*CD8A* groups based on the median *CD8A* expression. Metastatic Melanoma: 41 cases of metastatic melanoma who received anti-PD1 therapy, and 31 cases who received combination anti-PD1 and anti-CTLA4 therapy. All cases had RNASeq performed on baseline samples [215] and had associated survival follow up and clinical response data. Raw sequencing data for baseline samples in this study were downloaded from the Sequencing Read Archive (BioProject accession PRJEB23709). Adapters were trimmed with Skewer [216] and gene expression quantified with kallisto (version 0.46.0) [217] using the GRCh38 Ensembl transcriptome reference (release 99). tximport [218] and limma [211] were used to process and normalise data for survival analyses. Adoptive cell therapy in Melanoma: 25 cases treated with adoptive cell therapy, with RNA Seq data and survival

follow up and response data available [149]. Processed gene expression data were obtained from the Gene Expression Omnibus (GSE100797). Exploration of the data using principal component analysis determined that a batch effect existed for biopsies of subcutaneous melanoma lesions compared to other sites. This was corrected using ComBat [219] prior to subsequent analyses. To score expression of the published *CBFB/RUNX1* signature [220], the mean expression of genes in the signature (*ROS1*, *ANO1*, *PNLIPRP3*, *DMBT1*, *MCF2*, *FHOD3*, *GDA*, *RNF165*, *B3GALNT1*, *MARC2*) was calculated for each sample. For all datasets, survival curves were produced with the Kaplan-Meier estimator, and compared with the Logrank test. Expression of individual genes or a signature was compared between groups using the Wilcoxon rank-sum test. All human datasets were analysed using R Core Team (2019).

12. Statistical analysis

Data are presented in this thesis are mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by unpaired Student's *t* test, analysis of variance (ANOVA) for more than two data sets, or by Log-rank (Mantel-Cox) test for survival as noted in figure legends. Statistical significance noted when the P value was less than 0.05. * denoted $P < 0.05$, ** <0.01 , *** <0.001 .

**CHAPTER 3: Investigating mechanisms of resistance to CAR T cell
therapy using genome-wide CRISPR-Cas9 screening**

1. Introduction

Despite robust responses against haematological malignancies, the efficacy of CAR T cell therapy against solid tumours in clinical trials remains limited, regardless of the target antigen, or the incorporated co-stimulatory domain. As discussed in Chapter 1, several reasons have been identified for poor CAR T cell efficacy against solid tumours, including poor homing and penetration, and immune exclusion and exhaustion mediated, in part, by the TME. In addition to these tumour-extrinsic factors that limit CAR T cell efficacy, in this chapter we sought to investigate tumour resistance to CAR T cell therapy, and uncover tumour-specific genes that limit CAR T cell efficacy in a solid tumour model.

Genome-wide CRISPR-Cas9 screening has emerged in recent years as a powerful tool to screen all protein-coding genes in the genome that provide resistance, or sensitise to, a specific selection pressure. In this chapter, we utilised a series of unbiased, whole-genome, loss-of-function CRISPR-Cas9 screens designed to investigate the tumour-intrinsic mechanisms of resistance to CAR T cell killing.

1.1 CRISPR-Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR) is a powerful RNA-guided DNA cleavage tool that enables the efficient editing of genomic DNA. The CRISPR gene-editing tool is based upon the endogenous ability of bacteria to protect themselves from the insertion of viral DNA. Bacteria are constantly exposed to exogenous DNA, and as a result have developed defense mechanisms that are able to distinguish between “self” and “non-self” DNA. In bacteria, CRISPR forms an endogenous hyper-variable loci that is generated from the genetic material of invasive DNA, which is used to build acquired DNA-encoded immunity [221].

The bacterial CRISPR-associated protein (Cas9) is an endonuclease that generates double-strand breaks in DNA at a target genome locus. Following the first viral DNA insertion, the Cas complex recognises exogenous DNA as

“non-self” and integrates the novel repeat-spacer unit into the CRISPR locus [221]. This CRISPR repeat-spacer array is used to guide the Cas complex to induce a double stranded break in corresponding DNA sequences upon second viral invasion [222], and provides acquired immunity against viruses and plasmids.

This mechanism has been manipulated to enable efficient DNA editing in eukaryotic cells, which can be used to study a gene of interest or in some cases correct a dysfunctional gene in the clinical treatment of genetic diseases [223]. In order to disrupt a gene of interest, Cas9 is guided to the target locus by single guide RNA (sgRNA) templates, which are short (typically 20 base) RNA sequences that correspond to the target DNA sequence [224]. Using endogenous error-prone non-homologous end joining (NHEJ) break repair mechanisms, the double strand break is repaired in a way that results in the formation of indels, which disrupt the target loci [225]. Importantly, a donor template with homology to the targeted locus may be included, which results in homology-directed repair (HDR), allowing for precise mutations or gene inserts to be made (Figure 11).

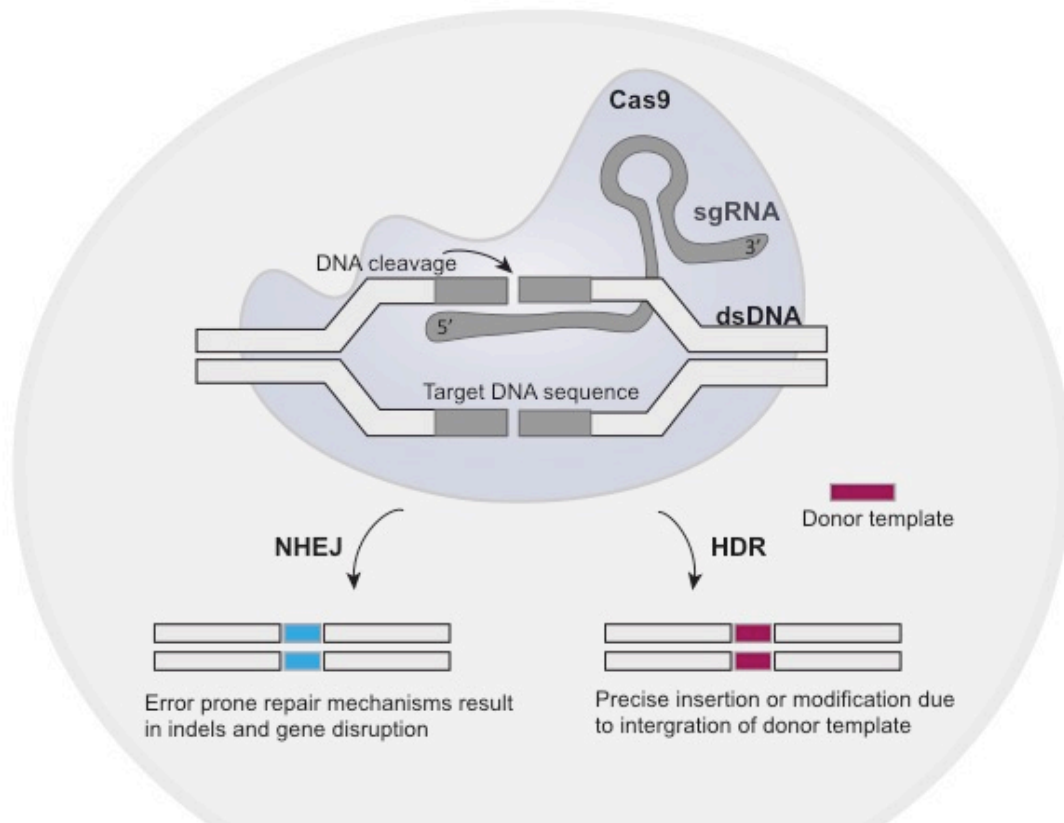


Figure 11. Schematic outlining mechanism of CRISPR-Cas9 gene editing by non-homologous end joining (NHEJ) or homology-directed repair (HDR). Adapted from Tang et al, (2019) [226].

1.2 Whole-genome CRISPR-Cas9 screening

The CRISPR-Cas9 system has been utilised for high-throughput functional screening assays known as genome-wide CRISPR-Cas9 screening. Lentivirus based pooled sgRNA libraries have been generated to target every gene in the mouse or human genome [227], with at least 4 individual sgRNA targeting each gene. This has proven to be a powerful technique that has been used in several elegant positive and negative selection screens, to uncover genes that propagate or abrogate a phenotype or biological process [228].

In this technique, a lentivirus encoding for the pooled sgRNA library is applied to the cell population of interest at a multiplicity of infection (MOI) that enables the integration of one sgRNA per cell [229]. A selection pressure, typically

immune pressure, drug selection or cell sorting on a specific cell surface marker, is then applied to the cell population and the sgRNA in the final population is compared to the starting population (T_0) or an untreated end population (T_{end}), where the sgRNA that are significantly enriched following the selection pressure are identified [212]. This technique has been successfully utilised by a number of groups, using a variety of immune-related selection pressures.

Most notably, several groups have reported the use of CRISPR-Cas9 screening approaches to identify critical mediators of T cell killing in the context of cancer immunotherapy. Overall, these screening approaches have identified antigen presentation and the IFN- γ signalling pathways as critical mediators of response to T cell based immunotherapies, and Patel et al, (2017) identified *Aplnr* as a novel regulator of Jak1 in this context [230]. Another group sought to identify genes that sensitised B16F10 tumour cells to T cell-mediated killing, and identified for the first time that targeting genes from the SW1/SNF chromatin remodeling complex could enhance T cell killing, proposing this complex as a future immunotherapy target [231]. Other genes that have been identified as critical mediators of T cell killing include *CMTM6*, which regulates tumour PD-L1 expression [232], and genes that make up the polycomb repressive complex 2 (PRC2), which mediate the transcriptional silencing of MHC I [233]. In addition, a number of groups have identified loss of TNF signalling as a key tumour resistance mechanism against T cell killing. CRISPR-Cas9 screening approaches have implicated *Ado* in tumour TNF response for the first time [202], and have led the identification of targetable genes, such as *Traf2* within this pathway to enhance the TNF response [203]. An *in vivo* CRISPR-Cas9 screen which sought to determine genes that confer resistance to α -PD-1 identified *Ptpn2* as a novel immunotherapy target, and identified that several genes from TNF signalling and antigen presentation pathways enhanced tumour sensitivity to immunotherapy [234].

Other CRISPR-Cas9 screens have sought to identify genes that confer resistance to, or sensitise to NK cell killing. Deletion of genes involved in TNF-

induced cell death have been identified as a key resistance mechanism to NK cell-mediated killing [202], while loss of various genes that are critical to the IFN- γ or antigen presentation pathways sensitise tumour cells to NK cell-mediated killing [91], showing in an unbiased way the dependency on NK cells to detection of “missing self”. Importantly, this suggests that NK cells may overcome T cell-resistance through loss of antigen presentation in the context of MHC I, highlighting the potential for NK cell-based immunotherapies to overcome this resistance mechanism.

Resistance to CAR T cell therapy has also been investigated using genome-wide CRISPR-Cas9 screen approaches. Recently, tumour-intrinsic mechanisms of resistance to CD19-CAR T cell therapy in the B-ALL cell line, Nalm6, was interrogated. In this context, loss of CD19 and death receptor signalling conferred resistance to CAR T cell killing [235] and will be discussed in greater detail in Chapter 5. Thus far, there have been limited reports of CRISPR-Cas9 screen approaches to identify mechanisms of resistance to CAR T cell therapy in solid tumours. Of note, Kearney et al, (2018) conducted a genome-wide CRISPR-Cas9 screen investigating mechanisms of resistance to HER2-directed CAR T cells, in the human MDA breast cancer cell line. In this case, since HER2 is endogenously expressed in this cell line, loss of HER2 expression was the key mechanism of resistance to CAR T cell therapy identified in this CRISPR-Cas9 screen [202]. In order to interrogate mechanisms of resistance aside from antigen loss, in this chapter, we conducted a mouse CRISPR-Cas9 screen using CAR T cells transduced with a truncated form of human HER2. Using this approach, we were able to avoid sgRNA enrichment of the target antigen.

2. Results

CRISPR-Cas9 screening reveals that loss of cytokine response pathways limit susceptibility to CAR T cell killing

In order to determine tumour-intrinsic mechanisms of resistance to CAR T cell therapy, we conducted a whole-genome loss-of-function CRISPR-Cas9

screen. First, we generated mouse HER2-directed CAR T cells. As with CAR T cell therapy in patients, bulk CAR T cells containing both CD4⁺ and CD8⁺ T cell populations were utilised, typically at a 30:70 ratio. The CAR T cells typically demonstrated an activated phenotype (CD44^{high}), and 30-60% transduction efficiency of the CAR was achieved, which was determined by the detection of Myc within the CAR construct (Figure 12). Next, we confirmed the ability of the CAR T cells to kill tumour cells in an antigen specific manner (Figure 13), and then proceeded to investigate the tumour-intrinsic mechanisms of resistance to this CAR T cell mediated killing.

Utilising a mouse adenocarcinoma cell line, transduced to express a truncated version of the human HER2 antigen (MC38-HER2), we conducted a genome-wide CRISPR-Cas9 screen to identify which genes, when disrupted, protect tumour cells from CAR T cell-mediated killing. Briefly, Cas9⁺ MC38-HER2 cells were transduced with the mouse “Brie” pooled lentiviral sgRNA library, and subjected to puromycin selection for 5 days. Following selection, MC38-HER2 cells were subjected to overnight CAR T cell killing, and allowed to recover between each co-culture. After 3 successive rounds of CAR T cell killing, the resistant population was sequenced to identify the remaining sgRNA, and therefore which genes when deleted resulted in protection against CAR T cell killing.

The resultant cell population showed significant enrichment for sgRNA targeting genes associated with cytokine signalling pathways, suggesting that loss of response to cytokine signalling was a key mechanism of resistance to CAR T cell therapy (Figure 14). We identified that loss of genes associated with TNF and IFN- γ signalling protected tumour cells from CAR T cell killing, underscoring the cytotoxic potential of CAR T cell derived cytokines in solid tumour killing in this model.

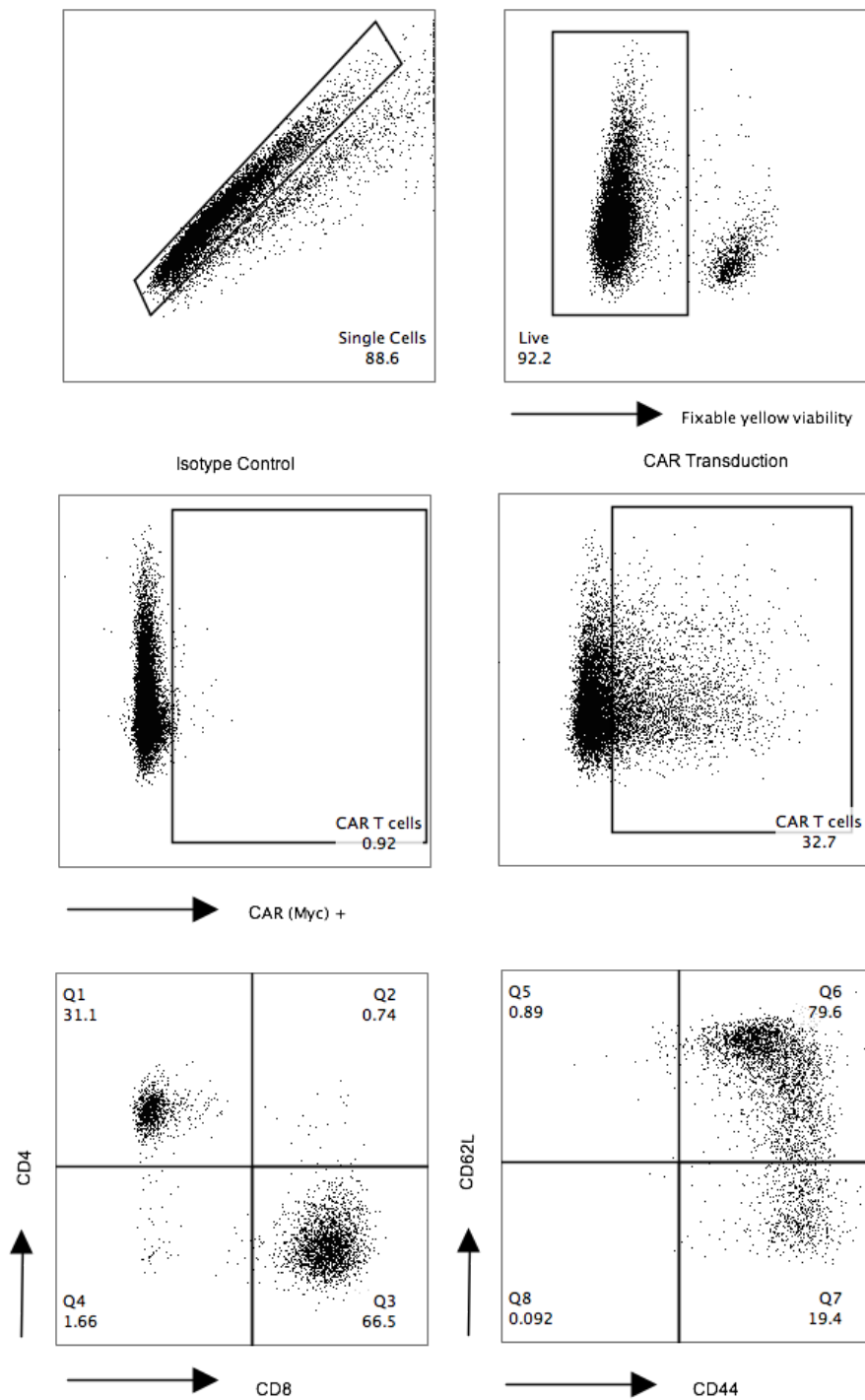


Figure 12. Generation of human HER2 specific mouse CAR T cells. The percentage of live cells positive for the CAR construct (myc), the CD4: CD8 ratio of the CAR T cells, and the activation phenotype based on CD62L and CD44 expression was determined, 4 days post-transduction by flow cytometry.

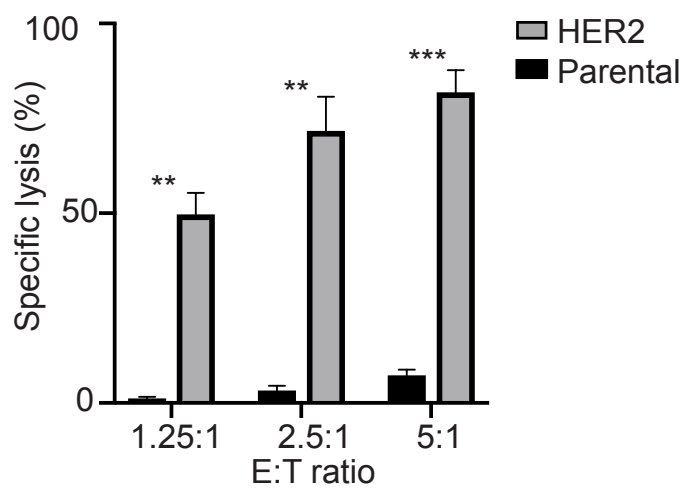


Figure 13. CAR T cells kill in an antigen specific manner. Overnight ^{51}Cr -release assay of CAR T cell killing of MC38 parental and HER2 cells at indicated E:T ratios. Data is mean $\pm$ SEM from a representative experiment.

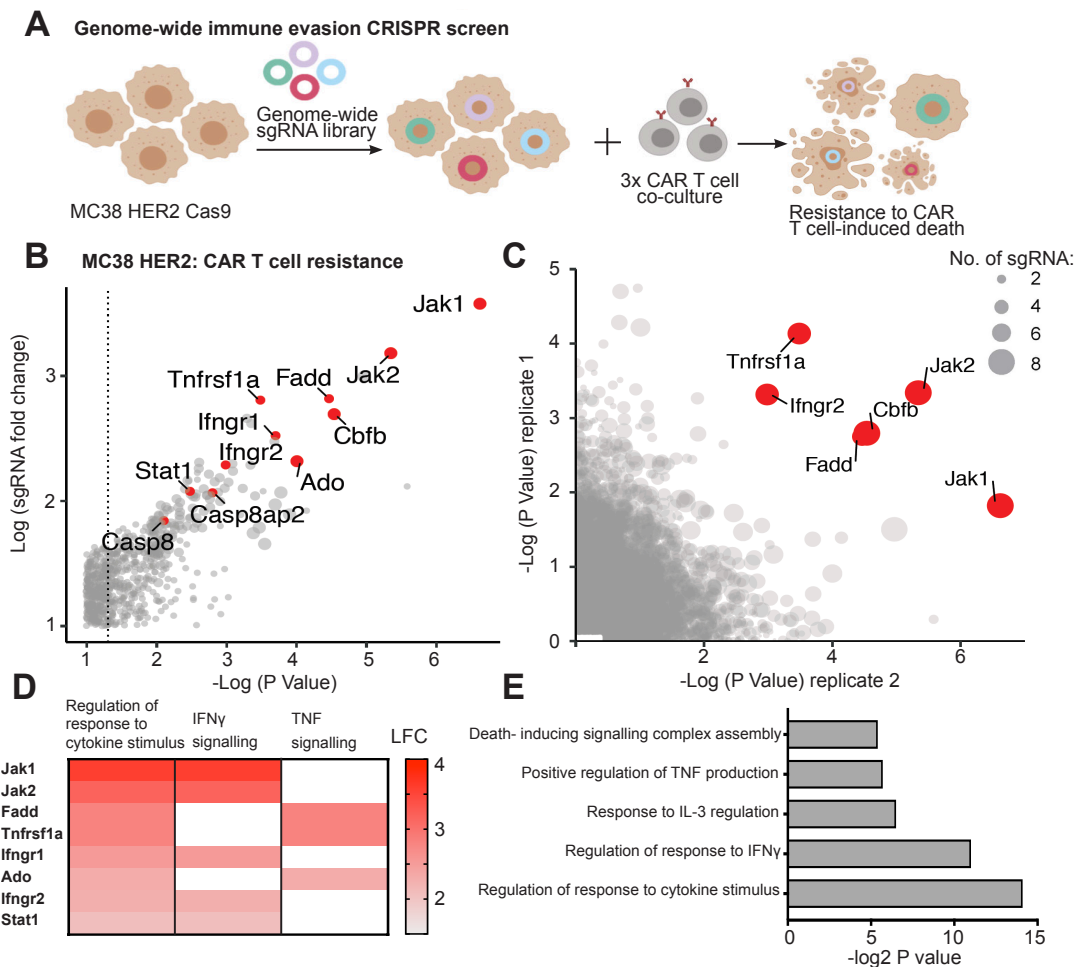


Figure 14. CRISPR-Cas9 screening reveals that loss of cytokine response pathways limit susceptibility to CAR T cell killing. (A) Schematic of the CRISPR-Cas9 screen methodology utilised. (B) Volcano plot showing p value and sgRNA fold change from CRISPR-Cas9 screen. (C) Scatterplot showing the shared top enriched sgRNA between biological replicates. (D) Heatmap showing LFC in genes from the IFN- γ and TNF signalling pathways from (B). (E) Gene Ontology (GO) term analysis showing enriched biological processes for top depleted genes identified in (B)

IFN- γ enhances TNF-mediated killing in MC38 cells

Next we investigated the mechanism by which loss of TNF and IFN- γ signalling protected tumour cells against CAR T cell killing. As discussed in Chapter 1, TNF is able to induce apoptosis directly in tumour cells, and therefore the protection conferred by loss of TNF signalling pathway genes was expected. Loss of IFN- γ signalling is protective against T cell killing [202], and is a known resistance pathway to immune checkpoint blockade in the clinic [131, 135]. However, this protective effect is due, at least in part, to the role of IFN- γ in upregulating antigen presentation in the context of MHC I. In contrast, MHC I is not required for CAR T cell killing, and the target antigen used for the CRISPR-Cas9 screen, HER2, is not upregulated by IFN- γ . Therefore, we assessed the role of IFN- γ signalling in CAR T cell killing. First, we stably depleted *Jak1*, a critical component of IFN- γ signalling which is downstream of the IFN- γ receptor (IFNGR1), and upstream of STAT1 and IRF1-mediated transcription, in MC38 cells and subjected them to CAR T cell killing (Figure 15A).

First, we identified that *Jak1*-deficient MC38 cells were more resistant to CAR T cell killing than control cells in an overnight killing assay (Figure 15A). Next we determined the role of IFN- γ signalling in mediating TNF-dependent apoptosis (Figure 15B). MC38 cells were treated with recombinant TNF overnight and percentage specific lysis determined. MC38 cells that were treated with both recombinant TNF and IFN- γ had significantly greater cell death than cells which were treated with TNF alone (Figure 15C), suggesting that in the context of IFN- γ exposure, tumour cell sensitivity to TNF is enhanced.

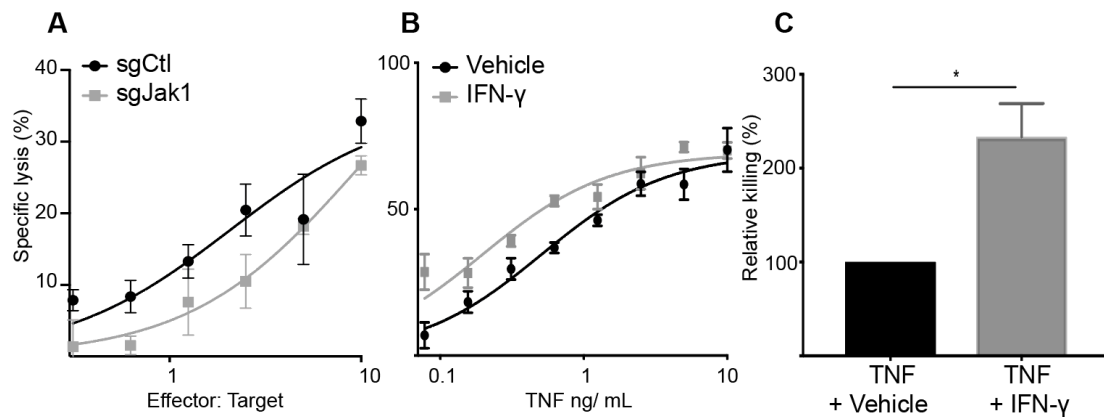


Figure 15. IFN- γ enhances TNF-mediated killing in MC38 cells. (A) Overnight ^{51}Cr -release assay showing CAR T cell-induced death of MC38-HER2 cells at the indicated E:T ratio. Data is mean $\pm$ SEM from a representative experiment. (B) Overnight ^{51}Cr -release assay showing recombinant TNF-induced death of MC38-HER2 cells at the indicated concentrations in the presence or absence of recombinant IFN- γ (1 ng/ mL). Data is mean $\pm$ SEM from a representative experiment. (C) Relative killing of TNF treated MC38 cells in the presence or absence of recombinant IFN- γ (1 ng/ mL). Data is pooled from three independent experiments.

Genome-wide CRISPR-Cas9 screening reveals that *Cbfb* protects tumour cells from T cell killing

In the genome-wide CRISPR-Cas9 screens (Figure 14A and 14B), we identified that loss of the transcriptional co-binding partner Core-binding factor beta (*Cbfb*) protected MC38-HER2 cells from CAR T cell-mediated killing. *Cbfb* is an essential co-binding partner of the *Runx* family of transcription factors, where it heterodimers in order to enhance the DNA-binding and transcriptional activity [236]. The role of *Cbfb* has been well characterised in hematopoiesis and various leukaemias [236-238], where mutations are present in 5-7% of AML [239]. However, the role of *cbfb* in solid malignancies remains poorly understood, although it has been implicated in patient prognosis in colorectal [240] and breast cancers [220]. *Cbfb* has been associated with regulating a diverse range of biological processes through mediation of both transcription and translation [220].

Our group has previously conducted CRISPR-Cas9 screening in MC38-Ova cells to investigate mechanism of resistance to killing by OT-I T cells [202], mouse T cells that recognise the chicken protein ovalbumin (Ova) presented in the context of MHC I. In order to investigate whether *Cbfb* also confers resistance against MHC-restricted T cells, as well as CAR T cells, we compared the significantly enriched sgRNAs from both CRISPR-Cas9 screens (Figure 16).

Analysis of the significantly enriched sgRNAs in the resultant MC38 population following CAR T cell or OT-I T cell killing, again identified that loss of genes associated with both IFN- γ and TNF signalling protected against T cell-mediated killing. In addition, *Cbfb* was significantly enriched in both CRISPR-Cas9 screens, suggesting a mechanism of action that is common to both CAR and OT-I T cell killing.

To validate that loss of *Cbfb* confers resistance to CAR T cell killing, we stably depleted *Cbfb* in MC38-HER2 cells (Figure 17A), and investigated the ability of CAR T cells to kill control or *Cbfb*-depleted MC38-HER2 cells, in short and

long term killing assays. We identified that loss of *Cbfb* did not protect against CAR T cell killing in a 4 hour assay (Figure 17B), which is mediated by perforin and granzyme B in the context of an immunological synapse. However, we observed that *Cbfb*-depleted MC38-HER2 cells were significantly less sensitive to CAR T cell killing in an 18 hour killing assay (Figure 17C), which includes cytokine mediated killing, despite equal concentrations of TNF and IFN- γ being detected in the supernatant (Figure 18).

In addition, we sought to validate that loss of *Cbfb* confers protection against OT-I T cell killing, and CAR T cell killing in another model. First, we conducted overnight killing assays using OT-I T cells and MC38-Ova targets and observed that *Cbfb*-depleted MC38-Ova cells were more resistant to OT-I T cell-mediated killing (Figure 19A). Then we investigated CAR T cell killing of control or *Cbfb*-depleted E0771 mouse breast cancer cells transduced to express human HER2, and observed a similar, albeit less striking, phenotype (Figure 19B).

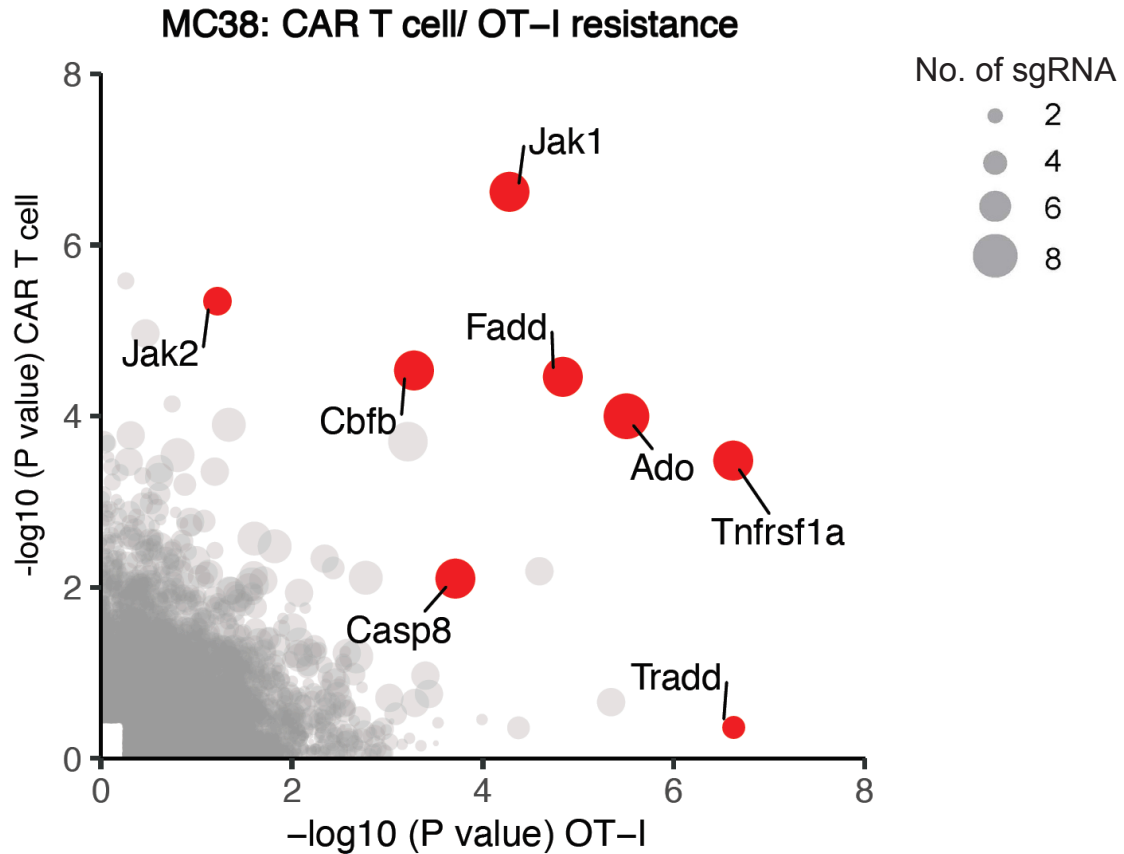


Figure 16. Genome-wide CRISPR-Cas9 screening reveals *Cbfb* protects tumour cells from T cell killing. Scatterplot showing significantly enriched sgRNA in the CAR and OT-I T cell screens. Genes relating to response to cytokine signalling are shown in red. Significantly overlapping genes of note between the two screens include *Cbfb*, *Fadd*, *Jak1*, *Ado* and *Tnfrsf1a*.

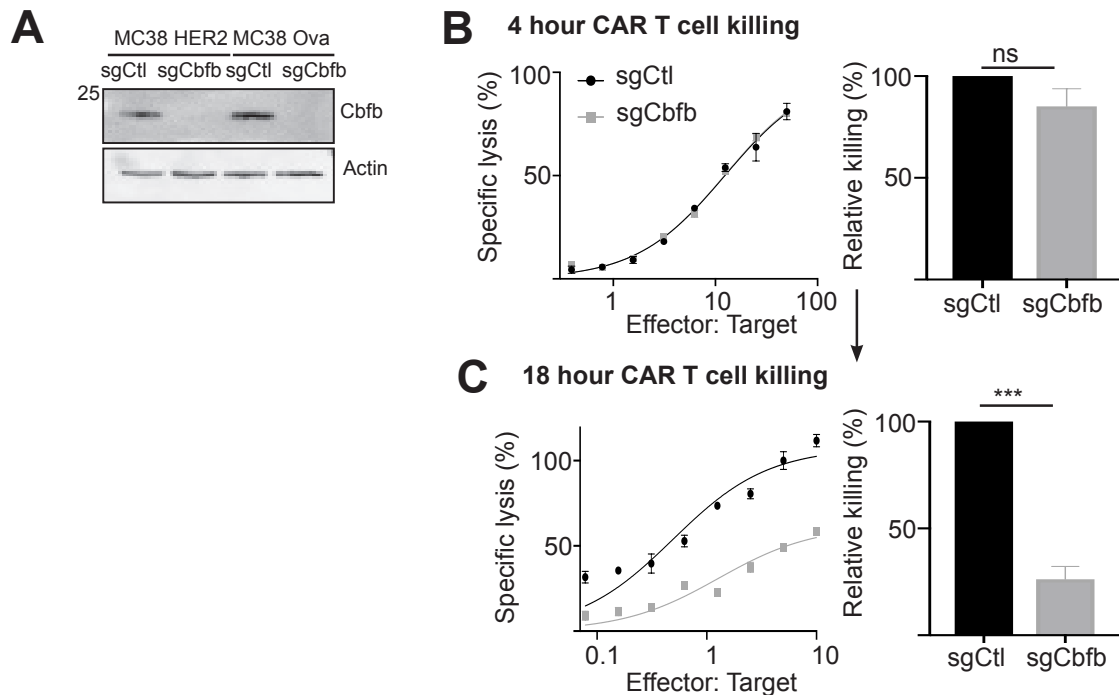


Figure 17. Loss of *Cbfb* protects against CAR T cell killing. (A) Western blot showing *Cbfb* depletion in MC38-HER2 and MC38-Ova cells. Two *Cbfb*-specific synthetic sgRNA were used simultaneously to achieve efficient depletion, and used at least 7 days post-transfection. (B) 4-hour ^{51}Cr -release assay of CAR T cell killing of MC38-HER2 cells at indicated E:T ratios presented on a log10 scale. (C) 18-hour ^{51}Cr -release assay of CAR T cell killing of MC38-HER2 cells at indicated E:T ratios presented on a log10 scale. Data is mean $\pm$ S.E.M of triplicate determinations. Relative killing data on right is pooled from three independent experiments.

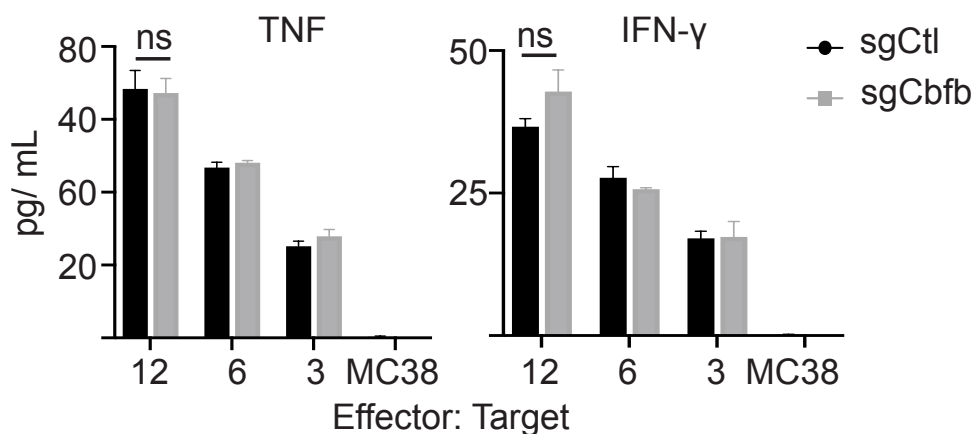


Figure 18. Equivalent CAR T cell cytokine secretion in co-culture with control or *Cbfb*-depleted MC38-HER2 cells. Cytokine bead assay measuring CAR T cell derived TNF and IFN-γ concentration in the supernatant in a 4 hour co-culture with control or *Cbfb*-depleted MC38-HER2 cells from Figure 17C above. Data is mean $\pm$ S.E.M of triplicate determinations, representative of two independent experiments.

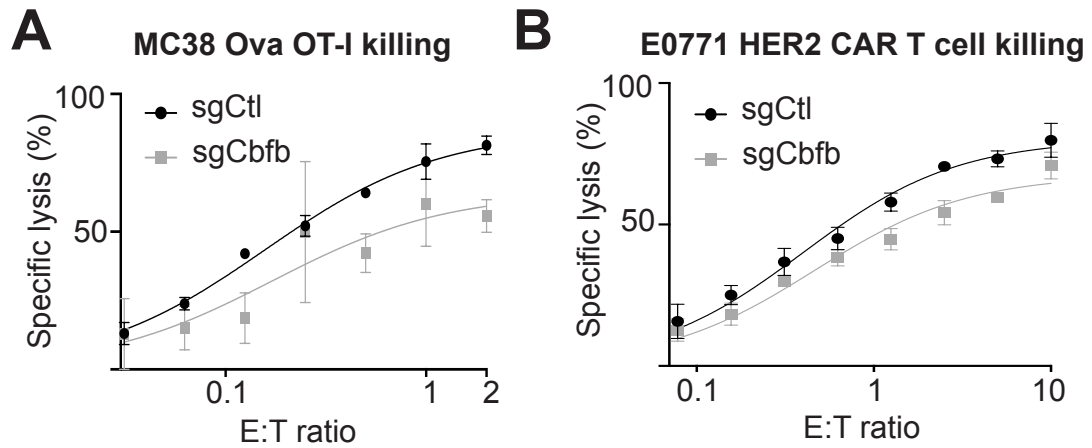


Figure 19. Loss of Cbfb protects against OT-I killing of MC38-Ova cells and CAR T cell killing of E0771-HER2 cells (A) 18-hour ^{51}Cr -release assay of OT-I killing of MC38-Ova cells at indicated E:T ratios presented on a log10 scale. (B) 18-hour ^{51}Cr -release assay of CAR T cell killing of E0771-HER2 cells at indicated E:T ratios presented on a log10 scale. Data is mean $\pm$ S.E.M of triplicate determinations, representative of two experiments.

***Cbfb*-depleted cells exhibit an altered transcriptional phenotype**

Based on the killing assay data, where *cbfb*-depleted cells demonstrated CAR T cell resistance only in a long-term killing assay, we hypothesised that *Cbfb*-depleted MC38-HER2 cells were more resistant to CAR T cell killing through disrupted cytokine signalling. To interrogate this further, we compared the transcriptional profile of control and *cbfb*-depleted MC38-HER2 cells co-cultured with CAR T cells (Figure 20).

We observed that *Cbfb*-depleted cells displayed significant transcriptional differences upon CAR T cell exposure compared to control cells, with Hallmark Gene Set Enrichment Analysis (GSEA) revealing that apoptosis was among the most significantly altered pathways (Figure 20A and E). Using a published MC38 transcriptional data set, treated with recombinant TNF or IFN- γ [202], we next generated stringent boutique MC38 TNF or IFN- γ signatures using the top upregulated genes ($p = <0.005$) and applied this to our RNA seq data set. These analyses revealed a significant enrichment in TNF response genes in control cells compared to *Cbfb*-depleted cells (Figure 20B and D) but no significant enrichment for IFN- γ response genes (Figure 20C), confirming specific modulation of the TNF signalling pathway in the absence of *cbfb*.

In addition to investigating differences in the transcriptional profile of *Cbfb*-depleted cells following CAR T cell treatment, we also investigated transcriptional differences in *Cbfb*-depleted cells compared to wild type cells at steady state (Figure 21). GSEA analysis of the transcription profile of these cells shows that control MC38 cells have a significantly enriched epithelial to mesenchymal transition (EMT) phenotype compared to control cells, suggesting that compared to control cells, *cbfb*-depleted cells maintain an epithelial-like phenotype (Figure 21).

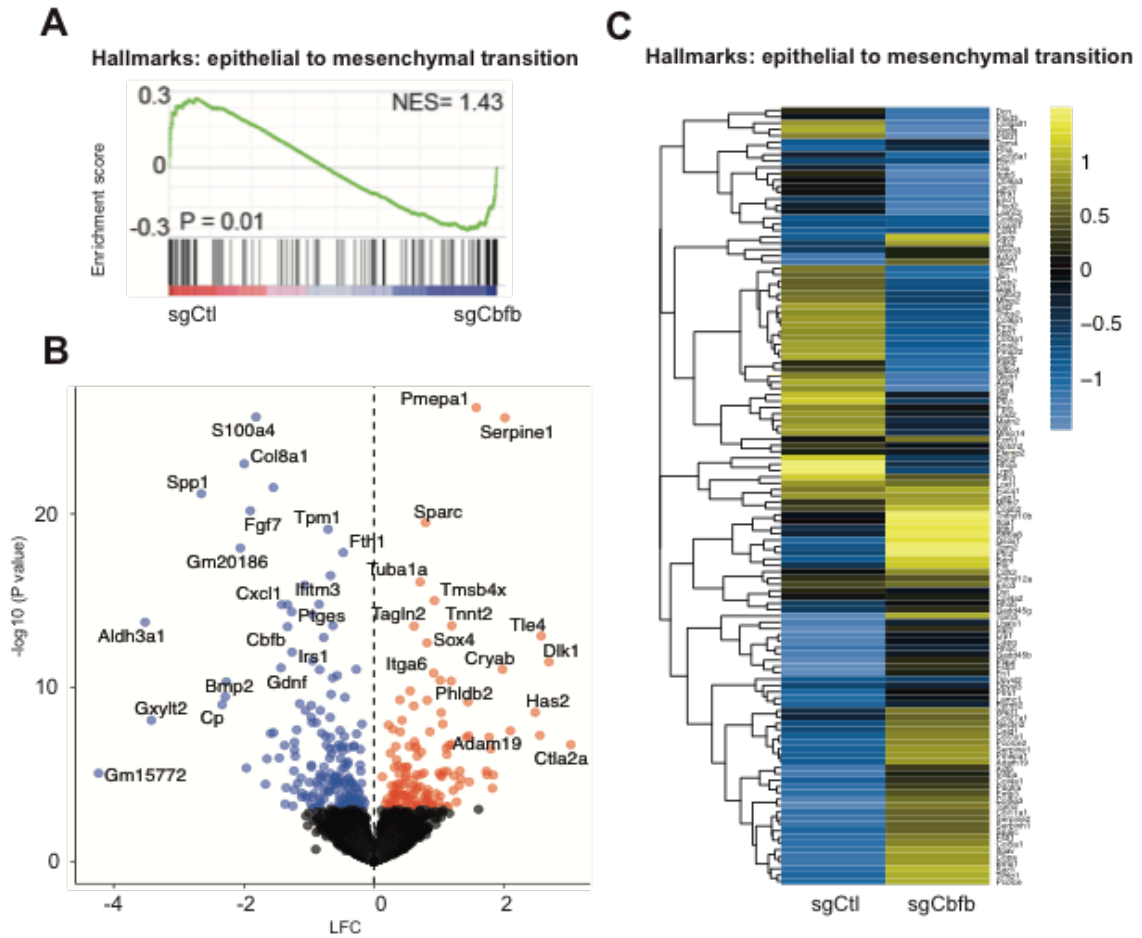


Figure 21. RNA sequencing reveals an altered epithelial-to-mesenchymal transition transcriptional phenotype. Control MC38 cells and Cbfb-depleted were subjected to 3' RNA sequencing. (A) GSEA analysis reveals a significant enrichment for Hallmark epithelial to mesenchymal transition in the transcription profile of control cells compared to *Cbfb* knockout cells. (B) volcano plot showing significantly changed genes. (C) Heatmap showing altered genes in the Hallmark epithelial to mesenchymal transition gene set.

***Cbfb*-deficient MC38 cells exhibit altered sensitivity to TNF-mediated apoptosis**

To functionally validate the transcriptional findings, we assessed the responsiveness of *Cbfb*-depleted MC38-HER2 cells to IFN- γ by assessing up-regulation of MHC I (H-2Kb) and PD-L1 (Figure 22A). We found that *Cbfb*-depleted tumour cells upregulated both MHC I and PD-L1 after IFN- γ stimulation to the same extent as control cells, while, as expected, *B2m*-depleted and *Jak1*-depleted MC38-HER2 cells failed to upregulate H-2Kb and PD-L1, respectively. To further assess *Cbfb* depletion-mediated resistance to CAR T cell killing via TNF, we mixed MC38-HER2 cells transduced with either control sgRNA crimson or *Cbfb* sgRNA at a 1:1 ratio and cultured the mixed population with CAR T cells, or recombinant TNF (Figure 22B). Analyses of the surviving tumour cell population by flow cytometry confirmed significant enrichment of the *Cbfb*-depleted tumour cells under both conditions (Figure 22C) suggesting that *Cbfb* plays a role in determining sensitivity to T cell-derived TNF. Indeed, MC38 cells, which are sensitive to low nanomolar concentrations of TNF, were highly resistant to cell death induced by recombinant TNF when *Cbfb* was stably depleted (Figure 23).

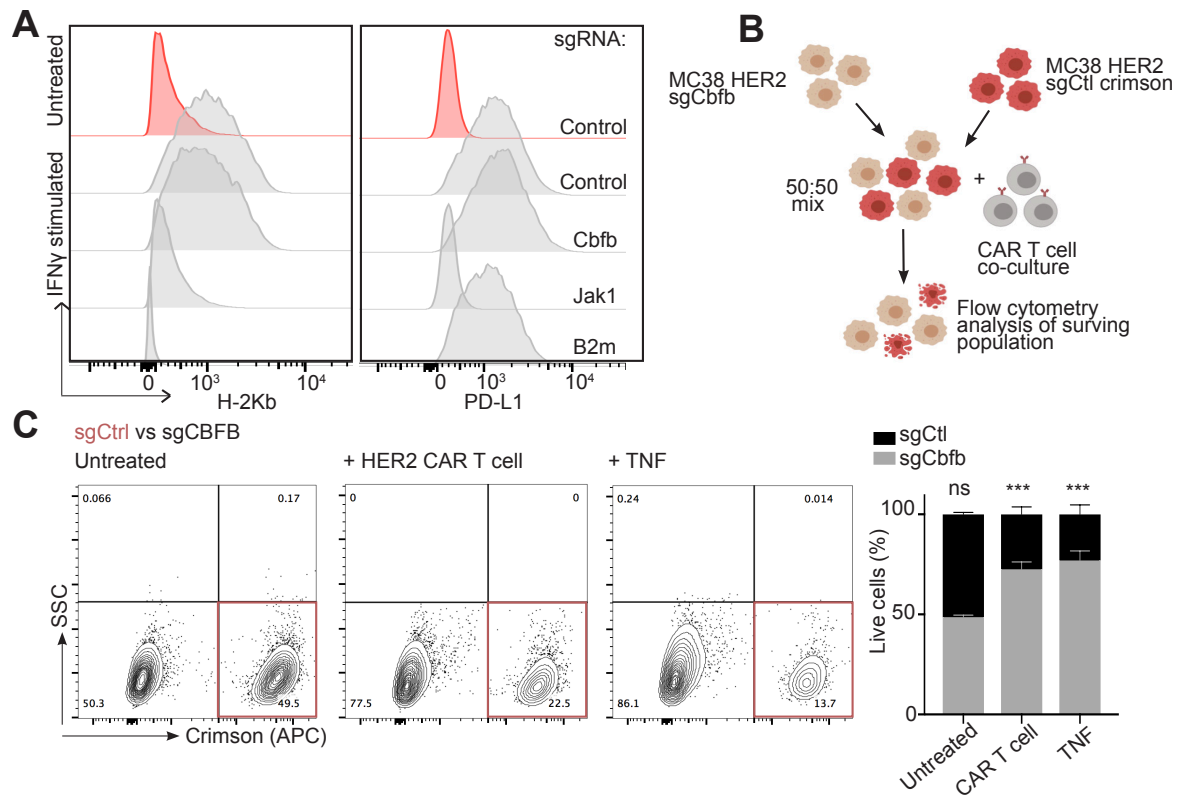


Figure 22. *Cbfb*-deficient cells functionally respond to IFN- γ but are less sensitive to TNF-mediated apoptosis. (A) Expression of MHC-I and PD-L1 in control or *Cbfb*-, *B2m*- or *Jak1*-depleted MC38 cells treated with mouse IFN- γ (10 ng/mL). (B) Schematic of competition assay. (C) Representative plots showing proportion of control (sgCt^l) crimson+ and *Cbfb*-depleted MC38 cells in untreated, CAR T cell treated (2:1 E:T ratio), or TNF (5 ng/mL) treated groups, quantitated on right. Data is mean $\pm$ SEM pooled from 3 experiments.

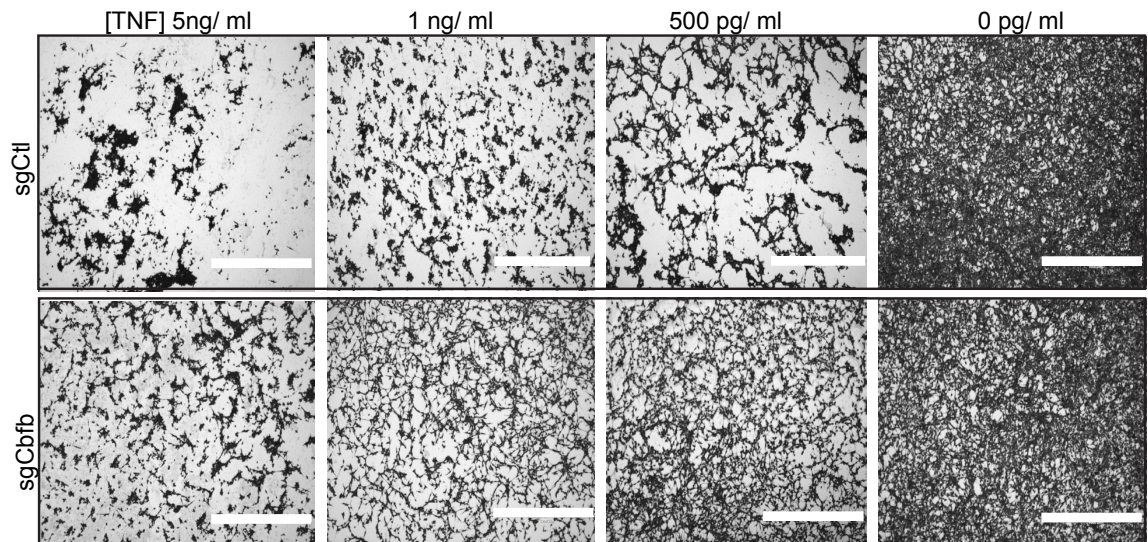


Figure 23. *Cbfb*-deficient cells are more resistant to recombinant TNF. Control or *Cbfb*-depleted MC38 cells were cultured in TNF containing media at the indicated concentrations for 48 hours. Cells were stained with Crystal Violet and visualised under a light microscope. Scale bar is 1000 μ m.

***Cbfb*-depleted tumour cells exhibit diminished chemokine secretion**

In addition to immune cells, within the TME the tumour cells themselves are major producers of chemokines, typically in response to IFN- γ and TNF exposure. Having identified that *Cbfb*-depleted cells had an attenuated TNF response, but intact IFN- γ signalling, we investigated the ability of *Cbfb*-depleted cells to secrete chemokines following TNF and IFN- γ stimulation or CAR T cell co-culture (Figure 24). Additionally, *Jak1*-depleted cells were used as a control, given their inability to respond to IFN- γ .

We observed that the secretion of MIP-3A, CXCL10 and CXCL1 was dependent on TNF exposure, and that secretion of MIP-3A and CXCL10 was diminished in *cbfb*-depleted cells, compared to control or *Jak1*-depleted cells. In addition, secretion of MCP-1 following CAR T cell co-culture was diminished in both *Jak1* and *Cbfb*-deficient cells. These data show that chemokine secretion in *Cbfb*-deficient cells is impaired, providing further function evidence for impaired TNF-signalling, given the critical need for TNF exposure in chemokine transcriptional up-regulation.

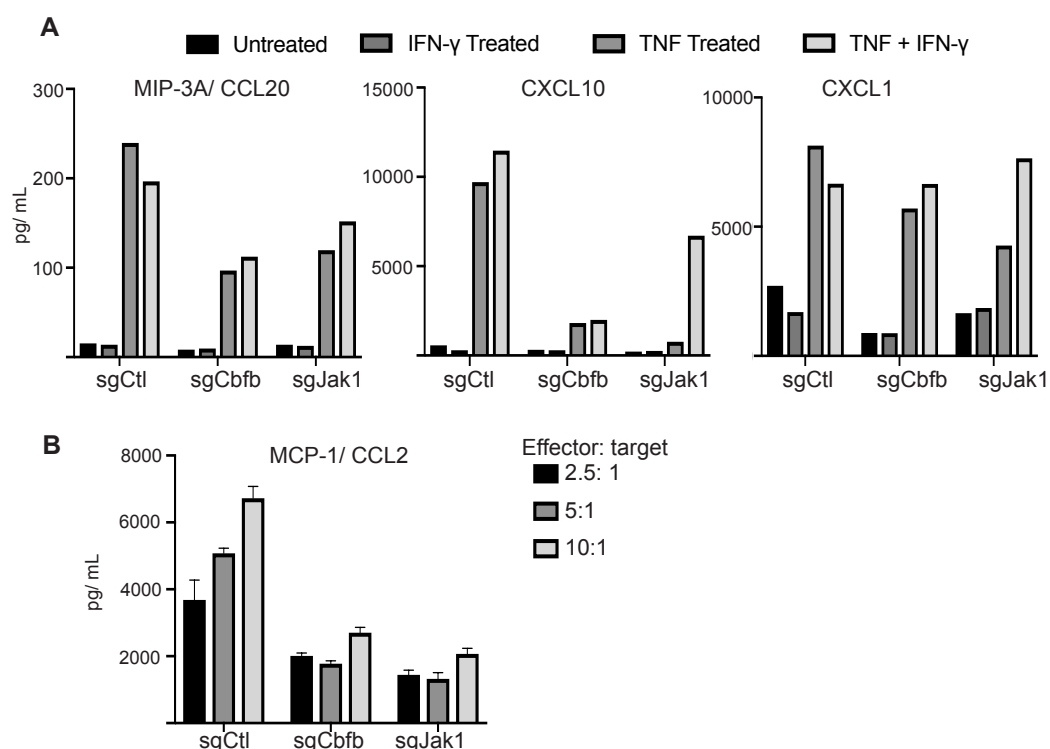


Figure 24. Chemokine secretion of *Cbfb* or *Jak1*-depleted cells following TNF and/ or IFN-γ exposure or CAR T cell co-culture. (A) Secretion of CCL20, CXCL10 and CXCL1, following exposure to recombinant TNF (10 ng/mL), IFN-γ (10 ng/mL) or both, for 6 hours. Chemokines were detected by chemokine CBA. (B) MCP-1 secretion of MC38-HER2 cells following CAR T cell co-culture at the indicated E:T ratios. MCP-1 was detected by mouse inflammation CBA. Data is mean ± S.E.M from triplicate determinations.

***Runx1* and *Cbfb*-depleted cells exhibit reduced TNF sensitivity**

Next we considered the mechanism by which loss of *Cbfb* reduces sensitivity to TNF. Reduced expression of *Cbfb* confers resistance to MEK inhibitors in colorectal cancer [241], a phenotype that was dependent on *Runx2* transcription. As an essential co-binding partner of the *Runx* family of transcription factors, *Cbfb* does not bind to DNA directly, but forms heterodimers to enhance the DNA-binding and transcriptional activity of *Runx* family members [236]. Interestingly, no *Runx* family genes scored significantly in our genome-wide CRISPR-Cas9 screens, so we investigated which *Runx* transcription factor was responsible for the observed phenotype, or to identify any *Cbfb*-dependent and *Runx*-independent transcriptional activity.

Chromatin-Immunoprecipitation sequencing (Ch-IP seq) data is presented in this thesis as an appendix. Despite some confounding technical challenges, Homer Known Motif Enrichment analysis suggested that in MC38 cells, *Cbfb* facilitates *Runx1*-mediated transcriptional activity (appendix), however this experiment will be repeated to ensure the reliability of the data.

Based on this information, we investigated a role for *Runx1* in TNF-mediated apoptosis by stably depleting *Runx1*, *Cbfb*, or both, in MC38 cells and assessed their sensitivity to TNF-mediated cell death. Both *Cbfb* and *Runx1*-depleted cells exhibited reduced sensitivity to TNF-mediated cell death compared to control cells (Figure 25A), suggesting a potential link between the role of *Cbfb* in mediating tumour cell sensitivity to TNF and *Runx1* transcriptional activity. Furthermore, sensitivity to TNF was restored by re-expressing *Cbfb* in *Cbfb*-depleted cells (Figure 25B), confirming an on-target phenotype.

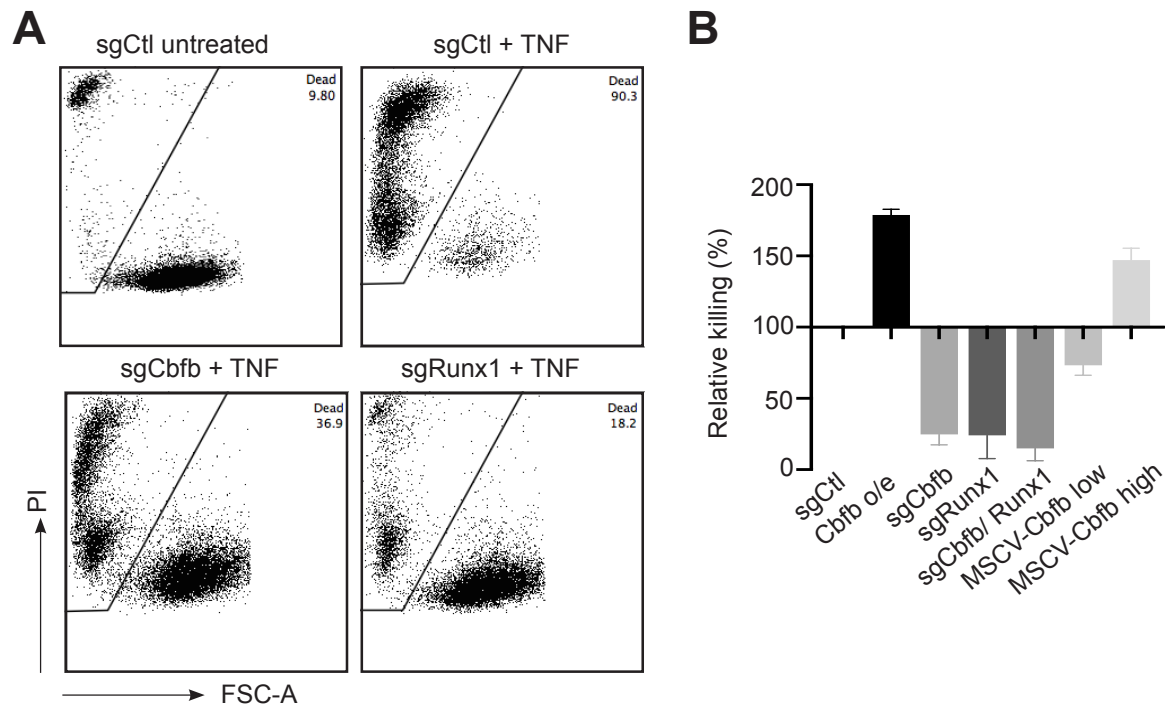


Figure 25. *Runx1* and *Cbfb*-depleted cells exhibit reduced TNF sensitivity (A) Representative flow cytometry plots of control, *Cbfb* or *Runx1*-depleted MC38 cells treated with mouse TNF (1 ng/mL). Dead/ dying cells were determined by PI-positivity and morphology. (B) Relative killing data from MC38 control, *Cbfb*, *Runx1* or double knockout, or *Cbfb* over-expressing (*Cbfb*^{o/e}), or *Cbfb*-knockout MC38 cells re-expressing *Cbfb* at low (MSCV-*Cbfb*^{low}) or high (MSCV-*Cbfb*^{high}) levels. Relative killing data is calculated by proportion of dead/dying cells following TNF treatment after subtraction of background death. Data is pooled from two independent experiments.

Cbfb*-depleted tumour cells are less responsive to immune checkpoint blockade *in vivo

Given that immune checkpoint blockade is a T cell-mediated immunotherapy that relies in part on TNF-mediated cytotoxicity [203], and loss of *cbfb* protected tumour cells against T cell-derived TNF, we next investigated whether *Cbfb*-depleted MC38 cells responded to α -PD-1 therapy *in vivo* (Figure 26). We observed that α -PD-1 therapy in mice bearing control MC38 tumours resulted in significantly reduced tumour burden at day 12 post-treatment compared to mice bearing *Cbfb*-depleted tumours, which did not respond to treatment (Figure 26). Accordingly, α -PD-1 treated mice bearing control tumours had a significant survival advantage compared to mice bearing *Cbfb*-depleted tumours.

Based on our *in vivo* data, and previous reports that *CBFB* expression may be a prognostic indicator in colorectal cancers [240] and in breast cancer [220], we hypothesised that tumour expression of *CBFB* and mutation status may have significant implications for cancer immunotherapy. We therefore interrogated clinical data from melanoma patients, a cohort in which ICB has had remarkable success, to identify the predictive value of *CBFB* tumour expression and overall survival in these patients. Using the publicly available TCGA melanoma data set [213] which comprises a mixture of primary and metastatic tumours [242] we found that, while high *CD8a* expression in the tumour is predictive of improved survival, this effect is attenuated when a *CBFB* homozygous or hemizygous deletion is present (Figure 27), suggesting that the CD8⁺ T cell-mediated anti-tumour responses are less effective in this setting.

Next, we interrogated clinical data from immunotherapy treated melanoma patient cohorts to dissect the predictive value of *CBFB* expression on response to therapy. As *CBFB* is highly expressed in immune cells, we assessed clinical trial data only where adequate sequencing allowed us to interrogate tumour *CBFB* expression only [215]. We observed that low *CBFB* expression in tumours prior to anti-PD-1 therapy was associated with reduced

overall survival (Figure 28A) although this was not statistically significant due to the low numbers of patients in this cohort with matched tumour transcriptome data. However, low expression of *RUNX1* was associated with a significantly poorer progression-free survival in dual checkpoint (anti-PD-1/anti-CTLA-4) treated patients (Figure 28B and C).

Finally, we assessed the association of tumour *CBFB* expression and responses to adoptive cell therapy (ACT) in a melanoma patient cohort [149]. Low *CBFB* tumour expression was again associated with poorer overall survival following therapy, although this was not statistically significant due to the low numbers of patients in this study, with only 1/11 patients with low *CBFB* expression surviving >36 months compared to 7/14 with high *CBFB* expression (Figure 29A). We also applied a previously described *CBFB*/*RUNX1* signature [220] to the patient cohort, and stratified the patients based on progression following ACT. We observed a significant difference in both *CBFB* gene signature and *RUNX1* expression in progressers vs. non-progressers (Figure 29B and C). Together, this data suggests that dysregulation of *CBFB* and *RUNX1* genes confers resistance to both ICB and ACT. This finding is clinically relevant because there is an unmet clinical need for biomarkers to determine responders and non-responders to ACT and ICB prior to onset of treatment.

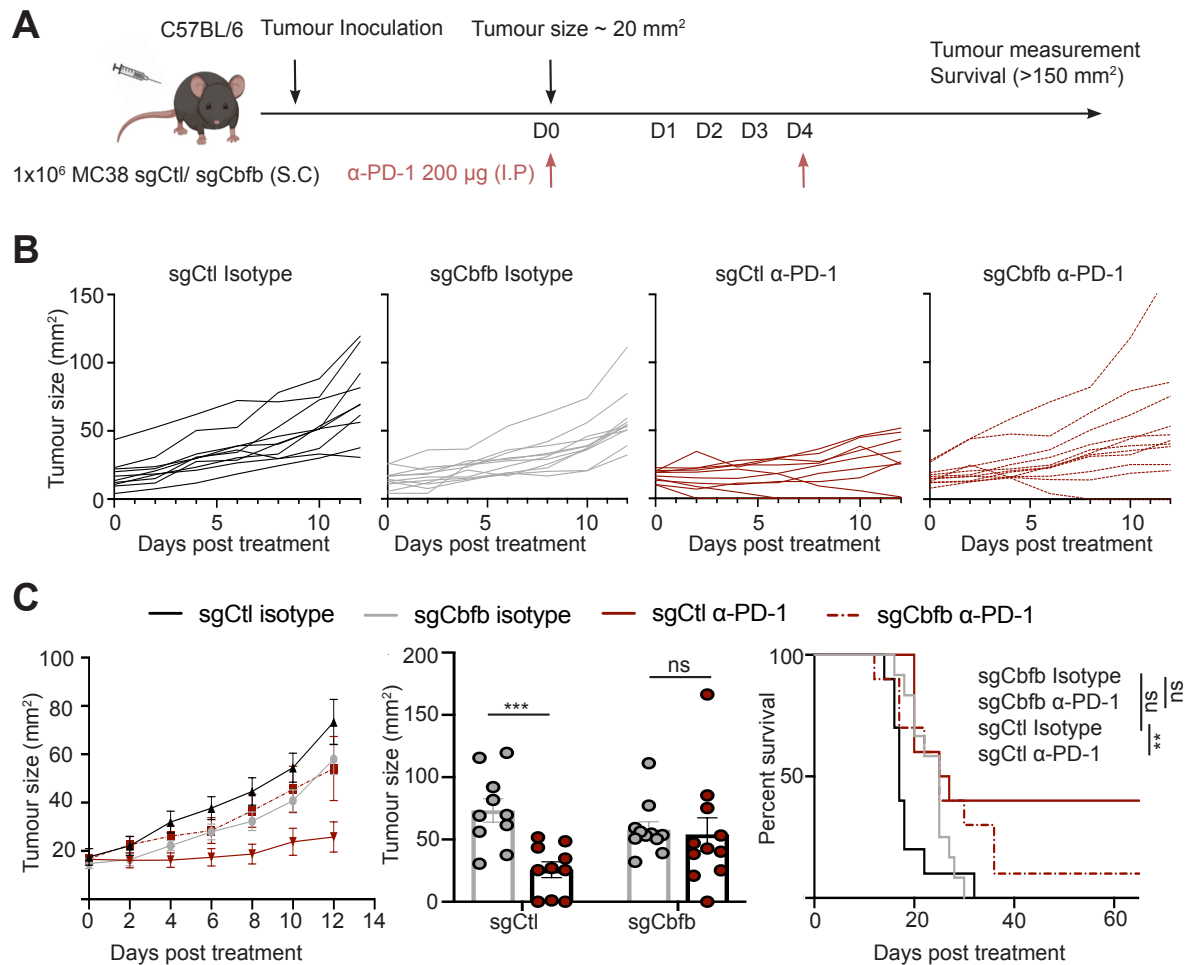


Figure 26. *Cbfb*-depleted tumour cells are less responsive to immune checkpoint blockade in vivo. (A) Schematic representation of *in vivo* experiment, MC38 control or *Cbfb*-depleted tumour bearing C57BL/6 mice were treated with two doses of anti-PD-1 (200 µg, I.P) or isotype control, 4 days apart. (B) Tumour growth of individual mice, pooled data from two independent experiments, n= 10-11/ group. (C) Left, growth of MC38 control or *Cbfb*-depleted tumours. Centre, tumour size at 12 days post-treatment. Data are mean ± SEM of 10-11 mice/group, pooled from 2 independent experiments. P < 0.001 by Student's t test. Right, survival of mice to day 65 after treatment. P < 0.01 by log-rank (Mantel-Cox) test.

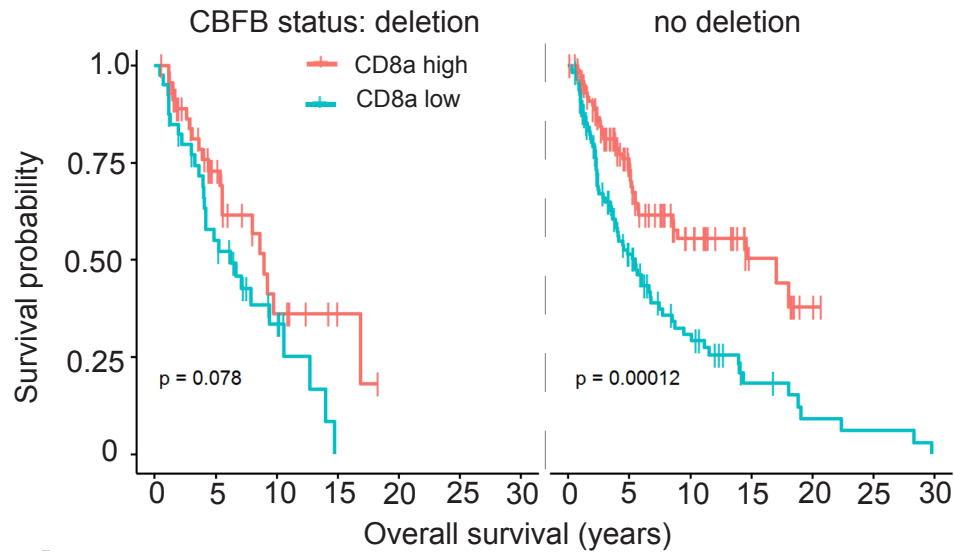


Figure 27. *CBFB* copy number deletion status abrogates prognostic advantage of high CD8a expression. Survival probability of melanoma patients from TCGA melanoma cohort data, stratified by CD8a expression and *CBFB* copy number deletion status.

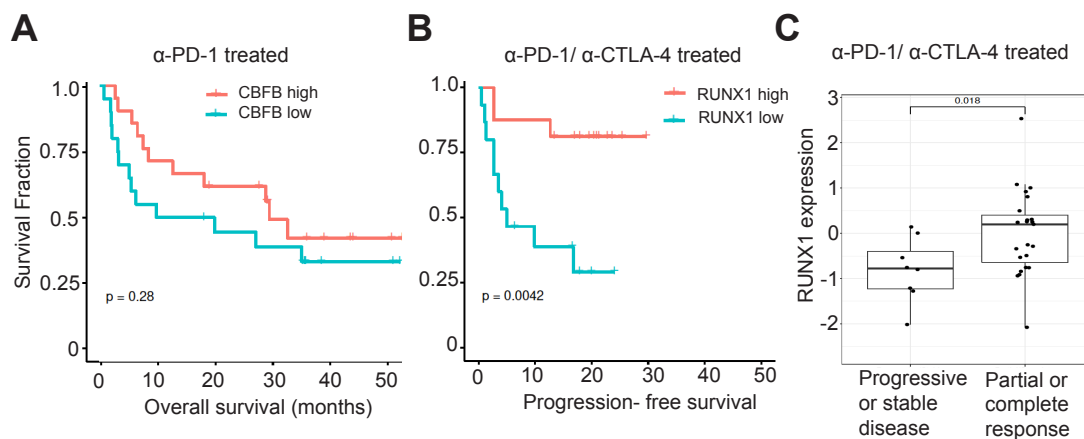


Figure 28. Patients with low *CBFB* and *RUNX1* are less responsive to immune checkpoint blockade. (A) Overall survival of melanoma cohort treated with anti-PD-1, stratified by tumour-specific *CBFB* expression from RNA sequencing from [215] (B) Progression-free survival of melanoma cohort treated with anti-PD-1 and anti-CTLA-4, stratified by tumour-specific *RUNX1* expression. (C) Progressive/stable disease and partial/complete response in anti-PD-1 and anti-CTLA-4 treated melanoma cohort treated, stratified by *RUNX1* expression.

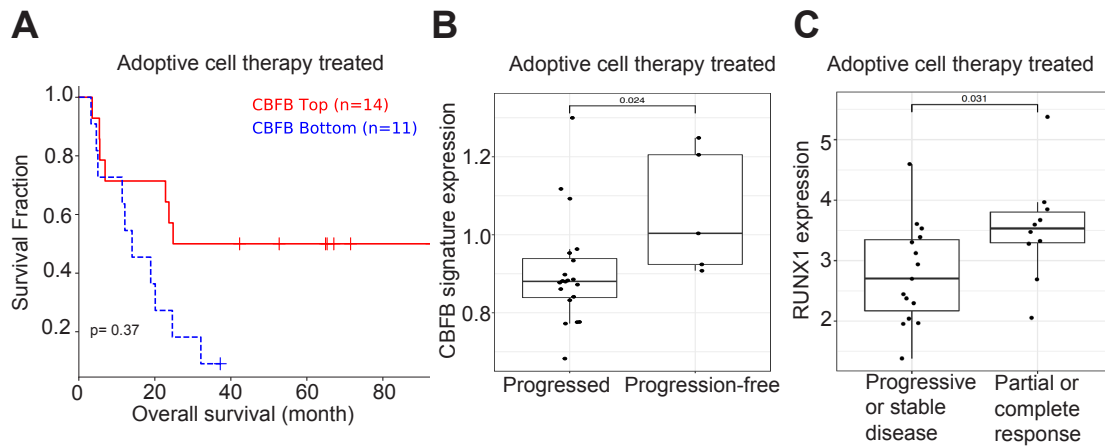


Figure 29. Patients with low *CBFB* and *RUNX1* are less responsive to adoptive cell therapy. (A) Survival of melanoma patient cohort treated with ACT, stratified by tumour-specific *CBFB* expression. (B) Progression status of ACT treated melanoma cohort, stratified on *CBFB*/*RUNX1* signature from [220] (C) Progressive/stable disease and partial/complete response in ACT treated melanoma cohort, stratified by *RUNX1* expression.

CRISPR-Cas9 screening reveals genes essential for protecting tumour cells from CAR T cell killing

In addition to the CRISPR-Cas9 screens presented in Figure 14 of this chapter where we sought to investigate tumour-intrinsic genes that confer resistance to CAR T cell killing, we also conducted a CRISPR-Cas9 screen to identify genes that sensitise tumour cells to CAR T cell killing. In this dropout CRISPR-Cas9 screening approach, MC38-HER2 cells transduced with the pooled sgRNA library were subjected to one overnight co-culture with CAR T cells. The sequenced population was then compared to the untreated T_{end} population for sgRNA that were no longer present, as disruption of these genes render MC38 cells more susceptible to CAR T cell killing (Figure 30).

The data from the dropout CRISPR-Cas9 screen identify a large number of novel genes that might sensitise MC38 cells CAR T cell killing. Of note is *Eed*, a member of the evolutionarily conserved polycomb repressive complex 2 (PRC2), which is involved in the transcriptional silencing of MHC I and drives T cell resistance in the context of immunotherapy [233]. Additionally, depletion or pharmacological inhibition of *Eed* re-establishes effective T cell mediated anti-tumour immunity [233].

Another enriched gene of interest from the CRISPR-Cas9 screen is *Rnf31*, a gene that is responsible for the ubiquitin ligase activity of the linear ubiquitin chain assembly complex (LUBAC), which was introduced in the publication in chapter 4, section 1. LUBAC promotes activation of the NF- κ B pathway, and therefore is critical to the tumour TNF response [243]. Small molecule inhibitors of LUBAC have been developed in order to inhibit NF- κ B signalling [244] and enhance TNF sensitivity. As with the smac-mimetics, previously discussed in detail, augmenting NF- κ B signalling with a LUBAC inhibitor results in broad immunomodulatory effects, particularly with respect to innate-immunity [245]. *Rnf31* has previously been identified in other CRISPR-Cas9 screens, where depletion of *Rnf31* enhanced T cell killing against B16F10 tumours [231], and sensitised tumours to α -PD-1 in vivo [234].

Encouragingly, this suggests that data from CRISPR-Cas9 screening approaches can provide insight in to future drug targets. Therefore, novel genes identified by this CRISPR-Cas9 screen might be useful to enhance CAR T cell killing in the context of a combination therapy. Follow up experiments based on the novel genes identified in this CRISPR-Cas9 screen are warranted, as there is an unmet clinical need for pharmacological enhances of CAR T cell therapy.

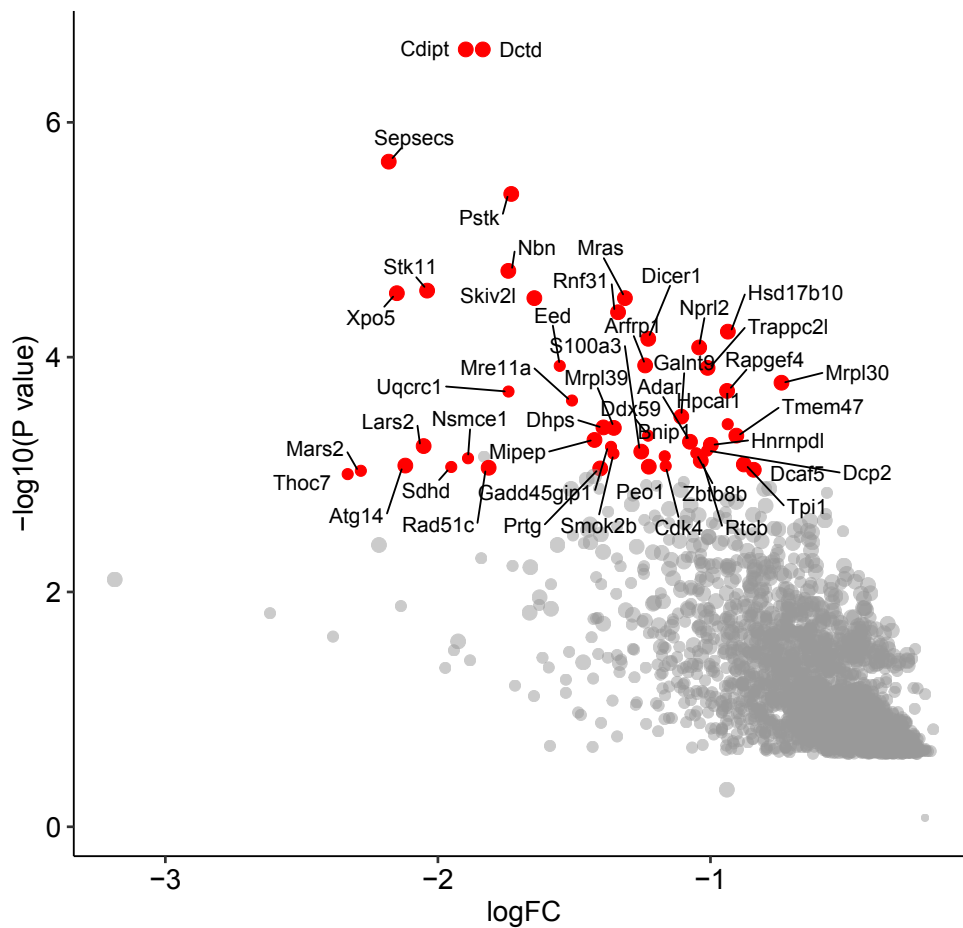


Figure 30. CRISPR-Cas9 screening reveals genes that sensitise MC38-HER2 cells to CAR T cell killing. Volcano plot showing p value and sgRNA fold change from dropout CRISPR-Cas9 screen.

3. Discussion

In this chapter we sought to investigate tumour-intrinsic mechanisms of CAR T cell resistance using a whole-genome loss-of-function screening approach. We identified that loss of response to TNF and IFN- γ signalling were the key mechanisms of immune evasion that protected MC38-HER2 cells from CAR T cell-mediated killing. Loss of IFN- γ signalling has been identified as a mechanism of acquired resistance to T cells [202] and checkpoint blockade in the clinic [131, 135], and neutralising IFN- γ with a blocking antibody reduces CAR T cell efficacy *in vivo* [197]. In addition, TNF has been identified as a critical effector molecule for immunotherapies, and augmenting tumour-TNF response in an area of intensive research [246].

Functional cooperativity between TNF and IFN- γ is integral to the orchestration of an effective anti-tumour immune response. TNF and IFN- γ synergistically up regulate MHC I [247] and ICAM-1 [248] expression, and the secretion of chemokines such as RANTES [94] and CXCL10 [249], following independent activation of the distinct transcription factors STAT1, IRF1 and NF- κ B, which lead to altered and synergistic gene expression. TNF and IFN- γ can also synergistically induce expression of mRNA encoding IRF-1 [250]. In addition, TNF and IFN- γ can elicit synergistic cytotoxic effects on tumour cells, where intracellular cooperation between TNF and IFN- γ can induce classical caspase-dependent apoptosis, and IFN- γ confers sensitivity to TNF induced apoptosis by STAT1 and IRF1 activation [251]. TNF-dependent activation of NF- κ B is stronger and persistent in the presence of IFN- γ [252], as IFN- γ can enhance TNF-induced NF- κ B nuclear translocation by inducing the degradation of I κ B β [253]. Finally, the nuclear localisation of STAT1 due to IFN- γ signalling precludes it from being recruited to TNFR1, which enhances TNF-induced NF- κ B activation, and IFN- γ signalling can up regulate cell surface expression of TNFR1 directly [252].

Additionally, we identified for the first time that loss of the transcriptional co-binding factor *Cbfb* protected MC38 cells against CAR and OT-I T cell killing, and identified for the first time a role for *Cbfb* in mediating tumour

responsiveness to TNF. The role of *Cbfb* has been well characterised in hematopoiesis and various leukaemias [236-238], however the role of *cbfb* in solid malignancies remains poorly understood. *Cbfb* has been implicated in patient prognosis in colorectal [240] and breast cancers [220], and *Cbfb* has been associated with regulating a diverse range of biological processes through mediation of both transcription and translation [220].

As an essential co-binding partner of the *Runx* family of transcription factors, *Cbfb* does not bind to DNA directly, instead it heterodimers in order to enhance the DNA-binding and transcriptional activity of *Runx* transcription factors [236]. In mammals, three *Runx* genes have been identified: *Runx1*, *Runx2* and *Runx3*, which together are essential regulators of biological processes including proliferation, differentiation, apoptosis and cell lineage [254]. *Cbfb* has recently been implicated in conferring resistance to MEK inhibitors in colorectal cancer [241], which was dependent on *Runx2* transcription. Therefore, we attempted to uncover the *Runx* transcription factor involved in mediating responsiveness to TNF. In this chapter we identified that *Runx1*-depleted MC38 cells exhibited reduced sensitivity to TNF-mediated apoptosis, suggesting that *Cbfb* and *Runx1* interact in order to facilitate a functional TNF signalling response.

Runx1 has been previously implicated in facilitating TNF signalling [255], where it functions as a cytoplasmic attenuator of NF- κ B signalling through interactions with the I κ B kinase complex [256], and interacts directly with the p50 subunit of NF- κ B in macrophages [257]. However, the precise mechanism of *Runx1* facilitated TNF signalling in our model remains unclear, and will be addressed with future work, based on the clinical need to overcome resistance to TNF in immunotherapy.

Based on our data that demonstrated loss of *Cbfb* conferred resistance to T cell killing, we assessed the efficacy of α -PD-1, as a T cell-mediated immunotherapy, in *Cbfb*-depleted MC38 cells. We demonstrated that *Cbfb*-deficient cells were less responsive to ICB *in vivo*, and importantly showed that both *CBFB* and *RUNX1* may hold prognostic significance in the treatment

of melanoma with immunotherapy. It is likely that this effect is two-fold. Firstly, efficacy of immunotherapy depends, in part, on tumour TNF response [203], and secondly, the ability of tumours to secrete chemokines is an important prognostic factor [46]. As a functional readout of TNF response, we identified that *cbfb*-depleted cells exhibited diminished chemokine secretion following TNF and IFN- γ exposure, which may account for loss of response to checkpoint blockade *in vivo*. In addition, *Runx1* has been implicated in the regulation of chemokines, particularly CCL2 [258], which may account for this phenotype. A significant proportion of patients fail to respond to immunotherapy, and therefore our data may have clinical relevance, as there is currently an unmet need for reliable biomarkers that predict patient response.

Tumour epithelial to mesenchymal transition (EMT) in tumour cells plays an important role in tumour growth, invasion, dissemination [259] and is associated with resistance to therapy [260]. It was therefore unexpected when we identified an enriched EMT transcriptional phenotype in control MC38 cells compared to *Cbfb*-depleted cells. However, there is evidence that *Runx1* plays a critical role in the regulation of EMT [254, 261] and in colorectal cancer promotes metastasis and EMT [262]. Therefore, it is likely in the absence of *Cbfb*, and subsequently inadequate *Runx1* transcription, that this process is repressed, resulting in an epithelial-like cell phenotype. Given the association with mesenchymal phenotypes and metastasis [263] and immune evasion [264], it was an expected finding that the loss of *Cbfb* conferred resistance against T cell killing, yet a more epithelial phenotype compared to control cells.

Perhaps the most unexpected finding in this chapter was that no *Cbfb* co-binding partners were highly enriched in the CRISPR-Cas9 screen, given the transcriptional dependency of *Cbfb* on *Runx* genes. We hypothesise that this may be due to a level of redundancy between the three mammalian *Runx* genes [265]. In addition, CRISPR-Cas9 screen blind spots have been identified as a limitation of this technology, where a 20% false-negative rate is common, and 50% of constitutively expressed genes are never significantly

enriched for [266]. Despite this, pooled library CRISPR-Cas9 knockout screens still offer the greatest sensitivity and specificity in comparison to other similar methodologies, such as RNA interference technology [228].

The data presented in the chapter highlight that tumour TNF and IFN- γ signalling is critical to the effector function of CAR T cells, and taken together, underscores the cytotoxic potential of CAR T cell-derived cytokines. There is currently an unmet clinical need for strategies to overcome these mechanisms of resistance, and this paves the way for therapeutic approaches that enhance tumour TNF response as a mechanism to enhance CAR T cell therapy. In the next chapter, we take the insight gained from the CRISPR-Cas9 screen in order to design a combination strategy to enhance CAR T cell-derived TNF-mediated killing.

Chapter 4: Antagonism of IAPs Enhances CAR T-cell Efficacy

Preface

The aim of this chapter was to investigate whether the efficacy of CAR T cell therapy for solid tumours could be enhanced with a smac-mimetic.

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The published manuscript and associated supplementary information are presented in Chapter 3 of this thesis. Additional related data not included in the manuscript are presented in this chapter as an addendum.

1. Introduction

Whilst CAR T cell therapy has proven remarkably effective against B cell haematological malignancies, with complete response rates in clinical trials of around 55% [176], results in the treatment of solid tumours, regardless of target antigen, remain poor. This is likely due to factors such as poor CAR T cell homing and penetration into the tumour, which results in low numbers of CAR T cells in the TME, T cell exhaustion in the presence of inhibitory ligands, and acquired tumour resistance. As a result, further research regarding how CAR T cell efficacy can be enhanced is necessary in order for CAR T cell therapy to be an effective clinical strategy for the treatment of solid tumours.

There is a strong rationale for combining CAR T cell therapy with different anti-cancer treatments in order to achieve synergistic effects and improve likelihood of a positive clinical outcome. Whilst several groups are attempting to overcome these obstacles with approaches such as by combining CAR T cell therapy with other immunotherapy approaches such as checkpoint blockade [192] or agonist antibodies [200], we considered the possibility of pharmacologically enhancing CAR T cell therapy by combining it with a small-molecule drug. Based on the CRISPR-Cas9 screen data presented in Chapter 3, which demonstrated that tumour cell resistance to TNF signalling limits CAR T cell efficacy, we considered pharmacological enhancers of tumour TNF signalling.

Antagonists of the Inhibitor of Apoptosis Proteins (IAPs), also known as smac-mimetics, have been described in detail in Chapter 1, section 4.1. Briefly, smac-mimetics are a class of small-molecule drugs that degrade the IAPs [201, 267], a family of endogenous proteins that modulate caspases in order to restrain apoptosis [268-270]. The IAPs are frequently upregulated in malignantly transformed cells [271], and modulate responsiveness to TNF mediated apoptosis [201, 267]. One of the most clinically advanced smac-mimetics, known as birinapant, has already demonstrated a favourable safety profile in clinical trials. While smac-mimetics have shown some efficacy as a

single-agent [272], their potential in the context of immunotherapy is their ability to sensitise tumour cells to TNF-dependent apoptosis, which supports the approach of combining birinapant with agents capable of increasing TNF concentrations within the TME [273]. It has previously been demonstrated that birinapant effectively synergises with TNF produced by cytotoxic lymphocytes to enhance tumour cell death [204]. Based on the knowledge that activated CAR T cells readily secrete inflammatory cytokines, we hypothesised that the smac-mimetic birinapant would enhance tumour cell killing mediated by CAR T cell-derived TNF (Figure 31).

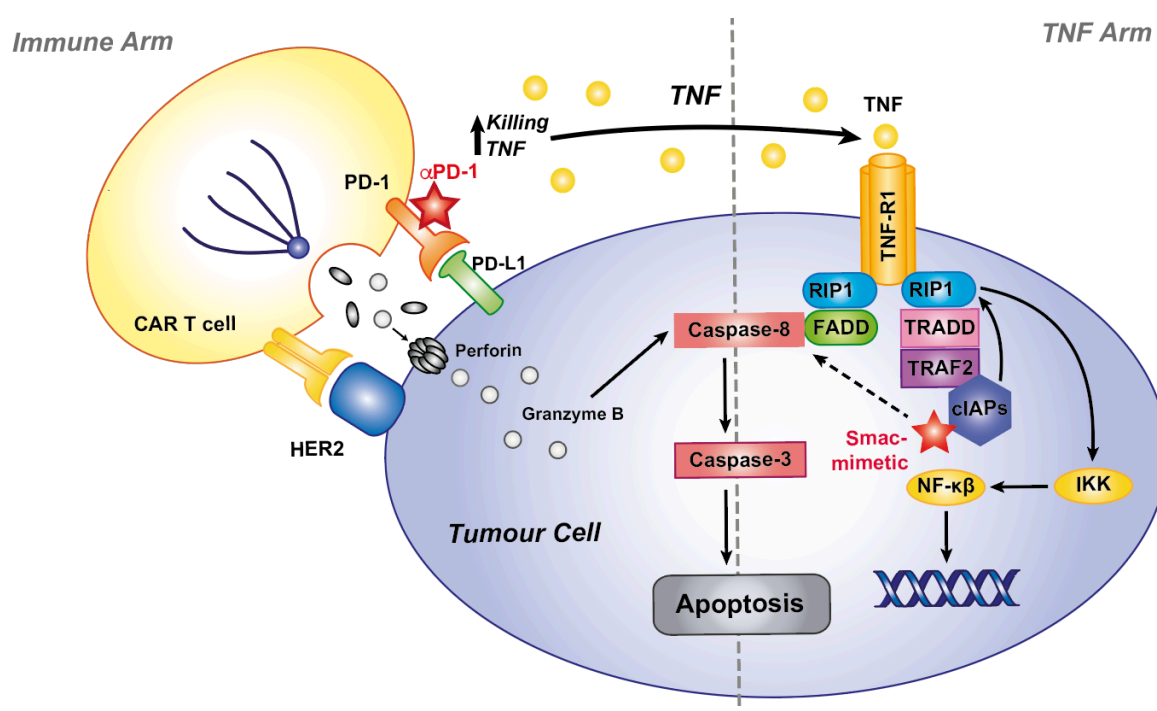


Figure 31. Mechanism of action of CAR T cell and birinapant combination therapy. Recognition of target antigen by CAR T cells promotes the secretion of inflammatory cytokines including TNF, in addition to granule-mediated cytotoxicity. Administration of birinapant sensitises tumour cells to TNF-mediated apoptosis through the TNF extrinsic pathway. Adapted from Kearney et al (2017) [204].

Upon tumour antigen recognition, activated CAR T cells form an immunological synapse with the tumour cell, resulting in direct T cell killing, via the release of granule-associated proteins perforin and granzyme B, as well as the release of inflammatory cytokines, including TNF. We hypothesised that concurrent administration with the smac-mimetic birinapant would degrade the IAPs, resulting in TNF-mediated tumour cell death via the TNF extrinsic pathway (Figure 31).

In this chapter, we demonstrate the ability of birinapant to enhance CAR T cell killing, and highlight the cytotoxic potential of CAR T cell-derived TNF in the TME. Furthermore, we demonstrate that the ability of smac-mimetics to sensitise tumour cells to TNF results in a phenomenon known as “bystander killing”, defined as tumour cell death that occurs independently of the formation of an immunological synapse between a cytotoxic lymphocyte a tumour cell, via the TNF extrinsic apoptosis pathway. Of note, we also demonstrate the ability of CAR T cell-derived TNF to kill antigen-negative tumour cells. This work identifies TNF-mediated bystander killing as mechanism for overcoming antigenic heterogeneity, which is associated with immune evasion and relapse due to antigen-negative clones.

In addition to *in vitro* and *in vivo* characterisation of this combination therapy, we developed the methodology for a CAR T cell cytotoxicity assay, utilising a patient biopsy-derived colorectal tumouroid model. The publication presented in this chapter was the first to use a tumouroid-based assay to quantify CAR T cell cytotoxicity, an approach that has since been utilised by other groups [274]. This colorectal cancer model is clinically relevant, as the use of both HER2-targeting CAR T cells (NCT03740256) and smac-mimetics [275, 276] are being investigated in clinical trials for this cancer type.

To our knowledge, this is the first publication that demonstrates the combination of CAR T cell therapy with a small-molecule drug to enhance anti-tumour efficacy, and since publication this finding has also been identified and replicated by other groups [205, 235].

2. Publication in *Cancer Immunology Research*

Priority Brief

Cancer
Immunology
Research

Antagonism of IAPs Enhances CAR T-cell Efficacy

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy has proven successful in the treatment of hematological malignancies, notably acute lymphoblastic leukemia and B-cell lymphoma. However, the efficacy of CAR T cells against solid tumors is poor, likely due to tumor-associated immunosuppression. Here, we demonstrated that antagonizing the "inhibitor of apoptosis proteins" with the clinical smac-mimetic, birinapant, significantly enhanced the antitumor activity of CAR T cells in a tumor necrosis factor (TNF)-dependent manner. Enhanced tumor cell death occurred

independently of the perforin-mediated granule exocytosis pathway, underscoring the cytotoxic potential of CAR T-cell-derived TNF. Combining CAR T-cell therapy with birinapant significantly reduced established tumor growth *in vivo*, where either therapy alone was relatively ineffective. Using patient biopsy-derived tumoroids, we demonstrated the synergistic potential of combining CAR T-cell therapy with smac-mimetics. Taken together, we identified CAR T-cell-derived TNF as a potent antitumor effector, which can be further harnessed by smac-mimetics.

Introduction

Adoptive cellular therapy using chimeric antigen receptor (CAR) T cells is showing great promise for the treatment of cancer (1, 2). However, although CAR T cells have been successful in treating hematological malignancies (2), results in the solid tumor setting have been less positive (3). One reason for the lack of efficacy of CAR T cells in solid tumors is the immunosuppressive microenvironment encountered (1). This includes the expression of ligands on tumor cells that bind to checkpoint receptors on the T cells and inhibit their function. For this reason, combining CAR T cells with additional therapies that target checkpoint receptor–ligand interactions may improve outcomes in solid cancers. PD-1 blockade, for example, has been shown to significantly enhance the efficacy of both CAR T-cell (4) and adoptive cell therapy (5). The

efficacy of CAR T-cell therapy may also be enhanced by agents that sensitize tumor cells to T-cell effector mechanisms, including cytokines. Smac-mimetics (SM) are a new class of anticancer agents that can sensitize tumor cells to tumor necrosis factor (TNF)-mediated cell death (6). Because TNF is key to the antitumor effector function of cytotoxic T cells (7), this class of small-molecule drugs may also enhance CAR T-cell efficacy.

During apoptotic cell death, the mitochondrial protein Smac/Diablo binds to and antagonizes the major "inhibitor of apoptosis proteins" (IAPs). Because IAPs are often upregulated in human cancer and facilitate resistance to tumor cell death following therapy, small-molecule mimetics of Smac/Diablo (SMs) were developed (6, 8). Antagonism of IAPs also results in formation of the death-inducing signaling complex upon TNF stimulation, rendering the tumor cell sensitive to TNF-induced cell death via the extrinsic pathway (9). Thus, therapies that boost immunity, particularly in the context of elevated TNF secretion, are likely to synergize with SMs. Indeed, IAP antagonism can promote antitumor immunity in multiple myeloma (10) and glioblastoma (11) when combined with checkpoint inhibition, in part due to TNF secreted by immune cells such as CD8⁺ T cells.

Based on the positive results from preclinical studies (12, 13), several SMs have completed phase I/II trials in patients with advanced solid and hematological cancers, with the SM birinapant showing the most promise (8). Given the critical role of TNF in antitumor effector functions of cytotoxic T cells (7) and the ability of SMs to sensitize tumor cells to TNF-dependent apoptosis, we investigated the potential of this class of drugs to enhance CAR T-cell efficacy. We demonstrated that IAP antagonism by birinapant sensitized tumor cells to CAR T-cell-derived TNF, significantly enhancing the antitumor activity of CAR T-cell therapy both *in vitro* and *in vivo*. The results pave the way for a combination therapy that may improve the efficacy of adoptive cell therapy in solid malignancies.

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

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Materials and Methods

Mice

All animal studies were performed in accordance with the NHMRC Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) and with approval from the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee. C57BL/6 human HER2 transgenic mice (14) and C57BL/6 perforin knockout mice (15) were bred in-house. C57BL/6 and C57BL/6 TNF knockout mice were obtained from the Walter and Eliza Hall Institute (Parkville, Victoria). All mice were housed in the Peter MacCallum Cancer Centre Animal Core Facility under specific pathogen-free conditions.

Human samples

Human PBMCs were isolated from healthy donor buffy coats supplied by Australian Red Cross Blood Service. Biopsy samples from 2 patients with HER2⁺ metastatic colorectal cancer were included in this study. All human biopsy studies were conducted in accordance with the Declaration of Helsinki under a protocol approved by the Peter MacCallum Cancer Centre Human Ethics Committee, which included informed written consent from all patients.

Antibodies, cytokines, and drugs

Neutralizing antibodies: mouse anti-TNF (BioLegend; clone MP6-XT22), human anti-TNF (BioLegend; clone MAb11). Enbrel (etanercept) was obtained from the Walter and Eliza Hall Institute of Medical Research (Parkville, Victoria). Antibodies for immunofluorescence: anti-TNF (BioLegend; clone MP6-XT22), IFN γ (eBiosciences; clone XMG1.2), Tubulin (Rockland Immunochemicals; clone 600-401-880). Secondary antibodies conjugated to Alexa fluorophores and ProLong antifade with DAPI were purchased from Molecular Probes. For cell stimulation, anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) antibodies were purchased from BD Biosciences and anti-myc tag (clone 2276) from Cell Signaling Technology. IL2 and IL7 were obtained from the NIH and PeproTech, respectively. Anti-human HER2 was purchased from BioLegend (clone 24D2). Birinapant was supplied by Medivir dissolved in 12% Captisol solution in water (vehicle).

Cell lines

The cell lines MC38 and E0771 were retrovirally transduced to express the human HER2 antigen under the control of the mouse stem cell virus LTR promoter as previously described (14). Mouse cell lines were cultured in DMEM medium and the human cell lines HeLa and AU565 in RPMI medium (Gibco), both supplemented with 10% FCS (Thermo Scientific), penicillin/streptomycin (Gibco), and incubated at 37°C in 10% CO₂. Tumor lines were verified to be *Mycoplasma*-negative by the Victorian Infectious Diseases Reference Laboratory by PCR analysis.

Generation of CAR T cells

To generate activated mouse T cells, splenocytes from C57BL/6 mice were filtered through an Acrodisc 70-nm filter with 10 mL red cell lysis buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA in 500 mL distilled water pH 7.4) and rinsed with 10 mL RPMI medium (Gibco; Invitrogen). Human PBMCs were isolated from buffy coats using a Ficoll gradient centrifugation. The cell suspension was pelleted at 1400 RPM for 4 minutes, washed twice in 20 mL RPMI, and resuspended in

10 mL enriched T-cell media [RPMI supplemented with 10% FCS (Thermo Scientific), penicillin/streptomycin (Gibco), L-glutamine, nonessential amino acids, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium pyruvate, and (Calbiochem)]. T cells were activated with anti-CD3 (0.5 μ g/mL) and anti-CD28 (0.5 μ g/mL) in the presence of IL2 (100 IU/mL) and IL7 (2 ng/mL) at a density of 5×10^6 cells/mL.

The generation of murine and human CAR T cells was done as previously described (14, 16). Briefly, retrovirus encoding a CAR composed of an extracellular scFv-anti-human HER2 fused to the transmembrane domains of CD28 and CD3 ζ was transduced into activated T cells from either the spleen of mice or from human PBMCs in the presence of IL2 (100 IU/mL) and IL7 (2 ng/mL; PeproTech). The viral packaging GP.E86 cell line containing empty (control) LXSN or LXSN-anti-HER2 CAR retroviral vector was generated as described previously (17). The CAR construct used in this study was a second-generation CAR composed of an extracellular single-chain variable fragment (scFv) specific for human-HER2, a CD8 hinge region, transmembrane and intracellular CD28 and CD3 ζ domains, and a c-Myc tag domain allowing for CAR T-cell FACS sorting (18). Primary mouse T cells were collected from splenocytes of C57BL/6 human-HER2 transgenic or wildtype C57BL/6 mice. T cells were retrovirally transduced as described previously (19). After transduction, T cells were maintained in supplemented RPMI media with IL2 (100 U/mL) and IL7 (2 ng/mL). *Tnf*^{-/-} or *perforin*^{-/-} CAR T cells were generated as above from C57BL/6 *Tnf*^{-/-} or *perforin*^{-/-} mice.

In vitro assays

CAR T-cell activity was measured using a standard chromium release assay as previously described (15). The percentage-specific killing was determined using the formula: (Sample ⁵¹Cr release - Spontaneous ⁵¹Cr release)/(Total ⁵¹Cr release - Spontaneous ⁵¹Cr release) $\times$ 100, and represented as a Michaelis-Menten kinetic trend. All assays were performed using triplicate wells. The rate of killing in the control is represented as 100% killing, and treatment groups were compared with this value. All killing assays were done over 18 hours unless otherwise specified. To generate relative killing bar graphs, relative killing at the E:T ratio that results in 50% maximal killing of the least cytotoxic condition was compared, using Michaelis-Menten trends, as done previously (15, 20). Cytokines were detected using a mouse inflammation CBA kit (BD Biosciences, 552364) as per manufacturer's instructions and analyzed on a FACSVerse (BD Biosciences). All assays were analyzed using triplicate determinations.

Flow cytometry

Cells assessed via flow cytometry were plated out in V-bottom plates in 100 μ L media before being pelleted by centrifugation at 1400 RPM for 4 minutes and resuspended in 100 μ L FACS buffer (PBS plus 2% FCS). Cells were then stained with an anti-human HER2 antibody (clone 24D2; BioLegend) for 30 minutes on ice before being washed twice in FACS buffer, resuspended in 200 μ L FACS buffer and transferred to bullet tubes for analysis. Analysis of samples was performed on a Fortessa X20 (BD Biosciences), and data were analyzed with FlowJo (Tree Star).

3'RNA sequencing (RNA-seq)

MC38 cells expressing human HER2 antigen and AU565 cells were left untreated or cocultured with CAR T cells at a 2:1 ratio at 37°C for 6 hours. Cells were washed thoroughly

with PBS to remove contaminating CAR T cells, and cells were lysed. Total RNA was isolated as per the kit manufacturer's instructions (Nucleospin RNA extraction kit, Macherey-Nagel).

Single-end 75-bp RNA-seq was performed in-house at the Peter MacCallum Cancer Centre Molecular Genomics Core on the NextSeq 500 (Illumina). Demultiplexing of the reads was performed using CASAVA v1.8.2, and low-quality reads $Q < 30$ were removed. Cutadapt (v1.9) was used to trim polyA-derived sequences and biased 3' reads resulting from random hexamer priming. HISAT2 was used to map the resulting reads to the human reference genome. Read counting was performed using featureCounts; part of the subread package. Voom-LIMMA workflow was used to normalize data for differential gene expression. Gene set enrichment analysis was performed using GSEA2 (v3.0) for identification of enriched signatures obtained from the MSigDB Hallmarks data sets (7). Sequencing data have been deposited into the Gene-Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE124140.

Fixed and time-lapse microscopy

Microscopy of T-cell–tumor cell conjugates was done as previously described (15). The slides were imaged using a FV1000 confocal microscope (Olympus). CAR T cells selected for quantitation had a single contact site with one tumor cell, indicating a single synapse event. The percentage of T cells that were positive for the indicated cytokine in a synapse or not was quantitated manually by confocal microscopy. A minimum of 100 cells were counted in each condition.

For time-lapse imaging, CAR T cells were sorted for double-positive CD8 and CAR (anti-myc) cells and labeled with CellTrace Violet (CTV, Molecular Probes) as follows: 1×10^7 T cells were stained with 5 $\mu\text{mol/L}$ CTV in PBS for 20 minutes at 37°C and washed for 5 minutes with media. Cells were then pelleted and resuspended in prewarmed media for 10 minutes. Labeled T cells were then added to adherent targets in media containing propidium iodide (PI) and 1 $\mu\text{mol/L}$ birinapant or vehicle control (Captisol). Chamber slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C, and constant CO₂ concentration of 5% was infused using a gas incubation system with active gas mixer ("The Brick"; Ibidi). Optical sections were acquired through sequential scans or Brightfield/DIC on a TCS SP5 confocal microscope (Leica Microsystems) using a $\times 40$ (NA 0.85) air objective and Leica LAS AF software. Image analysis was performed using Meta-Morph Imaging Series 7 software (Universal Imaging).

Mouse experiments

C57BL/6 human HER2 transgenic mice were injected subcutaneously with 2.5×10^5 MC38-HER2 cells. At day 7 after tumor injection, mice were preconditioned with total-body irradiation (4 Gy) prior to the administration of 1×10^7 CART cells on days 7 and 8. Mice were also treated with 50,000 IU IL2 on days 0 to 4 after T-cell transfer. Mice were treated with either vehicle (Captisol) or birinapant (12 mg/kg per mouse) on days 0, 4, and 8 after T-cell transfer. Tumor growth was monitored approximately every second day using a caliper square to determine the product of 2 perpendicular tumor diameters. The mice were culled when the tumor size reached the ethical limit (150 mm²) and recorded for the survival plot.

Patient-derived tumoroids

Tumoroids were grown from tumor cells derived from colorectal patient biopsies. Tumor cells were plated on μ -Plate 96 Well (ibidi) by adding 20 μL of Matrigel/cell suspension per well, and placed to set in 37°C incubator for 30–60 minutes. Following incubation, 350 μL of OB media supplemented with 500 nmol/L A8301 (Torcis, Bioscience), 2 $\times$ B27 (Gibco, Thermo Scientific), epidermal growth factor (50 ng/mL; Sigma-Aldrich), gastrin (1 $\mu\text{g/mL}$; Sigma-Aldrich), 1 mmol/L N-acetyl Cyst (Sigma-Aldrich), 5 $\mu\text{mol/L}$ SB202190 (Sigma-Aldrich), 10 $\mu\text{mol/L}$ SB431542 (Sigma-Aldrich), 10 $\mu\text{mol/L}$ Y27632 (Sigma-Aldrich) was added to each well, and tumoroids were grown in hypoxic conditions (37°C, 5% O₂, 5% CO₂) for 5 to 7 days. Tumoroids were grown to >50 μm and checked for viability using a light microscope on the day of assay, before proceeding. Tumoroids were deemed suitable for coculture after 10 to 14 days after initial biopsy.

A total volume of 350 μL supplemented OB media was applied to the Matrigel-embedded tumoroids. PI (Sigma-Aldrich) was added to a final concentration of 2 $\mu\text{g/mL}$. CAR T cells/well (2.5×10^5) were then resuspended in this media. Wells were set up with the following conditions: tumoroids + vehicle control, tumoroids + CAR T cells/TNF (10 ng/mL), tumoroids + birinapant (1 $\mu\text{mol/L}$), tumoroids + CART cells/TNF + birinapant, CAR T cells + birinapant + TNF-neutralizing antibody (80 $\mu\text{g/mL}$). Tumoroids were incubated at 37°C, 5% CO₂ for 24 hours, and then end-point images were acquired. U-Plate 96-well plates were mounted on a heated stage in a temperature-controlled chamber, maintained at 37°C, 5% CO₂ ("The Brick"; ibidi). Using the cellSens software (Olympus), imaging of the plate was obtained on an UPLSAPO 4X (NA 0.16) air objective using a Hamamatsu ORCA-Flash 4.0 camera. Z-stack images were acquired by taking 25 sequential images (Z spacing 50 μm) of PI (emission 656 nm, exposure 368.644 ms) through the Matrigel, and Z stack images converted to one image in EFI format. Image quantitation was conducted using Meta-Morph Imaging Series 7 software. An identical region of interest was identified within the Matrigel, and integrated morphometry analysis was used to filter out individual T cells based on size. A threshold was applied to delineate PI-positive pixels, and integrated intensity was measured. Identical conditions were applied to compared images.

Statistical analyses

Statistical significance was determined using an unpaired Student *t* test, ANOVA, or log-rank (Mantel–Cox) test for mouse survival data using GraphPad Prism 7 software. Differences were considered significant when $P < 0.05$.

Results

CAR T cells produce cytokines upon tumor cell recognition

TNF is a key inflammatory cytokine produced by cytotoxic T cells upon target recognition that is critical for their antitumor effector function (7). Here, we generated mouse CAR T cells specific for the human HER2 antigen (Fig. 1A; ref. 21) and found that upon recognition of HER2-expressing MC38 colon carcinoma cells (MC38-HER2), TNF and interferon gamma (IFN γ) were key cytokines produced (Fig. 1B). Human HER2-directed CAR T cells also secreted TNF and IFN γ when exposed to HeLa cells that overexpressed HER2 (Supplementary Fig.

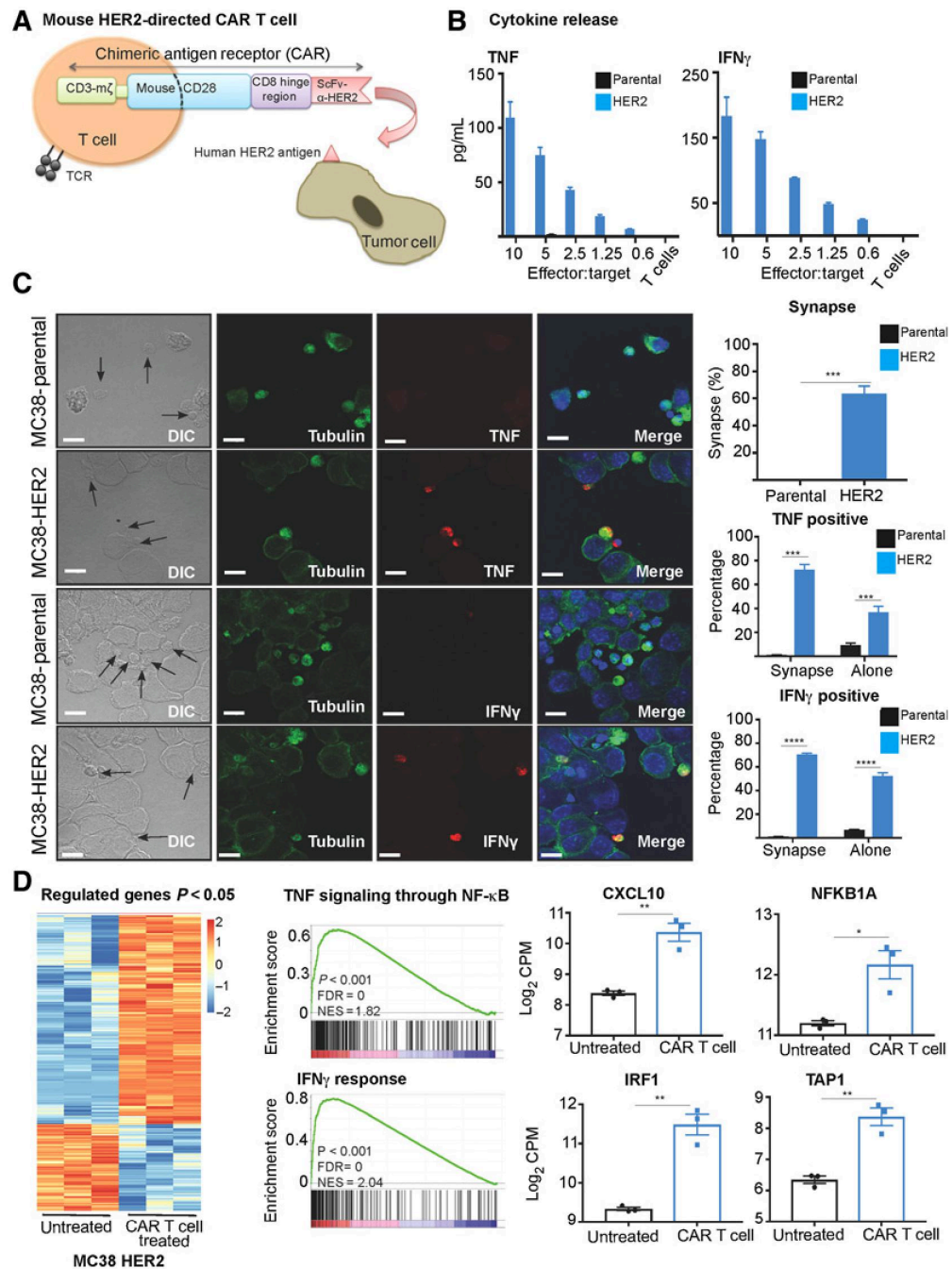


Figure 1.

CAR T cells produce inflammatory cytokines upon tumor cell recognition. **A**, Schematic representation of the CAR T-cell. **B**, MC38-HER2 cells were exposed to CAR T cells at the indicated effector-to-target (E:T) ratio. After 4 hours, cytokines in supernatants were measured by cytometric bead array. **C**, Left, MC38-HER2 or parental cells were seeded in chamber slides, then overlaid with CAR T cells. After 2 hours, cells were fixed and stained as indicated, then visualized by confocal microscopy. Scale bar, 10 μ m. Right, the percentage of CAR T cells that formed an immunologic synapse was quantitated by confocal microscopy (>100 cells). The percentage of CAR T cells positive for the indicated cytokines was quantitated by confocal microscopy (>100 cells). MC38-HER2 cells were left untreated or incubated with CAR T cells (in triplicate) at low effector-to-target ratio for 6 hours followed by **(D)** 3' RNA-seq of viable cells. Left, heat map showing significantly regulated genes following CAR T-cell treatment ($P < 0.05$). GSEA enrichment score plots from RNA-seq data. Right, log₂ counts/million cells from RNA-seq data in **D**. Error bars, mean $\pm$ SEM of triplicate determinations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by an unpaired Student *t* test.

S1A). Confocal microscopy of mouse CAR T cells cocultured with MC38-HER2 or parental MC38 cells (Fig. 1C, left) revealed an increase in immunological synapse formation between CAR T cells and MC38-HER2 tumor cells and a significantly increased number of synapses positive for TNF and IFN γ (Fig. 1C, right).

We also detected significant transcriptional changes in the MC38-HER2 tumor cells in response to CAR T-cell treatment (Fig. 1D, left), with GSEA analysis revealing significant gene enrichment in pathways relating to TNF signaling through NF- κ B and the IFN γ response (Fig. 1D, center). Further analysis of the data confirmed upregulation of specific genes known to be induced by TNF and IFN γ signaling, including *CXCL10*, *NFKB1A*, *IRF1*, and *TAP1* (Fig. 1D, right). Transcriptional analysis of the human breast cancer line AU565 (which endogenously expresses HER2) cocultured with human CAR T cells also showed gene enrichment pathways relating to TNF signaling (Supplementary Fig. S1B). These data demonstrated that CAR T cells can produce the inflammatory cytokines, TNF and IFN γ , which elicits a transcriptional cytokine response in tumor cells.

Birinapant enhances tumor cell death in the presence of CAR T cells

Because the SM, birinapant, has previously been shown to enhance the death of tumor cells expressing a model antigen in the presence of transgenic OT-I T cells (15), we wanted to determine if birinapant could enhance the antitumor activity of CAR T cells to tumor cells expressing the tumor antigen HER2. Mouse CAR T cells were cocultured with MC38 or breast cancer E0771 tumor cells (both engineered to express human HER2), and human CAR T cells with HeLa or AU565 tumor cells (both endogenously express HER2), and cell death was measured using a chromium release killing assay. In all cell lines, a significant increase in cell death in the presence of birinapant compared with vehicle was observed (Fig. 2A). Birinapant did not induce cell death of the CAR T cells (Supplementary Fig. S1C), as has been previously observed for other T cells (15, 22).

To investigate the mechanism of this enhanced tumor cell death in the presence of birinapant, we monitored the interaction of mouse CAR T cells with MC38-HER2 cells by time-lapse imaging. We observed a significant increase in the death of MC38-HER2 cells, indicated by red fluorescence, in the birinapant-treated cocultures (Fig. 2B; Supplementary Movies S1–S2). Further analyses of the imaging revealed a significant increase in the percentage of cell death that occurred independently of direct CAR T-cell interaction, described as "bystander killing" (refs. 7, 15; Fig. 2B, right). To confirm this, we conducted a killing assay with a 50:50 mix of unlabeled MC38-HER2 and chromium-labeled MC38 parental cells. CAR T cells do not recognize non-HER2-expressing tumor cells (14), and therefore, any chromium release occurred independently of direct CAR T-cell interaction (Fig. 2C). These data demonstrated a significant increase in bystander killing in the presence of CAR T cells and birinapant (Fig. 2D).

Birinapant enhances CAR T cell efficacy in a TNF-dependent manner

We next wanted to determine the mechanism by which birinapant enhances tumor cell death in the presence of

CAR T cells. Given the ability of birinapant to sensitize cells to TNF-mediated cell death, we first added an anti-TNF-neutralizing antibody to the cocultures of both mouse and human CAR T cells and their cognate target tumor cells (Fig. 3A). In all cases, the addition of anti-TNF significantly reduced the ability of birinapant to enhance tumor cell death in the presence of CAR T cells.

To confirm this observation, we also utilized TNF-deficient (*Tnf*^{−/−}) CAR T cells, which had significantly decreased effector function compared with wild-type (WT) CAR T cells, an effect that was not ameliorated by the administration of birinapant (Fig. 3B). We detected no TNF production from the *Tnf*^{−/−} CAR T cells, as expected, but equivalent amounts of IFN γ were secreted between *Tnf*^{−/−} and WT CAR T cells (Fig. 3C). To confirm that birinapant did not enhance tumor cell death via the perforin-mediated granule exocytosis pathway, we repeated the assay with *perforin*^{−/−} CAR T cells. *Perforin*^{−/−} CAR T cells had decreased effector function compared with WT CAR T cells at 4 hours, and tumor cell death was unaffected by the addition of birinapant (Fig. 3D, left). However, over an 18-hour assay, tumor cell death in the presence of *perforin*^{−/−} CAR T cells was increased, and this was further augmented in the presence of birinapant (Fig. 3D, center). This increase in cell death was significantly reduced by the addition of a TNF-neutralizing antibody (Fig. 3D, right), demonstrating that the increase in tumor cell death in the presence of CAR T cells and birinapant is perforin-independent and TNF-dependent.

Combination CAR T-cell therapy and birinapant enhances tumor control

Using a human-HER2 transgenic mouse model (14), we next investigated the combination therapy of CAR T cells and birinapant *in vivo*. MC38-HER2 tumor-bearing C57BL/6 transgenic human-HER2 mice were treated with 1×10^7 CAR T cells on days 0 and 1, and birinapant (12 mg/kg) on days 0, 4, and 8. Control mice established robust tumors, which were not cleared by CAR T-cell or birinapant therapy alone. However, the combination of CAR T-cell therapy and birinapant significantly reduced tumor growth, with complete tumor clearance observed in 50% mice. Long-term overall survival was significantly increased following the combination therapy (Fig. 4A), demonstrating that the addition of birinapant enhanced CAR T-cell therapy *in vivo*.

Birinapant enhances CAR T-cell efficacy using patient tumoroids

To determine if the combination of CAR T cells and birinapant would enhance tumor cell death in a human cancer, we utilized patient biopsy-derived colorectal tumoroids that expressed HER2 (Supplementary Fig. S2A). The tumoroids were not sensitive to TNF or birinapant alone, but the combination significantly enhanced tumor cell death, as indicated by red fluorescence (Fig. 4B; Supplementary Fig. S2B). To test the effect of birinapant on CAR T-cell efficacy using this model, human CAR T cells were applied to Matrigel-embedded tumoroids (Fig. 4C, top), which endogenously expressed HER2 (Fig. 4C, bottom). The addition of CAR T cells resulted in minimal tumoroid death, whereas the combination of CAR T cells and birinapant resulted in a significant increase in tumoroid death compared with CAR T-cell treatment alone (Fig. 4D). The addition of an anti-TNF-neutralizing antibody to the combination-

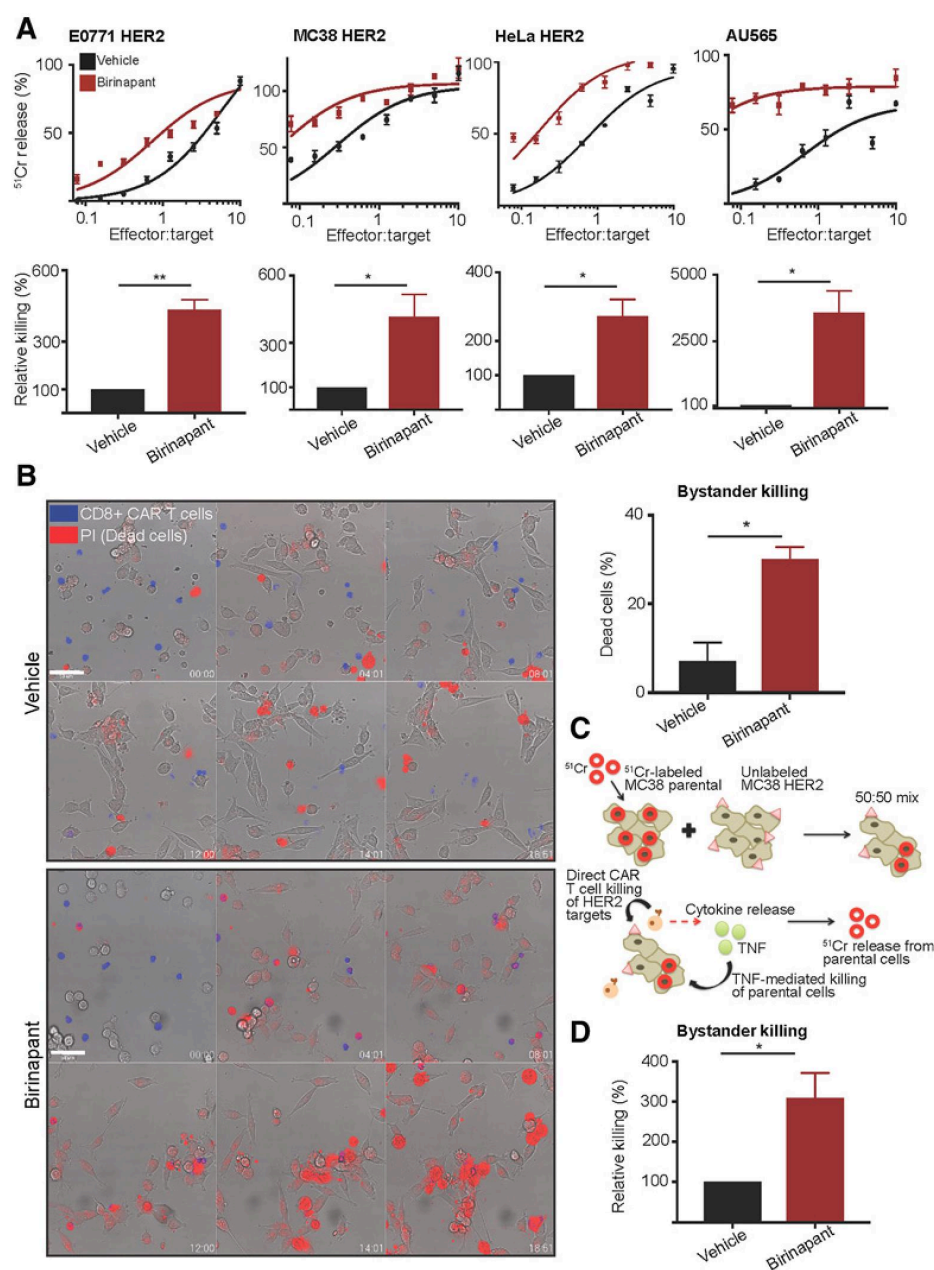


Figure 2.

IAP antagonism enhances tumor cell death in the presence of CAR T cells. **A**, Chromium release assay (18 hours) using mouse CAR T cells with E0771-HER2 and MC38-HER2 targets and human CAR T cells with HeLa-HER2 and AU565 targets (at the indicated E:T ratios) in the presence of vehicle control (Captisol) or birinapant (1 $\mu\text{mol/L}$). **B**, Left, MC38-HER2 cells were seeded in chamber slides, then overlaid with mouse CAR T cells (2:1 ratio) labeled with CTV in PI-containing media. Representative still images at the indicated time points are depicted (hr:min). Scale bar, 50 μm . Right, percentage PI-positive cells without direct CAR T-cell contact was manually quantitated by live imaging. The number of tumor cells that died throughout the duration of the movie (as measured by PI uptake) without contact with a T-cell was quantitated and presented in the graph. Quantitation data are pooled from 3 separate camera positions. **C**, Schematic of chromium release assay to quantify bystander killing. **D**, Relative killing of MC38 parental cells by bystander killing in the presence vehicle control (Captisol) or birinapant (1 $\mu\text{mol/L}$), as depicted in C. All relative killing data are pooled from 3 independent experiments. Error bars, mean $\pm$ SEM; *, $P < 0.05$; **, $P < 0.01$ by an unpaired Student t test.

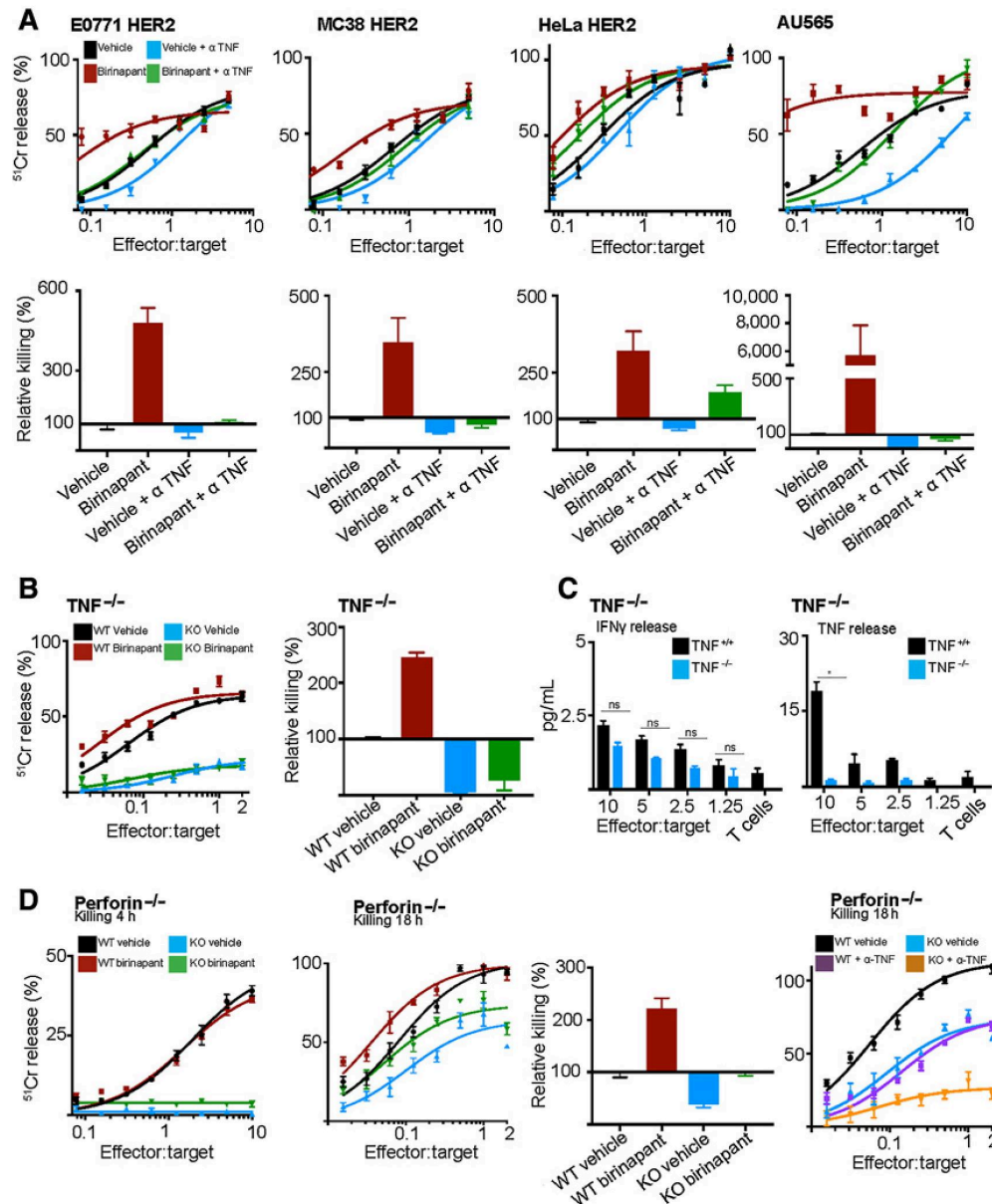


Figure 3.

Birinapant enhances tumor cell death in the presence of CAR T cells in a TNF-dependent, perforin-independent manner. **A**, Chromium release assay (18 hours) using mouse CAR T cells with E0771-HER2 and MC38-HER2 targets and human CAR T cells with HeLa-HER2 and AU565 targets (at the indicated E:T ratio) in the presence of vehicle control (Captisol) or birinapant (1 $\mu\text{mol/L}$) and presence or absence of a TNF-neutralizing antibody (10 $\mu\text{g/mL}$). **B**, Chromium release assays using WT or *Tnf*^{-/-} CAR T cells and MC38-HER2 cells as targets (at the indicated E:T ratio) in the presence or absence of vehicle control (Captisol) or birinapant (1 $\mu\text{mol/L}$). **C**, WT or *Tnf*^{-/-} CAR T cells were exposed to MC38-HER2 cells at the indicated E:T ratio. After 4 hours, cytokines in supernatants were measured by CBA. **D**, Left, chromium release assay (4 hours) using WT or *perforin*^{-/-} CAR T cells and MC38-HER2 cells as targets (at the indicated E:T ratio) in the presence of vehicle control (Captisol) or birinapant (1 $\mu\text{mol/L}$). Center, chromium release assay after 18 hours. Right, chromium release assay using MC38-HER2 cells and WT or *perforin*^{-/-} CAR T cells (at the indicated E:T ratio) untreated (vehicle) in the presence or absence of anti-TNF-neutralizing antibody (10 $\mu\text{g/mL}$). Error bars, mean $\pm$ SEM of triplicate determinations from a representative of 2 independent experiments. *, $P < 0.05$ by an unpaired Student *t* test.

treated tumoroids significantly reduced the amount of tumoroid death, suggesting that TNF-mediated bystander killing was the effector pathway of combination CAR T-cell and birinapant

therapy, not direct T-cell contact (Supplementary Fig. S2C). Together, these data demonstrate that the addition of birinapant to CAR T-cell treatment can significantly enhance tumor cell

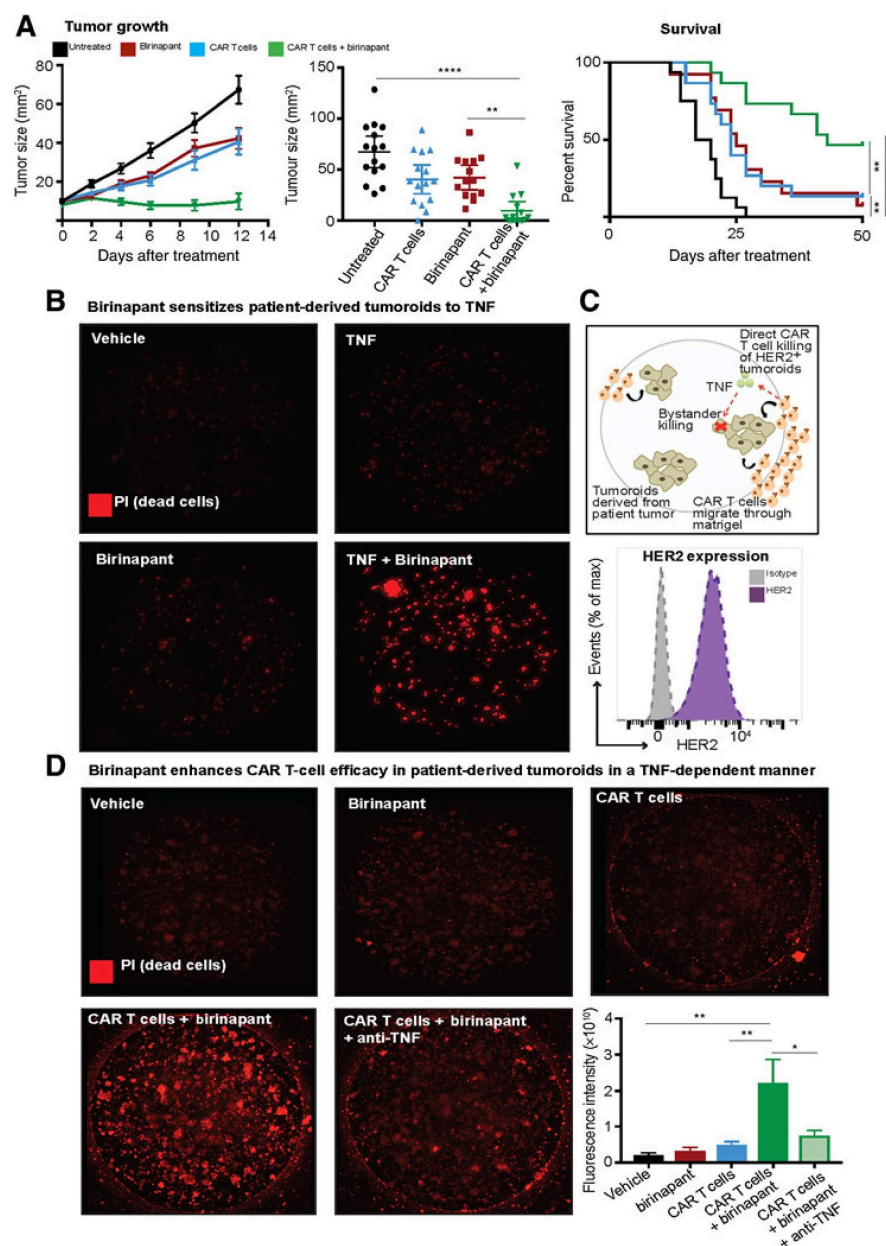


Figure 4.

Birinapant enhances CAR T-cell therapy *in vivo* and in patient-derived tumoroids. C57BL/6 human HER2 transgenic mice were injected subcutaneously with MC38-HER2 cells (2.5×10^5 cells/mouse) and tumors were allowed to establish for 7 days. Mice received total body irradiation (4 Gy) before adoptive transfer of anti-HER2 CAR T cells intravenously (1×10^7 cells/mouse), and a total of 5 doses of intraperitoneal IL2 (50,000 IU/injection) over 5 consecutive days. Birinapant was administered at 12 mg/kg by intraperitoneal injection on days 0, 4, and 8 after CAR T-cell injection. **A**, Growth of MC38 tumors at indicated time points and tumor size at 12 days after treatment. Data are mean $\pm$ SEM of 14–15 mice/group, pooled from 2 independent experiments. *, $P < 0.05$ by ANOVA. Right, survival of mice to day 50 after treatment. Data are mean $\pm$ SEM of 14–15 mice/group, pooled from 2 independent experiments. **, $P < 0.01$; ****, $P < 0.0001$ by log-rank (Mantel-Cox) test. **B**, Fluorescent microscopy of patient-derived colorectal tumoroids embedded in Matrigel and administered vehicle control (Captisol) or soluble TNF (10 ng/mL) in the presence or absence of birinapant (1 μ mol/L) in PI-containing media. **C**, Top, schematic representation of CAR T-cell treatment of Matrigel-embedded tumoroids. Dashed lines, production of TNF by CAR T cells and bystander killing of tumoroids by TNF-mediated apoptosis. Bottom, tumoroids were dissociated into single-cell suspensions and analyzed for HER2 expression by flow cytometry compared with isotype control. **D**, Tumoroids were treated with vehicle control (Captisol) or CAR T cells in the presence or absence of birinapant (1 μ mol/L), or in the presence of birinapant and a neutralizing TNF antibody, in PI-containing media. Total fluorescent intensity was quantified using Meta-Morph software. Data are mean $\pm$ SEM from tumoroids derived from 2 individual patients in 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$ by ANOVA.

death and is likely to be a combination for more effective adoptive cellular therapy.

Discussion

Although the development of CAR T-cell therapy has been revolutionary within the field of immunotherapy, the clinical success has been limited to hematological cancers (2) and a handful of solid cancers (23, 24). A limited number of studies have reported combination therapies that enhance CAR T-cell therapy in solid cancers. Here, we demonstrated that the SM, birinapant, significantly enhanced the efficacy of CAR T cells in a solid tumor setting, in a TNF-dependent manner.

Birinapant is currently undergoing clinical trials as a single agent for a number of hematological and solid tumor cancers (25, 26). Many tumors upregulate IAPs (27) to facilitate resistance to apoptosis following standard chemotherapies (28). The degradation of IAPs by SMs results in the secretion of TNF, which can then induce apoptosis in an autocrine manner via the extrinsic pathway (6). Tumor cells that do not secrete TNF following IAP degradation can also be killed by the exogenous addition of TNF (29). We have previously shown the potential of cytotoxic lymphocytes as a source of TNF that can be utilized by SMs *in vitro* (15). Using a human-HER2 self-antigen mouse model, we demonstrated here that HER2⁺ MC38 tumor-bearing mice treated with CAR T cells and birinapant had a significant survival advantage compared with either monotherapy, with complete remission achieved in half the mice. Given the lack of robust responses to CAR T-cell therapy observed in this model (14), these data may have significant implications for previously difficult-to-treat solid malignancies.

In order to demonstrate the potency of this combination therapy on patient tissue, we utilized HER2⁺ patient-derived colorectal tumoroids embedded in Matrigel as a model of a solid tumor. We have previously uncovered a critical role of the TNF-mediated apoptotic pathway as a mechanism of inducing tumor cell death in the absence of direct T-cell contact (7, 15). We demonstrated that, even with low CART-cell penetration, in the presence of birinapant, CAR T-cell-derived TNF can penetrate through the Matrigel and trigger apoptosis of buried tumor cells through this "bystander" effect (7, 15). This may therefore represent a therapy that overcomes the limitation of

small numbers of CAR T cells reaching and penetrating the tumor efficiently, while allowing for potent killing of antigen-negative tumor cells by TNF. Capitalizing on the enhanced TNF produced by T cells following checkpoint blockade (15) may also provide a therapy by combining CAR T-cell administration along with birinapant and anti-PD-1 treatment. Given all 3 therapies are either in the clinic or currently being assessed in clinical trials, we expect there can be rapid translation of this therapy into patients.

Disclosure of Potential Conflicts of Interest

R.W. Johnstone reports receiving commercial research grants from Roche and AstraZeneca and is a consultant/advisory board member for MecRx. No potential conflicts of interest were disclosed by the authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Michie, S.J. Vervoort, K.M. Ramsbottom, V. Narasimhan, N. Lalaoui, R.G. Ramsay, P.K. Darcy, I. Voskoboinik, J. Oliaro
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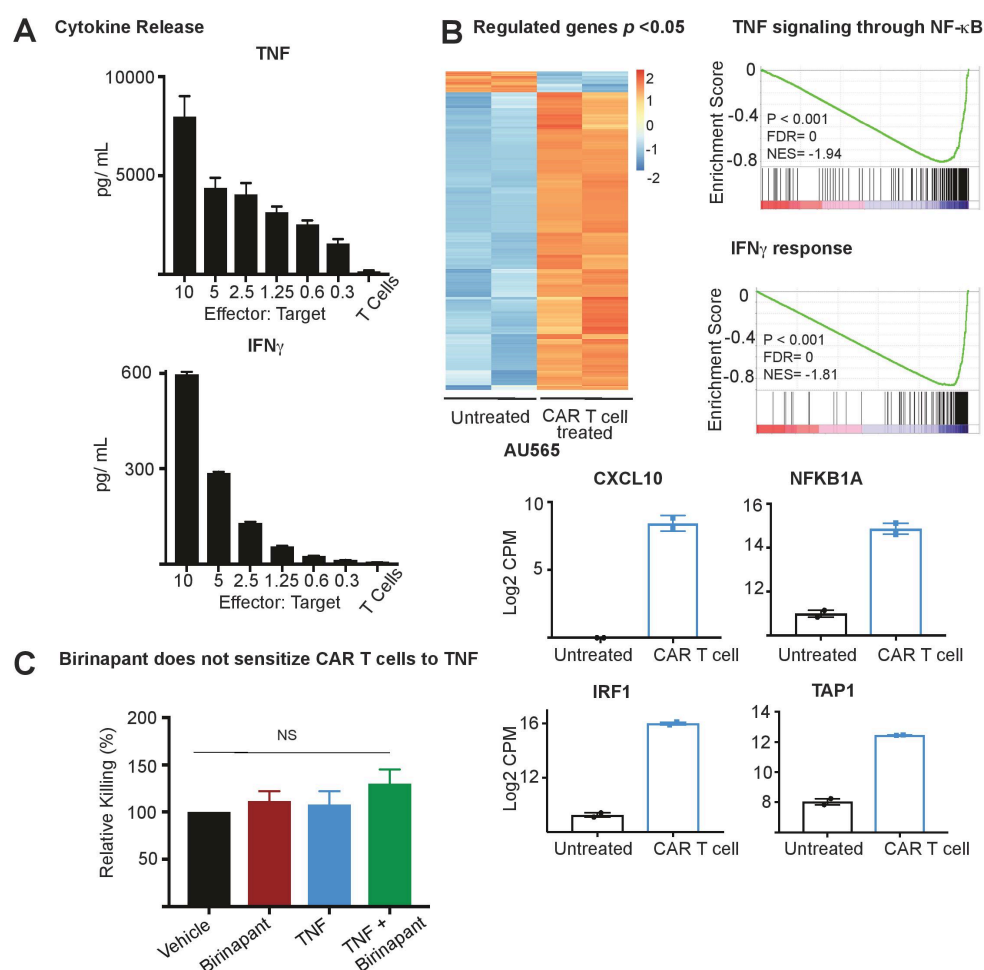
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3. Supplementary information associated with *Cancer Immunology Research* publication

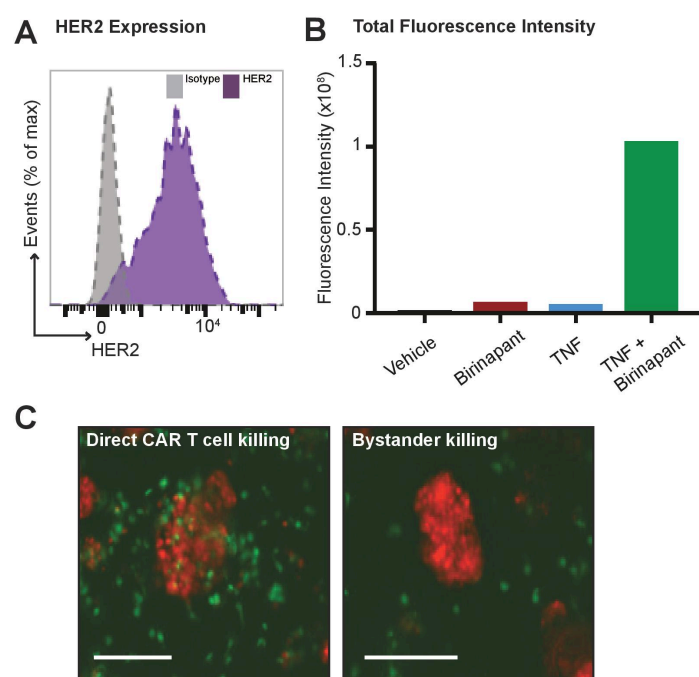
Supplementary Figure 1



Supplementary Figure 1. (A) HeLa cells over-expressing HER2 were exposed to CAR T cells at the indicated effector to target (E:T) ratio. After 4h, cytokines in supernatants were measured by CBA. Error bars represent mean $\pm$ S.E.M of triplicate determinations of a representative experiment. (B) CAR T cells were treated with birinapant (1 μ M), TNF (10 ng/mL) or the combination for 24 hours and cell death measured by flow cytometry. NS = not significant. C) AU565 cells were either left untreated or incubated with CAR T cells (in duplicate) for 6 hrs followed by 3' RNA-Seq with heatmap showing significantly regulated genes following CAR T cell treatment ($P < 0.05$). GSEA enrichment score plots from RNA-Seq data showing significant

correlation between CAR T cell treatment and genes regulating the indicated cellular processes (TNF signalling through NF κ B, IFN- γ response); Log2 counts per million from RNA-Seq data in (B). Error bars represent mean $\pm$ S.E.M of triplicate determinations.

Supplementary Figure 2



Supplementary Figure 2. (A) Total fluorescence intensity from Fig. 4C, quantitated by Meta-Morph software. (B) HER2 expression on tumouroids from a second patient (C) Single image of a tumouroid undergoing apoptosis (red PI) following contact with CAR T cells labelled with CFSE (green) (direct killing) or a tumouroid undergoing apoptosis in the absence of CAR T cell contact (bystander killing). Scale bar represents 100 μ m.

4. Addendum

Based on evidence from previous publications that demonstrated that smac-mimetics sensitised various cell lines to TNF, we screened mouse tumour cell lines transduced to express human HER2 (MC38-HER2, E0771-HER2), and human cell lines that endogenously express HER2 (HeLa, AU565) for their sensitivity to TNF-dependent apoptosis, and assessed whether this effect was enhanced by the administration of birinapant.

All the tested cell lines were sensitive to TNF-mediated apoptosis to some extent; with the mouse cell lines E0771-HER2 and MC38-HER2 demonstrating enhanced TNF sensitivity compared to the human cell lines HeLa and AU565. In addition, the presence of birinapant significantly enhanced TNF-mediated killing of all the cell lines tested, and therefore were used throughout this chapter to assess the efficacy of CAR T cell and birinapant combination therapy.

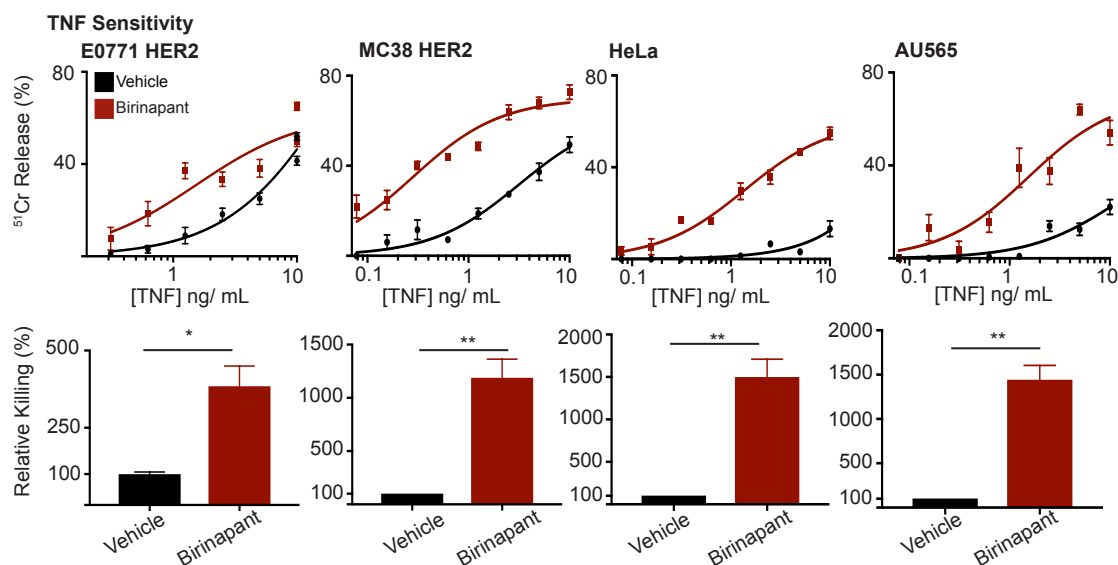


Figure 32. Identification of mouse and human cell lines that are sensitive to TNF-dependent apoptosis following the addition of birinapant. Chromium release assays utilising the mouse cell lines E0771 and MC38 transduced to express human HER2, and human cancer cell lines HeLa and AU565, and recombinant TNF +/- 1 μ M birinapant at the indicated concentrations. Chromium release assays are mean of triplicate determinations +/- SEM from a representative experiment. Relative killing is pooled data from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Generation of wild type and TNF knockout CAR T cells

To ensure that the TNF-deficient CAR T cells used in this study were comparable to wild-type CAR T cells for the functional assays used in this study, we assessed their phenotype by flow cytometry. Compared to wild type cells, TNF knockout CAR T cells exhibited comparable expression of the chimeric antigen receptor, indicating efficient transduction efficiency, and comparable CD4⁺ to CD8⁺ ratio. In addition, expression of surface markers CD62L and CD44, shows a similar activated phenotype, however TNF knockout CAR T cells had a slightly higher proportion of T_{em} (CD44^{high}, CD62L^{low}) compared to T_{cm} (CD44^{high}, CD62L^{high}) than wild type CAR T cells (Figure 33).

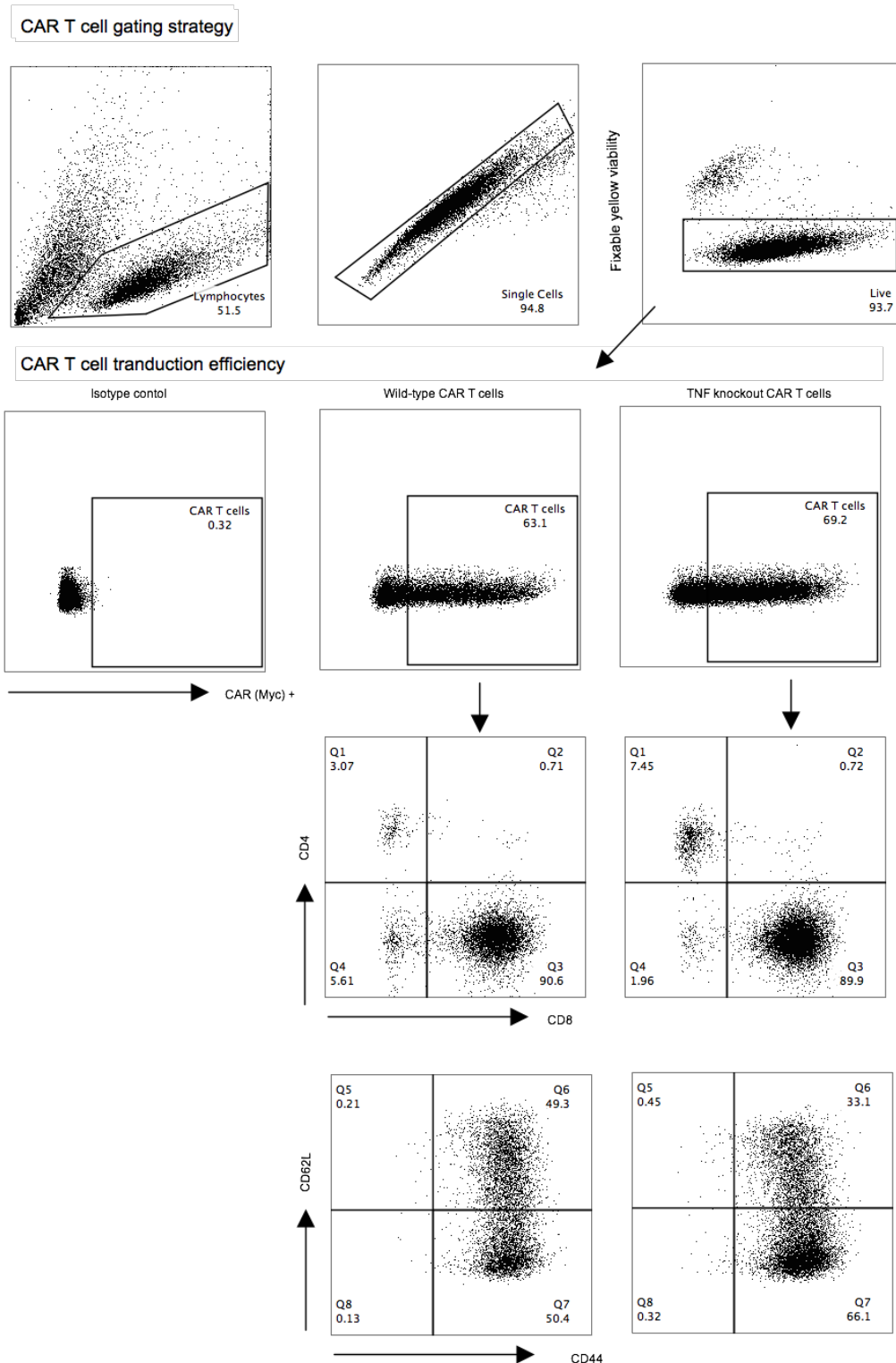


Figure 33. Analysis of wild type and TNF knockout CAR T cells by flow cytometry. FACS plots illustrate the gating strategy used in order to identify viable CAR T cells, CAR transduction efficiency and phenotype of CAR T cells, determined by the expression of cell surface markers CD62L and CD44.

Mechanisms of resistance to CAR T cell and birinapant combination therapy

Given that acquired resistance to immunotherapy approaches is a considerable problem, we performed a genome-wide CRISPR-Cas9 screen, as previously described in Chapter 3, to identify genes that, when deleted, provide protection against CAR T cell and birinapant combination therapy (Figure 34).

The results of this CRISPR-Cas9 screen demonstrated that loss of response to TNF signalling was the main mechanism of resistance to CAR T cell-birinapant combination therapy. When compared to CRISPR-Cas9 screen data from CAR T cell treatment alone, IFN- γ signalling was less important for determining responsiveness to treatment in the combination therapy. In addition to providing further evidence for the TNF-dependent nature of the efficacy of this combination therapy, this CRISPR-Cas9 screen identified that loss of function mutations in genes such as *Tnfrsf1a*, *Tnfrsf1b*, and *Casp8* which might confer resistance to CAR T cell and birinapant therapy. As discussed in Chapter 3, mutations do occur in these genes in cancer patients, many of which have prognostic significance [202]. These data therefore provide an insight into which genes are critical for an effective cytotoxic response, and mutations that would render patients non-responsive to this therapy. Therefore, it is unlikely that CAR T cell-birinapant combination therapy would be efficacious in patients whose tumour already have loss-of-function mutations in any of these genes, or indeed any genes critical to TNF signalling.

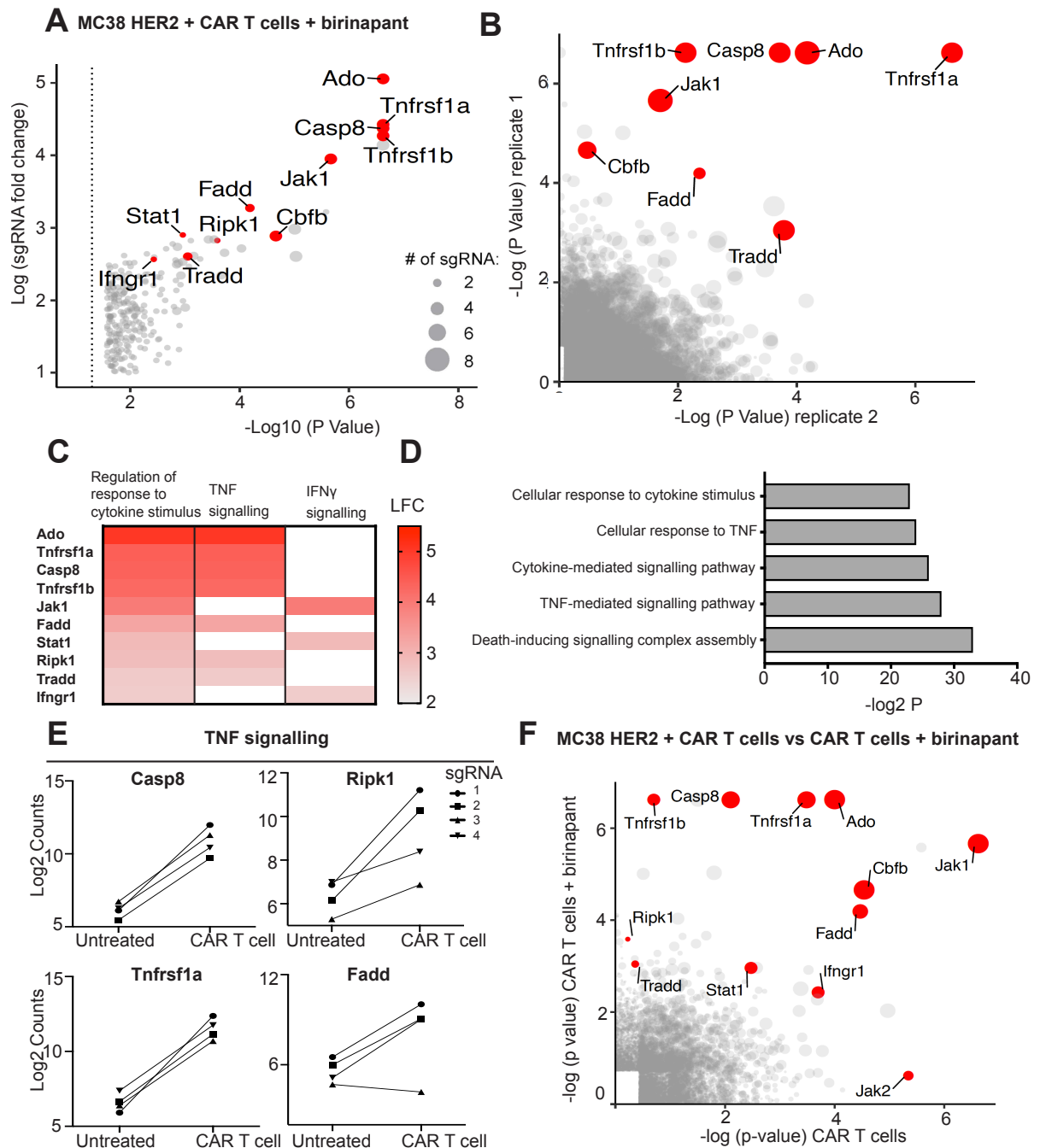


Figure 34. CRISPR-Cas9 screening reveals that loss of TNF response limit susceptibility to CAR T cell and birinapant combination therapy-induced killing. (A) Volcano plot showing P value and sgRNA fold change from genome-wide CRISPR-Cas9 screen, showing the top enriched genes after 2 rounds of 24 hour CAR T cell and birinapant co-culture. (B) Scatterplot showing the shared top enriched tumour genes after 3 rounds of overnight CAR T cell selection pressure, between two biological replicates. (C) Heatmap showing LFC in genes from the TNF and IFN- γ signalling pathways

from (A). (D). Gene Ontology (GO Term) analysis showing enriched biological processes based on the top enriched genes from (A). (E) Stick plots showing individual sgRNA enrichment of genes from the TNF signalling pathway from (A). (F) Scatterplot showing the shared top enrichment tumour genes between CAR T cell resistance CRISPR-Cas9 screen and CAR T cell + birinapant CRISPR-Cas9 screen.

5. Discussion

Taken together, the work presented in this chapter describes a novel combination therapy for enhancing CAR T cell efficacy against solid tumours by augmenting the tumour TNF response. Critically, this work demonstrates the potential for TNF-mediated bystander killing as a mechanism of targeting antigen negative cells, one of the key challenges associated with CAR T cell therapy against solid tumours.

This approach is clinically relevant with excellent translational potential; given CAR T cell therapy is currently under investigation in clinical trials for the treatment of solid tumours. Since clinical trials investigating the use of smac-mimetics in combination with other anti-cancer approaches, such as HDAC inhibitors [277], and immunotherapies such as α -PD-1 [271] are ongoing, the combination of CAR T cell therapy and a smac-mimetic could be rapidly translated in to the clinic.

In addition, smac-mimetics are being investigated in clinical trials in combination with α -PD-1. Given the ability of smac-mimetics to broadly sensitise tumour cells to TNF, and evidence that α -PD-1 immunotherapy enhances CAR T cell TNF secretion [192, 197], it is possible that a triple combination of CAR T cell therapy, checkpoint blockade and smac-mimetics might yield favorable results in the clinic (Figure 35). Such an approach would likely enhance CAR T cell efficacy whilst engaging the endogenous immune system, which presents an attractive avenue for future investigation.

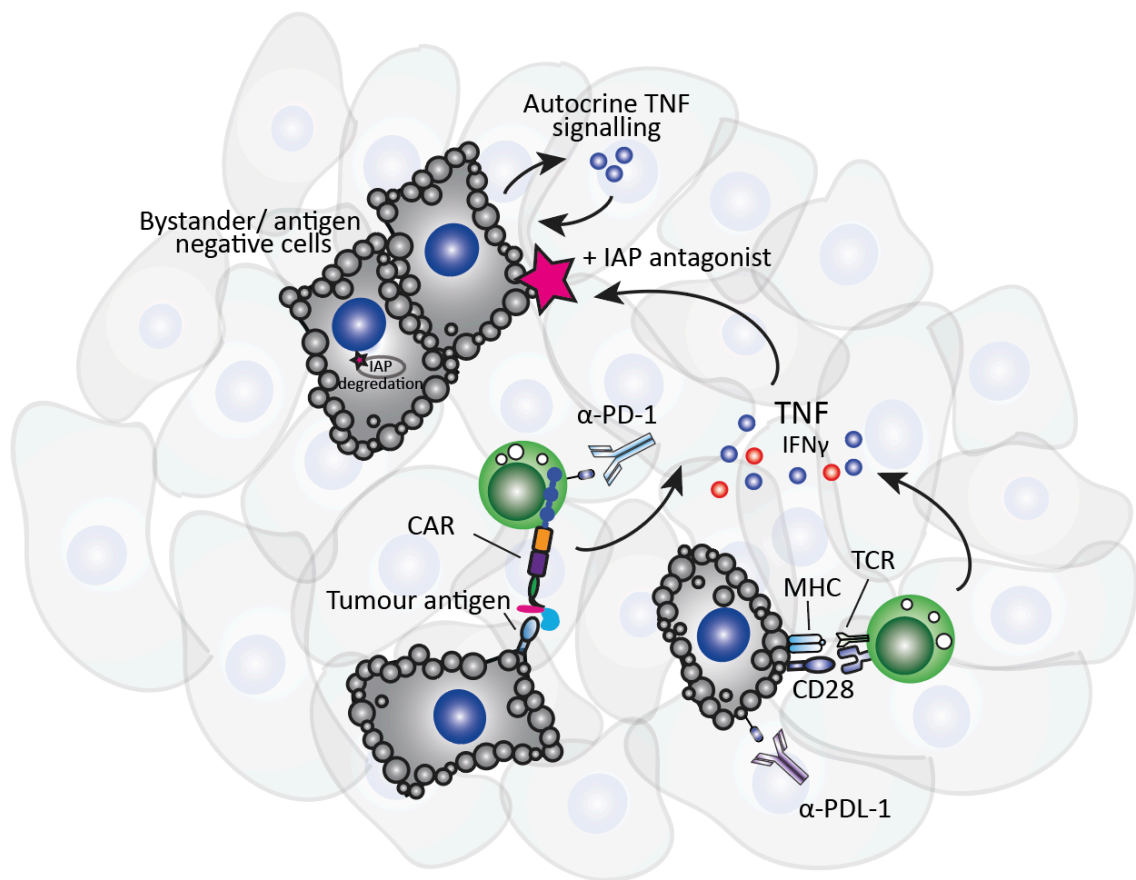


Figure 35. Birinapant enhances TNF dependent bystander killing. Following CAR T-tumour engagement, birinapant broadly sensitises tumour cells to CAR T cell-derived TNF-mediated apoptosis. Combining this approach with α -PD-1 might be a strategy to engage the endogenous immune system and enhance CAR T cell TNF-secretion. Adapted from Michie et al, (2020) [246].

**Chapter 5: Investigating mechanisms of CD19 antigen loss following
CAR T cell therapy in haematological malignancies**

1. Introduction

For the majority of work presented within this thesis, the role of CAR T cell therapy for the treatment of solid tumours was investigated, with a view to enhancing their efficacy in this context. As yet, there is no approved CAR T cell therapy available for solid tumours, although various antigenic targets have been identified, and clinical trials remain ongoing. Clinically however, CAR T cell therapy is efficacious in the context of haematological malignancies, in particular B cell malignancies such as acute lymphoblastic leukaemia (ALL) and B cell lymphomas. In this context, CAR T cells are generated towards the B cell surface marker CD19, a receptor that is expressed on mature B cells. CD19-directed CAR T cell therapy for the treatment of relapsed and refractory ALL has demonstrated robust remission rates of between 70-94% [157, 169, 278-280].

There are several reasons why CD19 is considered an ideal target for CAR T cell therapy. Following IV infusion, CAR T cells are rapidly exposed to the antigenic target with no trafficking required, as would be the case in solid tumours, and immediate antigen exposure appears to aid proliferation and persistence of the CAR T cells. In addition, blood-borne cancers do not have a TME that facilitates immune exclusion and exhaustion, which enhances anti-tumour immunity. In contrast to other antigenic targets, the “collateral damage” of total B cell aplasia and hypogammaglobulinaemia as a result of CD19-CAR T cell therapy can be treated clinically with antibody infusion [281], and therefore “on-target” but “off-cancer” effects are tolerable.

1.1 Resistance to CAR T cell therapy

However, like other immunotherapy approaches, resistance to CD19-CAR T cell therapy is an emerging problem, where up to 20% of ALL patients and 50% of diffuse large B cell lymphoma patients fail to respond to therapy, and around 35% of responding patients eventually relapse [171, 282, 283]. Several mechanisms of resistance to CAR T cell therapy have been identified, including poor CAR T cell persistence, primary tumour resistance and acquired resistance resulting in antigen negative relapse.

1.2 T cell intrinsic defects

Relapse following CD19-CAR T cell therapy can be broadly divided into CD19⁺ and CD19⁻ relapse variants. Of the CD19⁺ subtype, this typically occurs due to lack of CAR T cell persistence and primary tumour cell resistance. It is clear that in some circumstances, inherent T cell defects are responsible for failure of treatment, where T cells from non-responding patients typically exhibit upregulated transcriptional programs associated with effector differentiation, glycolysis, exhaustion and apoptosis [284]. In addition, sustained remission following therapy was associated with the therapeutic product exhibiting increased frequency of TNF secreting CD8⁺ CAR T cells [175]. Interestingly, one instance of CD19⁺ relapse was determined to be as a result of the unintentional transduction of a single leukemic B cell in the CAR T cell manufacturing process, masking it from recognition and conferring resistance to CAR T cell killing [285].

1.3 Primary resistance to CAR T cell therapy

However, it is clear that a sizable proportion of CD19⁺ B cells that fail to respond are intrinsically resistant to this therapy. Similar to the CRISPR-Cas9 screens presented in Chapter 3, whole-genome CRISPR-Cas9 screening has identified that loss of several pro-apoptotic death receptor signalling molecules such as FADD and CASP8 confer resistance to CAR T cell therapy in the Nalm6 ALL cell line [235]. This data suggests that dysregulation of the cellular apoptotic machinery may provide resistance to CAR T cell therapy in B cell malignancies, as we have observed in the solid tumour context. Since the secretion of TNF from CD19-CAR T cells is correlated with sustained disease remission [175], it is likely that TNF signalling is critical to the cytotoxic effector function of CD19 CAR T cells. Therefore, resistance to TNF signalling is a likely resistance mechanism to CAR T cell therapy in B cell malignancies as we have observed in solid tumour models.

1.4 Antigen negative relapse

Although CD19 is required for the development of mature B cells, it is not required for leukemic development and antigen negative relapse is a potential consequence of CAR T cell therapy, despite CAR T cell persistence. While

the majority of patients do initially respond to therapy, it is now clear that a significant proportion of CAR T cell-treated patients experience relapse due to acquired resistance via antigen loss [286]. In order to overcome this obstacle, considerable research efforts in to the mechanisms of antigen loss following CAR T cell therapy are underway.

1.5 Mechanisms of antigen loss

The level of antigen expression prior to treatment onset has been correlated with responses to immunotherapy [287], and the levels of CD19 surface expression is somewhat heterogeneous in B cell leukaemias [288]. In a subset of relapsing patients, CD19 isoforms that enable resistance to CAR T cell therapy were present from initial diagnosis, which lead to relapse due to the expansion of these clones. It is therefore considered necessary to confirm the CD19 isoforms present prior to onset of treatment [172].

However, the majority of patients that experience relapse do so following the acquisition of antigen negative clones that were not present prior to treatment. Analysis of one clinical trial showed that of the B-ALL patients that relapsed after treatment, 12/ 17 patients presented with CD19⁻ disease [289]. In some cases, alternative-splicing events in exon 2 resulted in expression of a CD19 variant that lacked the CAR T cell binding epitope [173], which enabled CAR T cell escape. This suggests that targeting alternative exons might be an effective strategy to overcome this resistance, however this approach has not been widely supported by other studies. Frame-shift insertion or deletion mutations, and occasionally missense single nucleotide variants, in exons 2-5 of CD19 have been identified which has led to the formation of a truncated protein lacking membrane anchorage [289].

In addition, acquired mutations that result in the loss of CD19 membrane trafficking may also be a mechanism of antigen loss in B cell malignancies. One of the critical molecules for CD19 membrane expression is CD81, a protein that belongs to the trans-membrane 4 superfamily of proteins [290], and forms part of the B cell co-receptor. CD81 associates with CD19 in the rough endoplasmic reticulum (rER) and is critical for the transport of CD19 to

the cell surface. Whilst inherited or *de novo* mutations in CD81 presents in humans as a humoral deficiency [291], acquired CD81 mutations have been identified in CD19⁺ escape variants following CD19-directed immunotherapy [292].

In some instances, selection pressure from CD19-directed CAR T cells resulted in the acquisition of a CD19⁺ malignancy with a myeloid phenotype, through a mechanism known as lineage switching. Rearrangements in the mixed lineage leukemia (MLL) gene on chromosome 11q23 is associated with poor prognosis in B-ALL treated with chemotherapy [293], however CAR T cell therapy has induced complete remissions in this leukemic subtype [174]. Whilst MLL rearrangement is often associated with aberrant cross-lineage myeloid antigen presentation on leukemic blasts, adopting a distinct lineage phenotype is a rare event that is associated with poor patient outcome [294]. However, following CAR T cell immune pressure, 2/7 patients with MLL rearranged B-ALL relapsed with CD19⁺ disease, which exhibited a complete loss of lymphoid lineage antigens and acquired expression of myeloid antigens, which was clonally related to their B-ALL disease [174]. Lineage switching is therefore considered a mechanism of CAR T cell immune escape, which may occur following mutations in the MLL gene.

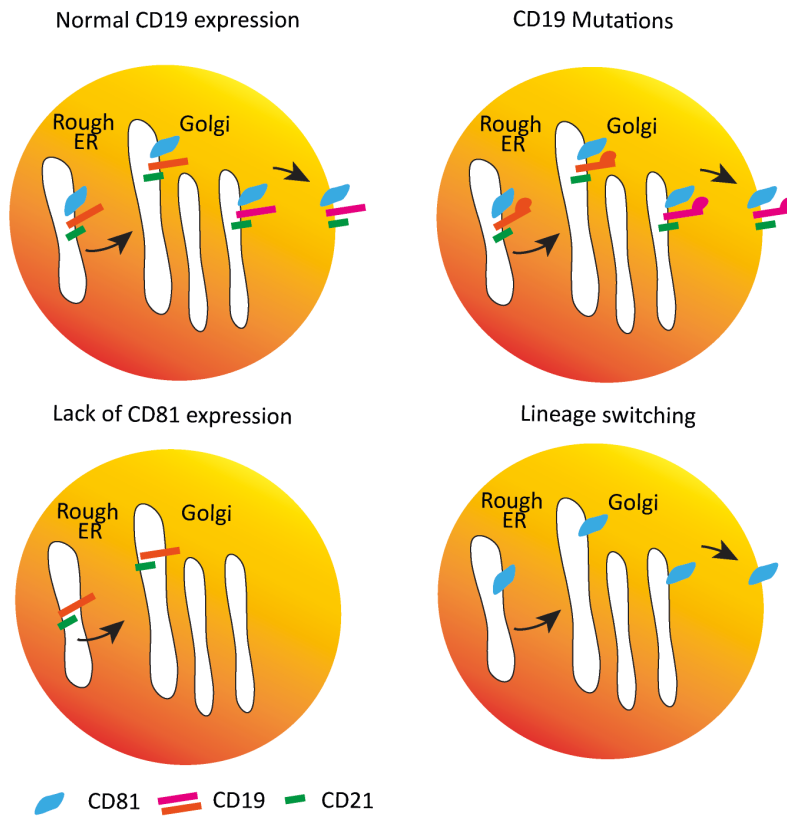


Figure 36. Known mechanisms of CD19 antigen loss in B-ALL. Adapted from Velasquez et al, (2017) [295].

While several mechanisms of resistance have been identified (Figure 36), we considered that many possible mechanisms of antigen loss following CAR T cell selection pressure remain unidentified, in particular those genes that control CD19 expression on an epigenetic level. Therefore, we sought to investigate the mechanism by which B cells are able to down-regulate surface CD19 expression in an unbiased way by utilising a genome-wide CRISPR screening approach.

Similar to previous CRISPR-Cas9 screens described in this thesis, the MHH-CALL4 cells were transduced with a pooled sgRNA library, this time targeting the human genome, again with 4 sgRNA per gene. In order to select for cells with diminished CD19 surface expression following sgRNA library transduction, we utilised a flow cytometry cell sorting technique, which has previously been demonstrated to be a particularly effective approach given

the stringent selection pressure of cell sorting [232, 233]. We hypothesised that enrichment of sgRNA for several epigenetic drivers of CD19 surface expression, which have not yet been identified, would result in loss of surface antigen expression. In addition, based on the published literature, we anticipated that genes involved in lineage switching such as MLL genes would score highly. In order to ensure the specificity of the CRISPR-Cas9 screening approach, significant enrichment for sgRNA targeting both CD19 and CD81 were considered critical to validate the results.

2. Results

Investigating mechanisms of CD19 antigen loss in B cell malignancies

First, we selected a B-ALL cell-line known as MHH-CALL4, which is a CD19⁺ B cell precursor ALL, obtained from a pediatric patient, since CD19 CAR T cell therapy is most commonly used to treat pediatric ALL and this patient would be a suitable candidate for CAR T cell therapy. The CRISPR-Cas9 screening approach we utilised involved flow cytometry cell sorting in order to stringently select for cells that had low CD19 surface expression. First, we confirmed the endogenous expression of CD19 on the surface of the MHH-CALL4 cells. Then we assessed several CD19 antibodies (Figure 37) in order to determine the most suitable antibody for use in the CRISPR-Cas9 screen. Based on this data, we selected the CD19 FITC-conjugated antibody clone, because it demonstrated reliable CD19 staining with adequate sensitivity to detect the lowest CD19 expressing cells. Additionally, unlike PE or APC, FITC has limited emission spectral overlap with mCherry, which was used to detect the presence of Cas9 in these cells.

The experimental design utilised for this genome wide CRISPR-Cas9 screen is outlined in Figure 38. Briefly, we virally transduced MHH-CALL4 cells with the Brunello sgRNA library, which targets the human genome with 4 sgRNA per gene. Following puromycin selection to ensure a pure population of transduced cells, the cells were stained with a FITC anti-CD19 antibody as selected for above (Figure 37), and the cells with the lowest 20% CD19

expression were sorted by flow cytometry.

After sorting on the lowest 20% of CD19 expressing cells, the cells were allowed to recover for a period of 3-4 weeks. We identified by FACS analysis of the sorted cells that the CD19 expression was reduced after each sort, however since CD19 is an actively trafficked and cycled receptor, a proportion of the cells regained high CD19 expression following sorting. Therefore, we sorted the cells 3 times in order to apply adequate selection pressure, and to obtain a true CD19^{low} population of cells. Over three successive sorts, the expression of CD19 in the bulk B cell population was reduced (Figure 39). We therefore sequenced the resultant population to interrogate the enriched guides that resulted in the CD19^{low} phenotype (Figure 40).

Analysis of the enriched sgRNA in the CD19^{low} population compared to the T₀ starting population identified both CD19 and CD81, which served to provide validation of the screen's specificity for CD19. In addition, there was significant enrichment for a variety of other genes (PRKCSH, KMT2D, COMMD3, AHR), a number of which had not previously been associated with CD19 expression. In order to better interpret the data, we conducted String and Gene Ontology analyses to interrogate the known connections between these genes of interest. String analysis from the top enriched genes from the CD19^{low} CRISPR-Cas9 screen (Figure 41) returned several clusters of related genes in addition to CD19 and CD81; including histone methylation, N-linked glycosylation, Aryl hydrocarbon receptor processing and E3 ubiquitin ligase activity.

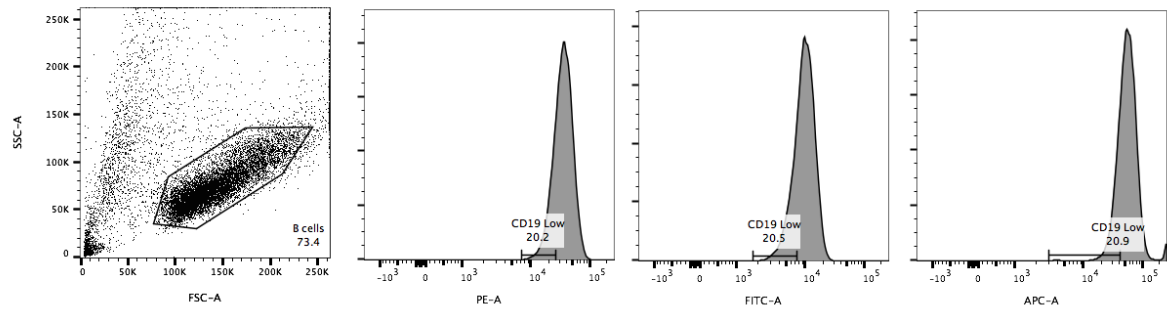


Figure 37. Expression of CD19 on MHH-CALL4 cells, using three antibody clones. MHH-CALL4 CD19 expression was investigated by flow cytometry using three antibody clones: SJ25C1 conjugated to PE, 340364 conjugated to FITC or H1B19 conjugated to APC, in order to select the most appropriate antibody for the CRISPR-Cas9 screen cell sorting approach.

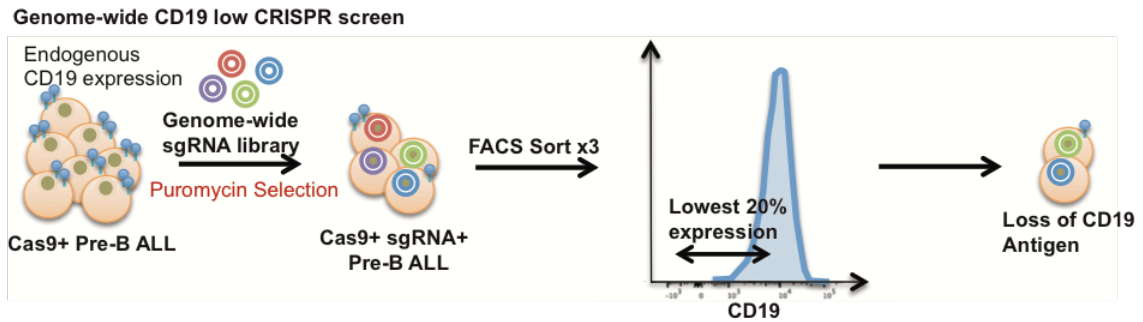


Figure 38. Schematic outlining the experimental design of the CD19^{low} genome-wide CRISPR-Cas9 screen. The MHH-CALL4 B-ALL cell line that endogenously expresses CD19 was transduced with a human whole-genome sgRNA library. Following puromycin selection, the cells were sorted 3 times on the lowest CD19 expression and the resultant CD19^{low} population was analysed for sgRNA enrichment.

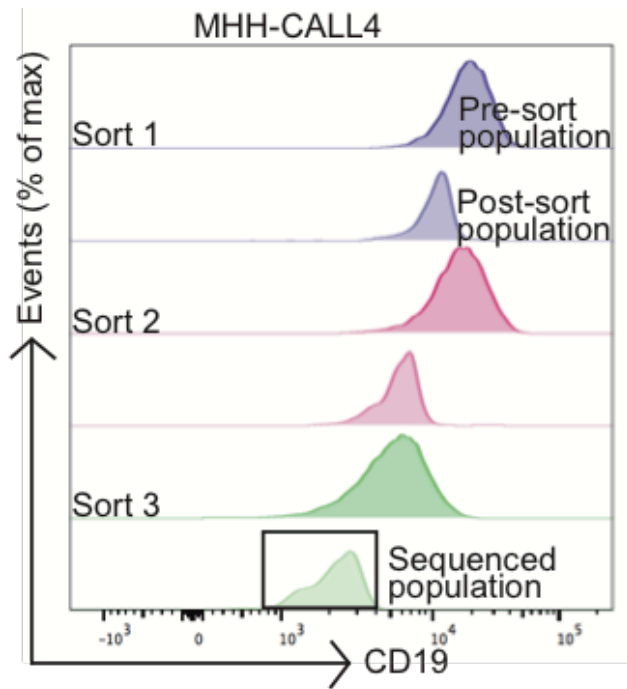


Figure 39. Decrease in surface CD19 expression on MHH-CALL4 cell line following successive cell sorts. Analysis of the CD19 expression on MHH-CALL4 cells pre- and post-cell sort for the lowest 20% CD19 expression. Surface expression of CD19 antigen was reduced following three successive sorts and the resultant CD19^{low} population was analysed.

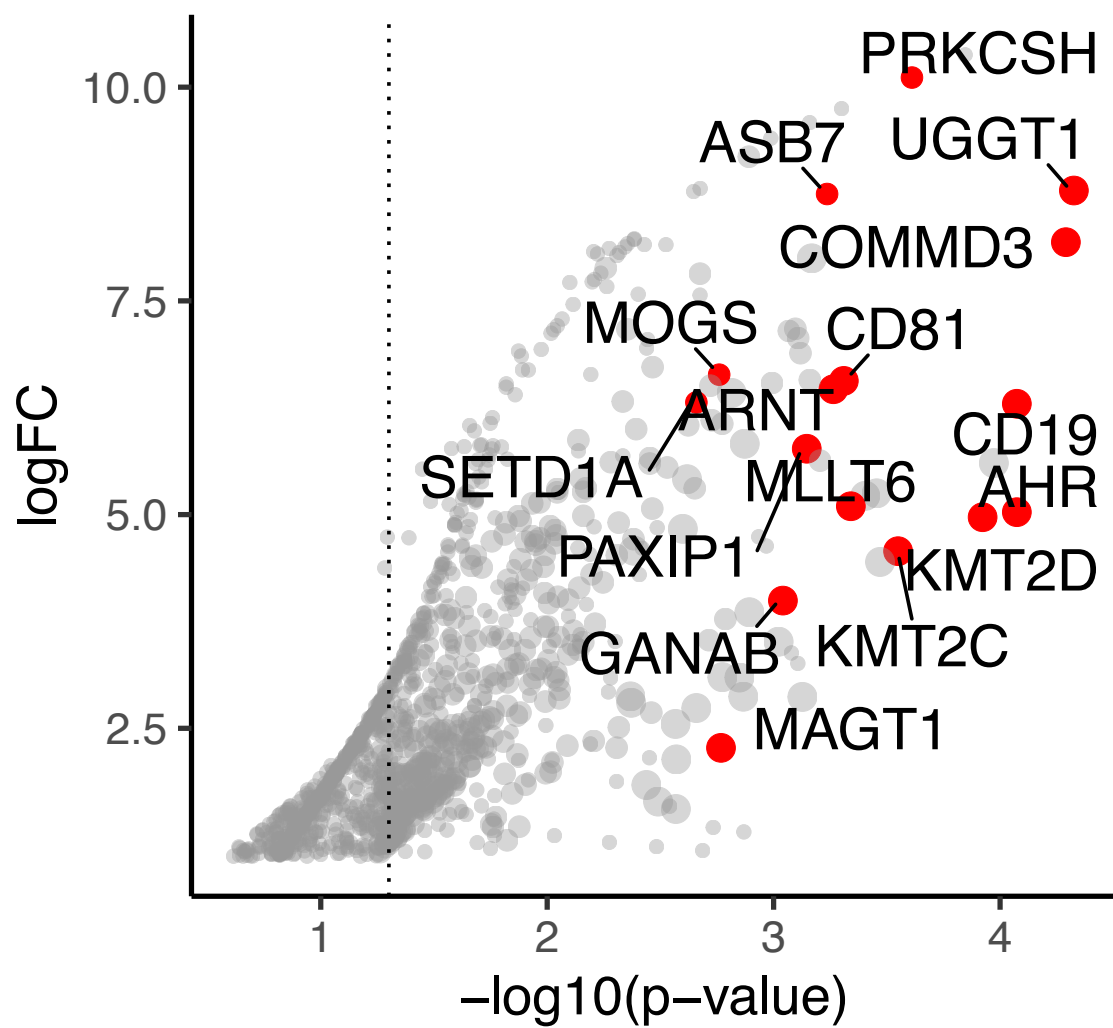


Figure 40. Enriched sgRNA from the CD19^{low} CRISPR-Cas9 screen.
Volcano plot presents LFC of individual sgRNA against -log P value.

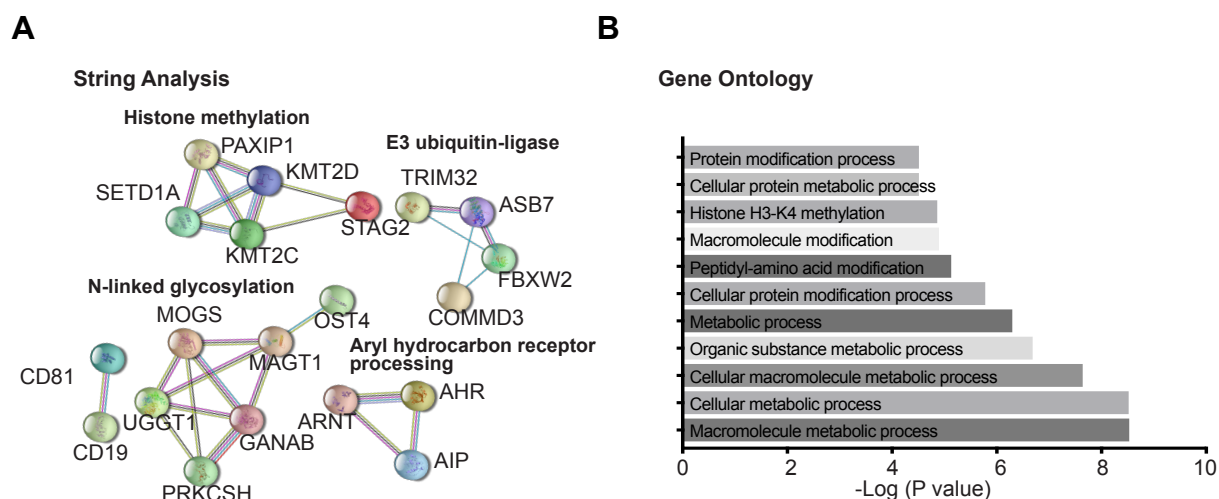


Figure 41. Analysis of shared pathways between top scoring genes in CD19^{low} CRISPR-Cas9 screen. (A) String analysis showing connections between top scoring genes related to histone methylation, E3 ubiquitin-ligase, N-linked glycosylation and Aryl hydrocarbon receptor processing. (B) Gene Ontology from Top 55 scoring genes.

Validation of the role of KMT2D in CD19 expression

We hypothesised that epigenetic regulation would play a role in the surface expression of CD19, and that epigenetic silencing may be a key mechanism for antigen loss in this context. Therefore, of the top scoring genes from the CRISPR-Cas9 screen, we considered the genes Histone-lysine N-methyltransferase 2C (KMT2C) and 2D (KMT2D), associated with histone modification, the most interesting and novel due to their potential to be involved in the epigenetic regulation of CD19.

Therefore, we investigated the role of KMT2D in the regulation of surface CD19 expression in MHH-CALL4 cells. In order to investigate this, we stably depleted KMT2D by virally transducing the cells with KMT2D sgRNA in a GFP-containing plasmid and analysed CD19 expression by flow cytometry (Figure 42). We identified that the GFP⁺ cell population of sgKMT2D cells exhibited a lower surface CD19 expression than the control guide-containing cells. We next examined the percentage of total GFP⁺ cells that had low surface CD19 expression (Figure 43). The data showed that the KMT2D-depleted MHH-CALL4 cell population contained a greater percentage of cells deemed to be CD19^{low}. However based on the CRISPR-Cas9 screen data, a greater difference in CD19 surface expression compared to control cells was expected. The nature of CRISPR-Cas9 screening approaches that select for low surface marker expression, as oppose to selecting for cells with no expression, does allow for the enrichment of guides that result in subtle decreases in protein expression. B cells that are able to escape from CD19-directed CAR T cells via antigen loss are typically antigen negative, and we did not see clear evidence of epigenetic silencing via loss of KMT2D or a population of KMT2D-depleted cells that were entirely CD19 negative. Therefore, we did not continue to pursue this CRISPR-Cas9 screen finding, as the importance of KMT2D in loss of CD19 expression was unclear.

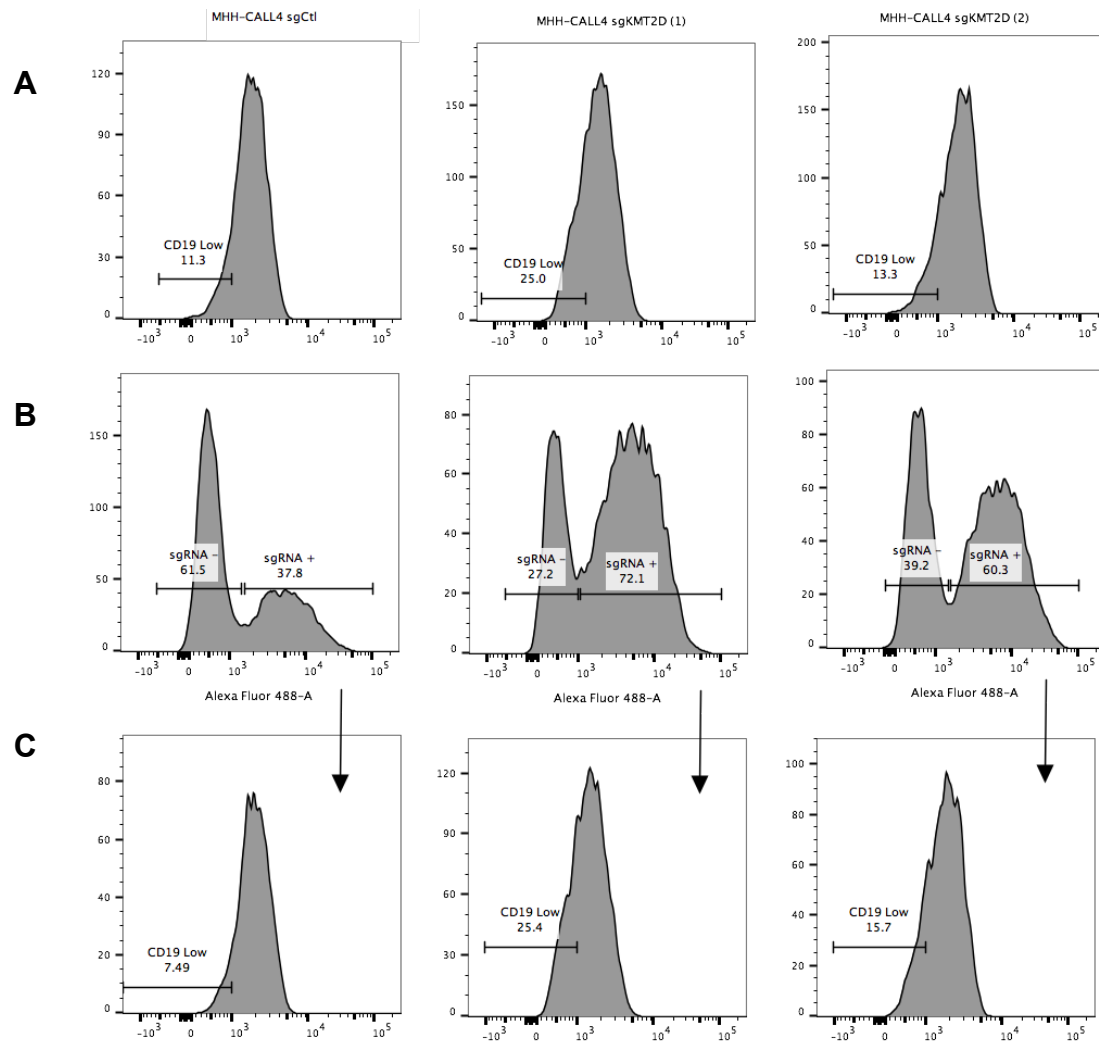


Figure 42. Surface CD19 expression in KMT2D-depleted MHH-CALL4 cells. (A) CD19 expression in the bulk B cell population following sgRNA incorporation. (B) Transduction efficiency of sgRNA by GFP expression. (C) Surface CD19 expression of GFP+ positive MHH-CALL4 cells. Data are representative of two independent experiments.

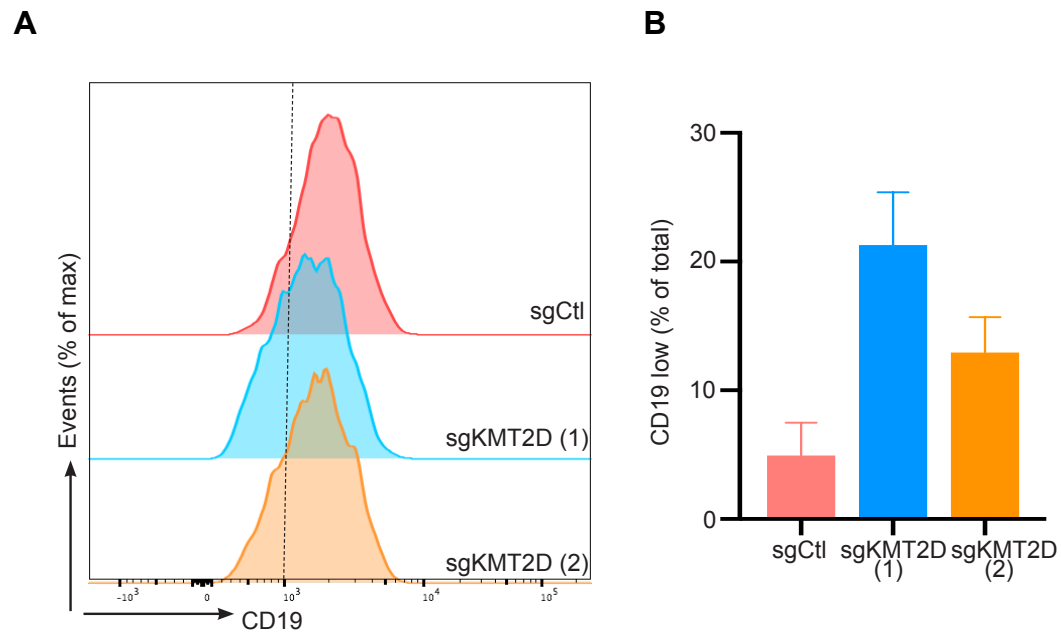


Figure 43. Proportion of CD19^{low} MHH-CALL4 cells assessed by flow cytometry. (A) Histogram of surface CD19 expression of control or KMT2D depleted MHH-CALL4 cells, gated on GFP⁺. (B) Percentage of cells designated CD19^{low} from (A), data are mean $\pm$ SEM from two independent experiments.

3. Discussion and future investigation

In this chapter, we conducted a genome-wide loss-of-function CRISPR-Cas9 screen in order to elucidate mechanisms of CD19 antigen loss in B cell malignancies, in order to better understand how CD19⁻ relapse occurs following CAR T cell therapy.

3.1 Known pathways identified by CRISPR-Cas9 screen

We observed significantly enriched sgRNA for CD19 and identified several known regulators of CD19 expression, such as CD81, and N-linked glycosylation.

Genes associated with N-linked glycosylation (MOGS, MAGT1, GANAB, UGGT1 and PRKCSH) emerged as the most significantly enriched in the CD19^{low} population. Proteins destined for the cell surface, including CD19, enter the endoplasmic reticulum where they undergo co-translational modifications, such as N-linked glycosylation [296]. This process involves the attachment of a carbohydrate oligosaccharide to the amide nitrogen of an asparagine residue of a protein. Following this process, proteins are transported to the golgi where mannose trimming and modifications occur, as well as the addition of complex glycans before proteins can be trafficked to the cell surface [297]. Mutations that alter protein folding and assembly can trigger ER retention of the misfolded protein [298]. CD19 is a glycosylated protein that contains 5 asparagine residues in its extracellular domain, including Asp86 in exon 2 [299], where alternative splicing events are known to occur in CD19⁻ relapsed patients [173]. Proper glycosylation is known to be critical to the surface expression of CD19 [300], and research in to the role of N-linked glycosylation defects in CD19⁻ relapse is ongoing. It is therefore likely that additional cases of CD19⁻ relapse will emerge that can be attributed to inappropriate N-linked glycosylation and ER retention.

3.2 Novel pathways identified by CRISPR-Cas9 screen

We hypothesised that CD19 might be subject to epigenetic silencing upon CD19^{low} selection pressure, therefore when we identified histone

methyltransferases KMT2D and KMT2C were significantly enriched in the CRISPR-Cas9 screen, we investigated the role of KMT2D in CD19 surface expression.

KMT2D is a histone mono-methyltransferase, an enzyme that catalyses the methylation of lysine 4 on histone H3 (H3K4), associated with transcriptionally active chromatin. KMT2D belongs to a family of six set-1 like H3K4 methyltransferases known as KMT [301], which includes KMT2C, which also scored highly in the CD19^{low} screen. Mutations in methyltransferases, and therefore alterations in the methylation of histones to control gene expression, along with acetyltransferases and histone proteins themselves, may result in epigenetic modifications, which have been implicated in tumour suppression and lymphogenesis [302].

KMT2D mutations are heavily implicated in B cell malignancies. KMT2D is the most frequently mutated gene in diffuse large B cell lymphoma (DLCL), accounting for 30% of all *de novo* cases [303], and around 90% of all follicular lymphoma diagnoses [304, 305]. Taken together, KMT2D mutations are implicated in 70% of all B cell Non-Hodgkin's Lymphoma diagnoses [302].

Stable depletion of KMT2D in MHH-CALL4 cells resulted in only a small decrease in surface CD19 expression compared to control sgRNA, therefore to what extent histone methylation plays in CD19 expression remains unclear. KMT2D loss-of-function mutations have been associated with oncogenesis and lymphogenesis, and therefore it is possible that KMT2D depletion results in enhanced cell proliferation and out-competes control cells as opposed to true CD19 loss.

In addition to the histone methyltransferases, we identified several novel pathways that have not previously been associated with CD19 expression, all of which warrant further investigation. Three genes of interest from the screen that had not previously been linked to loss of CD19 expression following CAR T cell therapy were ARNT, AHR and AIP, all of which are associated with Aryl hydrocarbon receptor processing. Aryl hydrocarbon receptor is a ligand-

induced nuclear receptor transcription factor, which is classically involved in mediating xenobiotic toxicity and acting as an environmental sensor for dioxins [306]. Aryl hydrocarbon receptor is highly induced upon B cell activation, controls cell-fate decisions in B cells [307] and is required for optimal B cell proliferation [308]. In addition, aryl hydrocarbon receptor contributes to the transcriptional program of IL-10 producing regulatory B cells [309] and suppresses PAX5 [310], a transcription factor upon which CD19 expression is dependent [311]. Whilst aryl hydrocarbon receptor processing has been attributed to several components of B cell physiology, it has not previously been directly linked to CD19 expression,

In addition, another cluster of genes including TRIM32, ASB7, FBXW2 and COMMD3 was identified in this CRISPR-Cas9 screen, all of which are associated with E3 ubiquitin ligase activity. Several E3 ubiquitin ligases are associated with normal B cell development [312], including TRAF2, 3 and cIAP2, however the E3 ligases identified in this CRISPR-Cas9 screen have not previously been associated with B cell development or CD19 expression. Therefore, both the Aryl hydrocarbon receptor and E3 ubiquitin ligase pathways present interesting findings from this work and attractive avenues for future investigation.

While loss of the CD19 antibody binding epitope has been identified as a mechanism of CAR T cell resistance, we were not able to identify any significantly enriched genes that might have resulted in a loss of antibody binding epitope, possibly because these are derived from alternative splicing events. Furthermore, we did not identify significant sgRNA enrichment for any genes that were clearly associated with B cell lineage switching. However, the gene MLLT6 (myeloid/ lymphoid or mixed lineage leukemia, translocated to 6) scored highly in the CRISPR-Cas9 screen, a gene from the mixed leukaemia lineage family. Mutations in the MLL genes are associated with leukaemias with a mixed lineage phenotype, and therefore it is possible that this gene is associated with lineage switch in these cells.

3.3 Technical challenges associated with genome-wide CRISPR-Cas9 screening in with B-ALL cell lines

Considering that depletion of KMT2D resulted in only subtle changes in the CD19 surface expression, this CRISPR-Cas9 screen could be repeated in order to apply a more stringent selection pressure. Repeating this screen with additional cell sorts or selecting the lowest 5 or 10% CD19 expression would enhance the stringency of this screening approach. However, this work is confounded by significant technical challenges associated with viral transduction and manipulation of B cells, which are slow growing and affected by stressors such as cell sorting. As a result, genome-wide CRISPR-Cas9 screening with the MHH-CALL4 cell line is technically difficult, requires large cell numbers and takes several months, which often results in sgRNA dropout over time. In addition, CD19 loss in a cell line may not accurately model this occurrence in primary B cells, and therefore repeating this screen using primary B cells would be an attractive strategy for future investigation, although technically challenging.

3.4 Future directions

Overall, this screening approach has identified several novel pathways for CD19 antigen loss in B-ALL, and the detection of known genes and pathways confirms the validity of the data. This work paves the way for other experimental work in order to elucidate the precise mechanisms by which leukemic B cells are able to downregulate CD19 expression in order to avoid immune pressure by CAR T cells. Identification of these mechanisms is critical to our understanding of CD19⁻ relapse, and potentially will aid the identification of novel drug targets to prevent the development of CD19⁻ clones during CAR T cell therapy.

Identification of TNF signalling as a key resistance pathway that limits efficacy of CAR T cells in solid tumours in Chapter 3 allowed for the development of the rational combination therapy presented in Chapter 4. Therefore, with additional validation, it is hoped that these uncovered genes and associated pathways might yield a potential combination therapy to overcome the observed resistance to CAR T cell therapy following the development of

antigen negative clones. In addition, an interesting approach might be to conduct another genome-wide CRISPR-cas9 screen in these cells selecting for cells with the highest 10% CD19 surface expression. Identification of any of any genes that, when disrupted, enhance CD19 expression might uncover novel drug targets in order to further enhance B cell CD19 expression prior to, or during CAR T cell therapy.

While the mechanisms of antigen negative relapse are still being uncovered, other strategies in order to overcome this are being proposed, including the targeting of other B cell antigens, such as CD20 and CD22, with mono- or dual-specific CAR T cells. However, there is no evidence that the mechanisms of CD19 downregulation are specific to CD19 and therefore it is likely that antigen negative relapse will occur with other surface markers, and therefore mechanisms of antigen loss should continue to be elucidated.

Chapter 6: summary, discussion and future perspectives

1. Summary

Immunotherapy has rapidly emerged as the fourth pillar of cancer care, with remarkable responses reported with immune checkpoint blockade and CAR T cell therapy against some cancer types. Such successes have added to the body of evidence implicating the immune system in the control of cancer. In particular, CD19-targeting CAR T cell therapy against B cell ALL demonstrated complete remission rates of up to 90% in early clinical trials [169], and overall has a response rate of around 55% [176]. In contrast these results have not been recapitulated against solid tumours, where the response rates, regardless of target antigen or co-stimulatory domain, are less than 10% [176]. There are several reasons why CAR T cell therapy is less effective in solid tumours than in haematological malignancies, including poor CAR T cell homing and penetration in to the TME, and T cell exhaustion due to soluble immunosuppressive molecules and expression of inhibitory ligands. Considerable research efforts are directed towards overcoming these challenges and enhance CAR T cell efficacy against solid tumours, either by re-designing the CAR T cell, or in a combination approach with other immunotherapies.

In Chapter 3, we sought to investigate the tumour-associated genes responsible for restraining CAR T cell efficacy in solid tumours, utilising a whole-genome CRISPR-Cas9 screening approach. Here, we identified that loss of cytokine response pathways was critical for CAR T cell immune evasion. Loss of IFN- γ signalling has previously been identified as a key tumour resistance mechanism to immunotherapy, where acquired mutations in critical IFN- γ signalling genes, such as JAK1 confer resistance to immune checkpoint blockade in the clinic [131, 135]. In addition, previous CRISPR-Cas9 screens have identified that IFN- γ signalling is critical for OT-I T cell killing [202, 231], and neutralising IFN- γ results in reduced CAR T cell efficacy *in vivo* [197]. As discussed in the published review in Chapter 1 section 4.1, TNF is a critical effector molecule of both T and NK cells, and strategies to augment tumour TNF response in the context of immunotherapy remain under investigation.

Additionally, we identified that loss of the transcriptional co-binding factor *Cbfb* protected MC38 cells against CAR and OT-I T cell killing. While *Cbfb* has been well characterised in hematopoiesis and various leukaemias [236-238], its role in solid malignancies remains poorly understood. *Cbfb* has previously been implicated in patient prognosis in colorectal [240] and breast cancers [220], and therefore we investigated the mechanism by which loss of *Cbfb* confers resistance to T cell killing, and the implications for immunotherapy. In Chapter 3 we identified for the first time, a role for *Cbfb* in mediating tumour responsiveness to TNF and observed that mice bearing *Cbfb*-depleted tumours did not respond to α -PD-1 *in vivo*. Finally, analysis of clinical trial data implicated both *Cbfb* and its associated co-binding partner *Runx1* in responsiveness to immune checkpoint blockade and adoptive cell therapy in melanoma patient cohorts. There is currently an unmet clinical need for biomarkers that predict responsiveness to immunotherapy, and therefore the potential prognostic significance of *Cbfb* should be considered.

These data presented in Chapter 3 identify that loss of response to TNF signalling in tumour cells limits CAR T cell efficacy in solid tumours, and highlight the critical need for tumour TNF signalling in CAR T cell therapy. This data also demonstrates the need for strategies that augment tumour TNF response to enhance the efficacy of immunotherapies against solid tumours. In Chapter 4, we utilised the cytotoxic potential of CAR T cell-derived TNF and identified that combining CAR T cell therapy with a small-molecule smac-mimetic, birinapant, enhanced CAR T cell therapy in a TNF-dependent manner.

Importantly, we also identified that a key mechanism of action of this combination therapy was TNF-dependent bystander killing. By sensitising tumour cells to death via the TNF-dependent extrinsic cell death pathway, we demonstrated the ability of birinapant in association with CAR T cell-derived TNF, to kill tumour cells independently of an immunological synapse between T cells and tumour cells. As a result, bystander killing is not dependent on tumour cell antigen expression, and therefore this might be a strategy to

overcome the challenge of tumour antigen heterogeneity. Taken together, the data presented in Chapters 3 and 4, highlight that tumour cytokine signalling is critical to the effector function of CAR T cells, and underscores the cytotoxic potential of CAR T cell-derived cytokines.

In Chapter 5, we considered mechanisms of resistance to CD19-directed CAR T cell therapy in B cell malignancies. Resistance to CD19-CAR T cell therapy is an emerging problem, and around 35% of patients that respond to therapy eventually relapse [171, 282, 283], with either CD19⁺ or CD19⁻ disease variants. Relapse with CD19⁻ disease is associated with acquired resistance, and uncovering the mechanisms by which B cells lose surface expression of CD19 in this context is critical for ensuring long-term patient survival. To investigate mechanisms of CD19 antigen loss and to simulate selection pressure for CD19^{low} variants, we conducted a genome-wide CRISPR-Cas9 screen in a B-ALL cell line, and sorted the lowest 20% of CD19 surface expression by flow cytometry. The screen revealed enrichment for sgRNA associated with histone methylation, N-linked glycosylation, Aryl hydrocarbon receptor processing and E3 ubiquitin ligase, in addition to CD19 and CD81 as predicted. Several of these pathways have not previously been linked to CD19 antigen loss, and therefore these data present attractive avenues for future investigation, and may constitute potential drug targets in the prevention of CD19⁻ relapse following CAR T cell therapy.

2. Discussion and future perspectives

The data presented in Chapter 3 and 4 of this thesis have demonstrated that loss of TNF signalling is a key resistance mechanism of solid tumours to CAR T cell immune pressure, and this can be overcome by augmenting the tumour TNF response with a smac-mimetic to enhance CAR T cell therapy. Furthermore, our data highlights the potential for CAR T cell therapy against solid tumours in the context of combination therapies. Future studies will be required to elucidate the optimal CAR T cell combination therapies for the treatment of different tumour types, and enhancing the efficacy of CAR T cell therapy for the treatment of solid tumours is currently under intense

investigation.

2.1 Enhancing CAR T cell efficacy using combination therapy approaches

2.1.1 Augmenting TNF response in CAR T cell therapy

TNF has emerged as a key effector molecule in immunotherapy and as such research efforts have been directed towards strategies that sensitize tumours to TNF response [203, 246, 313]. We, and others [203, 205, 235] have recently highlighted the use of a class of small-molecule drugs called smac-mimetics to harness the cytotoxic potential of TNF in immunotherapy, and their use continues to be investigated in clinical trials. Smac-mimetics degrade the IAPs [269, 270], a family of endogenous proteins that modulate caspases in order to restrain apoptosis [268]. IAPs are frequently upregulated in malignantly transformed cells [271] and this accounts in part, for many tumours being resistant to the apoptotic effects of TNF.

Key to the efficacy of smac-mimetics in immunotherapy is their ability to broadly sensitise malignantly transformed cells to TNF-mediated apoptosis [201]. The degradation of IAPs stimulates TNF secretion from a small subset of tumour cells directly, which can act in an autocrine or paracrine fashion [267, 314, 315], however the efficacy of smac-mimetics is optimal when combined with therapies that deliver a source of TNF directly to the tumour, such as oncolytic viruses [316] or in the present study, CAR T cell therapy. In addition, the ability of smac-mimetics to sensitise antigen negative cells to TNF-mediated apoptosis implicates their use as a strategy to enhance bystander killing and overcome tumour antigen heterogeneity in this context.

As extensively discussed in the publication in Chapter 1 section 4.1, smac-mimetics have broad immunomodulatory functions largely due to their ability to trigger non-canonical NF- κ B signalling. Importantly, non-canonical NF- κ B signalling promotes tumour chemokine secretion [317], and approaches that enhance chemokine production in the TME are an attractive therapeutic strategy to enhance the chemotactic gradient for T cell penetration. Given poor CAR T cell homing and tumour penetration is a key reason for the lack of efficacy of CAR T cells against solid tumours [42], combination therapies that

enhance the infiltration and activity of CAR T cells in solid tumours would be extremely clinically relevant.

In light of the clinical success of immune checkpoint blockade, α -PD-1 was one of the first combination therapies investigated for enhancing CAR T cell efficacy. In haematological malignancies, α -PD-1 in combination with CAR T cell therapy has been evaluated in clinical trials, as high PD-L1 expression is common in B cell non-Hodgkin lymphoma and serves as a prognostic indicator in this cancer type [318]. In combination with CD19-directed CAR T cell therapy, α -PD-1 has exhibited a favourable safety profile and was not associated with any dose-limiting toxicities [319], and therefore several clinical trials investigating the enhanced efficacy of this combination approach remain ongoing (NCT02706405, NCT03310619, NCT02926833, NCT03287817, NCT02650999).

In solid tumours, pre-clinical assessment has identified that α -PD-1 enhances CAR T cell efficacy *in vivo* [192], and can enhance CAR T cell-cytokine secretion [197, 320]. Based on this, α -PD-1 in combination with CAR T cell therapy is being investigated in clinical trials for a range of solid tumour targets. Additionally, smac-mimetics are being clinically investigated as a strategy to enhance the endogenous immune response in combination with immune checkpoint blockade (NCT03270176, NCT02587962), based on favorable pre-clinical efficacy [204, 271]. Therefore, the triple combination of CAR T cell therapy, smac-mimetic and α -PD-1 might be a strategy to deliver TNF directly in to the tumour microenvironment and engage the endogenous immune system, whilst simultaneously enhancing TNF secretion and broadly sensitising tumour cells to the T cell-derived TNF. As all of these components are either approved for use or have demonstrated favourable safety profiles in clinical trials, this is an approach that could be rapidly translated in to the clinic.

2.1.2 immune checkpoint blockade combination therapies

In addition to the use of α -PD-1 in combination with CAR T cell therapy, several other immunotherapy combinations have been identified. The other

approved immune checkpoint blockade α -CTLA-4 has demonstrated efficacy against melanoma through its ability to modulate the endogenous immune response by neutralising the inhibitory interaction between CTLA-4 on T cells and CD80/CD86 on APCs. Investigations into the benefits of combining CAR T cell therapy and α -CTLA-4 in order to overcome the challenges associated with the hostile tumour microenvironment [321] are ongoing, although as CAR T cells do not directly interact with APCs, it is unclear if CTLA-4 blockade enhances CAR T cells directly or indirectly through modulation of the endogenous immune system.

Another approach to combine the immunomodulatory properties of checkpoint blockade with CAR T cell therapy is the re-engineering of CAR T cells to secrete checkpoint inhibitors, which not only serves to enhance CAR T cell killing but also enhances the endogenous immune response. Pre-clinically, Anti-CAIX (carbonic anhydrase IX) CAR T cells secreting α -PD-L1 antibodies resulted in a 50-80% reduction in tumour weight compared to anti-CAIX CAR T cells alone, and demonstrated enhanced cytokine production and immune recruitment [195]. Another study has demonstrated enhanced efficacy of α -PD-1 secreting CAR T cells compared to combination therapy with PD-1 inhibitors in mouse models of ovarian cancer [322]. Based on this, MUC1, EGFR, EGFRvIII and mesothelin targeting CAR T cells that secrete PD-1, PD-L2 or CTLA-4 antibodies have begun clinical trials for the treatment of a range of solid tumours [323].

In addition to the currently approved therapies, it is likely that the arsenal of effective immune checkpoint immunotherapies will continue to grow, to incorporate other T cell receptor targets. Of interest is T cell immunoglobulin and mucin-domain containing-3 (Tim-3), which exhibits similar expression patterns to PD-1 and functions as a co-inhibitory receptor to suppress T cell proliferation and cytokine production. One of the ligands for Tim-3, Galectin-9 which is highly expressed on breast [324], gastric [325] and lung cancers [326] as well as melanoma [327] is a negative regulator of T cell immunity [328]. The use of α -TIM-3 as an immune checkpoint blockade is currently being investigated in phase I clinical trials as a monotherapy and in

combination with α -PD-1 for the treatment of both haematological and solid malignancies [329]. In addition, LAG-3, which is expressed on activated T cells and NK cells, is associated with an exhausted phenotype when concurrently expressed on TILs with PD-1. Blockade of LAG-3 synergistically enhances α -PD-1 therapy, suggesting that PD-1 and LAG-3 have complimentary functions in the dampening of T cell responses within tumours [28]. Such is the interest around targeting LAG-3 as an immunotherapy approach, there are currently 27 clinical trials investigating the use of LAG-3 blocking strategy [329], including LAG-3 antibodies as a single agent (NCT03489369), in combination with α -PD-1 (NCT03005782, NCT02061761), and dual affinity antibodies targeting both PD-1 and LAG3 (NCT03219268). It is therefore likely that in the future CAR T cell therapy in combination with multi-checkpoint blockade will be investigated.

2.2 CRISPR-Cas9 in immunotherapy

In this thesis, we used a series of genome-wide CRISPR-Cas9 screens in order to identify potential genetic targets for enhancing CAR T cell therapy. This approach has emerged in recent years as a powerful tool to screen all protein-coding genes in the genome that provide resistance, or sensitise to a specific selection pressure and has been extensively used to answer basic immunology questions.

2.2.1 Loss-of-function CRISPR-Cas9 screening

In the same manner that clinical response to immunotherapies is cancer-type specific, the cell line utilised and cancer type that it is derived from is critical to the outcome of CRISPR-Cas9 screening approaches, and is a major source of differences in results between published screens. Since immune checkpoint blockade is part of the current standard of care in melanoma, many CRISPR-Cas9 screening approaches investigating resistance to T cell mediated killing have used melanoma-derived cell lines. Genome-wide CRISPR-Cas9 screening has been conducted using the human melanoma cell lines Mel624 [230] and D10 [203], and the mouse melanoma cell line B16F10 has been utilised by several groups to identify genes that, when disrupted, confer resistance to T cell killing [202, 231]. In each of these

studies, resistance due to loss of IFN- γ signalling and antigen presentation were identified as mechanisms of resistance to T cell mediated killing. However, each screen identified genes that had not previously been linked to immunotherapy response, such as *Aplnr*, a novel regulator of Jak1 [230], and genes from the SW1/SNF chromatin remodeling complex that when deleted enhance T cell killing [231]. The insight gained from these studies has therefore identified several new immunotherapy targets, and while each of these studies targeted melanoma, differences between cell lines and human and mouse cells account for some of the variations between results of these CRISPR-Cas9 screens.

In this thesis we sought to uncover tumour-intrinsic resistance mechanisms to CAR T cell therapy using the mouse cell line MC38-HER2, as an alternative approach to the CRISPR-Cas9 screen conducted in the MDA human breast cancer cell line, which primarily identified loss of HER2 as the main resistance mechanism [202]. We used a mouse cell line transduced to express a truncated version of the human HER2 antigen in order to overcome this, as human HER2 disruption does not occur using a mouse sgRNA library. As a result, we were able to elucidate resistance mechanisms, such as *Cbfb*, which may have otherwise been obscured by antigen loss. Species differences therefore account for some differences between identified genes in these CRISPR-Cas9 screens, and highlight the importance of complimentary mouse and human CRISPR-Cas9 screening approaches.

One of the most striking differences between CRISPR-Cas9 screens is the relative importance of TNF signalling in protecting against immune pressure, which appears to be cell line dependent. Melanoma cell lines are typically more resistant to the apoptotic effects of TNF, which explains why sgRNA targeting genes involved in TNF signalling are often not highly enriched in melanoma-specific CRISPR-Cas9 screens [230, 231]. However, in one instance, a CRISPR-Cas9 screen designed to identify genes that when deleted sensitise IFNGR-depleted melanoma cells to T cell killing, showed that response to immunotherapy could be enhanced through augmenting the tumour TNF response [203], suggesting specific differences in tumour TNF-

responses between melanoma cell lines. In addition, the MC38 cell line used throughout this thesis is susceptible to TNF-mediated apoptosis, and therefore has been useful for identifying novel genes that modulate TNF sensitivity, such as *Cbfb* (Chapter 3), and *Ado* [202].

Interestingly, in every instance CRISPR-Cas9 screening approaches have highlighted the importance of cytokine signalling to the efficacy of immunotherapy. In some cases, deletion of pro-apoptotic members of the BCL-2 family such as BID have conferred resistance against T cell killing, due to disruption of the intrinsic apoptotic pathway [203, 235]. However the identification of genes that, when deleted, protect against direct perforin or granzyme B mediated killing have been rare. This is likely due to the loss of several genes being required to confer resistance against perforin and granzyme B-mediated killing, unlike cytokine pathways that can be disrupted by loss of a single receptor or transcription factor, therefore making acquired resistance via loss of cytokine response more likely. In addition, bystander effects occur following IFN- γ [330, 331] or TNF [163, 204] secretion, and therefore can act on many cells simultaneously, unlike the formation of an immunological synapse. Taken together, data from these CRISPR-Cas9 screens emphasise the importance cytokine signalling in the efficacy of immunotherapy, but also highlight the need for appropriate cell line selection and underscore the importance of tumour cell TNF sensitivity to the CRISPR-Cas9 screen results.

2.2.2 Other CRISPR-Cas9 screening approaches

In Chapter 3, we utilised both resistance screens, in order to identify genes that when disrupted confer resistance against CAR T cell killing, and dropout screens that identify genes that when deleted sensitise to CAR T cell killing. In addition to these methodologies, other CRISPR-Cas9 screening techniques that have been successfully employed include *in vivo* CRISPR-Cas9 screens, which use small boutique sgRNA libraries that are used to target genes of interest in a tumour cell population and subsequently injected in to mice. This approach has been used to identify genes that when disrupted confer protection against T cell adoptive transfer [202] or immune checkpoint

blockade [234]. *In vivo* CRISPR-Cas9 screening benefits from being a more physiologically relevant model system to answer fundamental immunology questions, however is less well suited to identify genes of interest from human cell lines. This approach would be an interesting and valuable future direction to this current study in order to identify new CAR T cell targets for solid tumours, however as CAR T cell therapy demonstrates poor efficacy *in vivo*, inadequate selection pressure severely limits the use of this this approach in the context of solid tumours.

Other CRISPR-Cas9 screening approaches that have been successfully utilised include activation screens, which use pooled libraries of modified sgRNA and catalytically inactive Cas9, known as dCas9 to recruit a synergistic activation mediator (SAM) complex to gene promoters resulting in target gene induction [332]. Within immunotherapy, activation screens have been used to identify tumour-intrinsic modulators of CD3 bi-specific antibody efficacy in B cell lymphoma, where up regulation of genes that encoded for surface proteins critical for cell-cell adhesion, such as SPN, CD52 and MUC1 limited the efficacy of this immunotherapy [333].

Taken together, a variety of genome-wide and boutique library CRISPR-Cas9 screening approaches have emerged as a powerful new tool for answering fundamental questions regarding genes than function to enhance or limit the efficacy of immunotherapies, and have been invaluable in identifying, in an unbiased manner, new genetic targets for immunotherapy.

2.2.3 CRISPR-Cas9 modification of T cells

In addition to the identification of novel targets, CRISPR-Cas9 is rapidly revolutionizing immunotherapy as an alterative strategy for the generation and genetic modification of CAR or transgenic T cells.

One of the first attempts at enhancing CAR T cell therapy utilising CRISPR-Cas9 engineering was the disruption of T cell checkpoint receptors. CD19-targeting CAR T cells have been generated with CRISPR-Cas9-mediated PD-1 disruption, where greater than 50% editing efficiency has been observed,

which resulted in enhanced tumour clearance *in vivo* [334]. Following this, a clinical trial investigating the use of PD-1^{-/-} CD19 CAR T cells in the treatment of B-ALL and Burkitt's lymphoma is underway (NCT03298828). In solid tumours, CRISPR-Cas9 disruption of PD-1 in CAR T cells has enhanced tumour clearance in pre-clinical models of glioblastoma [335] and triple-negative breast cancer [336]. In clinical trials, disruption of PD-1 has proven feasible and safe in cells engineered to express a transgenic TCR [154], although the efficacy of PD-1 deficient T cells requires further clinical investigation. Indeed, there are 20 registered clinical trials using CRISPR-Cas9 PD-1 knockout approaches in CAR or otherwise genetically altered T cells [337].

In addition to PD-1 disruption, deletion of receptors for immunosuppressive molecules has been investigated as an additional and complimentary approach. In order to overcome the exhaustion-promoting effects of TGF- β accumulation in the TME, disruption of the endogenous TGF- β receptor II in CAR T cells by CRISPR-Cas9 has been investigated. This approach demonstrated efficient CRISPR-Cas9 gene-editing and resulting in CAR T cells that were less susceptible to exhaustion and demonstrated better *in vivo* efficacy in TGF- β -rich tumours [199].

The use of retroviral vectors to transduce T cells is suboptimal because of random and varied integration in to the genome [338]. In contrast, insertion of the CAR by CRISPR-Cas9 directed homology-directed repair (HDR) presents an attractive strategy because of the potential for sequence-specific targeted gene delivery into a loci of interest [339].

Generating CAR T cells for each patient is a time consuming and labour intensive process that has considerable associated costs. As a result, there is on-going research efforts to generate a universal “off-the-shelf” CAR T cell product that could be generated from donor cells and given to any eligible patient in order to implement CAR T cell therapy as a standard of care. However, a major roadblock for the use of allogeneic CAR T cells is the risk of graft-versus-host-disease (GVHD), or rejection of the transferred T cells by

the host. CAR T cells have therefore been generated using CRISPR HDR, where the CAR was inserted in to the T-cell receptor α constant (TRAC) locus, an approach that simultaneously disrupts the endogenous TCR with the CAR insertion [340], and which results in uniform CAR expression, and improved efficacy associated with enhanced persistence and reduced exhaustion [341].

Other strategies to generate universal CAR T cells include multiplex disruption of the donor TCR and HLA class I via B2M depletion by CRISPR-Cas9. In animal models, these universal CAR T cells demonstrated reduced alloreactivity and did not cause GVHD [342]. Recently, transgenic NY-ESO-1-targeting T cells with a disrupted endogenous TCR were recently shown to be safe and able to persist in a phase I clinical trial [154] demonstrating the potential of this approach in adoptive cell therapy.

Finally, the unparalleled control of transgene genomic integration enabled by CRISPR-Cas9 HDR may be used in the future to enhance CAR T cell therapy by generating improved “armoured CARs”. These are CAR T cells that have been engineered to secrete additional soluble factors such as IL-12, to further enhance T cell function and inhibit T cell exhaustion [343]. Generating modified CAR T cells using HDR would enable precise integration into a determined locus, and therefore could be used to ensure the secretion of soluble factors only upon antigen-recognition, as opposed to constitutive expression. This approach would be an attractive strategy for the generation CAR T cells that are capable of secreting additional TNF, to enhance their efficacy when combined with birinapant and enhance bystander killing, but limit toxicity associated with systemic TNF. Therefore the TNF transgene might be integrated after the nuclear factor of activated T cells (NFAT) promoter as has been investigated for IL-12 armoured CARs [344], or in the locus of an exhaustion-associated gene, such as PD-1 to ensure TNF release only within the tumour.

2.3 Identification of appropriate target antigens

In addition to optimisation of the CAR T cell product, there are challenges in identifying suitable target antigens for CAR T cell therapy that are sufficiently expressed on malignant cells and not on normal cells, and antigen heterogeneity on tumours can lead to relapse.

In the case of CD19-CAR T cells, where relapse due to the development of antigen negative clones is an emerging problem, other B cell specific antigens are being investigated. Phase I clinical trials have reported success with CD20-directed CAR T cells for the treatment of B cell and mantle cell lymphomas [166]. In addition, a clinical trial utilising CD22-directed CAR T cells in patients refractory to CD19-CAR T cell therapy with CD19⁺ disease variants reported complete remission rates of 73%, however relapses were associated with diminished CD22 surface density [287], further highlighting the need to understand antigen loss in this context. CAR T cells specific to dual B cell antigens are being investigated as a strategy to overcome resistance associated with antigen loss. Three clinical trials have generated bi-cistronic retroviral vectors encoding both CD19 and CD22-targeting CARs simultaneously, with some durable remissions reported [345-347], despite low transduction efficiencies. Other studies have attempted to generate bi-specific CAR T cells by simultaneously [348] or sequentially [349] transducing the cells with separate lentiviral vectors, which was associated with complete remissions in 71% and 94% of patients, respectively, which was not associated with dose limiting toxicities. Long-term follow up data from these studies will shed light on whether these approaches results in improved patient outcomes and reduced incidence of antigen negative relapse.

While CD19 and CD22 expression is restricted to B cells, they are also expressed on normal and not exclusively malignant cells. Therefore, the result of successful CAR T cell therapy is the complete ablation of B cells, which is clinically tolerable because antibody replacement can be used to mitigate the resultant hypogammaglobuliemia. A considerable challenge has therefore been to identify CAR T cell antigenic targets; as such “collateral damage” is not clinically tolerable in other tissues.

Although a hematological malignancy, development of CAR T cell therapy to treat acute myeloid leukaemia (AML) has been considerably hampered by the lack of targetable surface antigens. Antigens associated with AML blasts include CD33 and CD123, which are ubiquitously expressed on malignant myeloid cells, but also expressed on normal myeloid cells and progenitors, the loss of which would result in complete loss of the myeloid compartment [350]. Therefore, complete responses to myeloid targeting CAR T cells are expected to require a rescue allogeneic stem cell transplant as a result of myeloablation [351].

Imaginative solutions to this problem have been sought, including the CRISPR-Cas9-mediated disruption of CD33 from allogeneic donor hematopoietic stem and progenitor cells (HSPC), which are engrafted in to AML patients prior to CD33 CAR T cell therapy. While this approach has been successful in primate models, and the deletion of CD33 has been shown to have no deleterious effects on the hematopoietic or immunological function of the graft [352], extensive research will be required before clinical trials can be conducted.

Limited clinical data is currently available for CD33-targeting CAR T cells although, one patient is reported to have a transient clinical response [353], and no anti-leukemic activity has yet been reported for CD123-targeting CAR T cells. Other antigens being considered to target malignant myeloid cells include Natural killer group 2D (NKG2D) ligands, which are expressed on malignant cells but have limited expression on healthy tissues. In early clinical trials of NKG2D targeting CAR T cells, persistence of the T cells was limited and one of the two treated patients achieved a clinical response [354], however the upregulation of NKG2D ligands on normal cells under conditions of cellular stress and inflammation might limit the use of this target antigen.

Unlike B cell malignancies, solid tumours rarely express one specific tumour antigen, which has limited CAR T cell usage against solid tumours thus far. In an attempt to recapitulate the success of CD19-directed CAR T cell therapy,

tumour associated antigens that are enriched on the tumour cell but expressed at low levels in normal tissue have been identified for solid tumours [178]. CAR T cells targeting a wide range of target antigens including HER2 [179], EGFR [180] and NY-ESO-1 [154, 181] have been developed and investigated in clinical trials, albeit to limited clinical success. In addition, a number of new antigens are being considered for CAR T cell therapy, including antigens that are highly expressed on a number of cancer types, and so might be effective against a number of malignancies. Tumour heterogeneity often results in poor antigen distribution through the solid tumour, and subsequent escape of antigen-negative variants [182, 183].

3. Concluding summary

CAR T cell therapy has demonstrated remarkable efficacy in the treatment of CD19⁺ B cell malignancies, where complete response rates are around 55%, leading to the approval of two CD19-targeting CAR T cell products. However, acquired resistance due to the development of antigen-negative clones is an emerging problem. In addition, the successes of CD19 CAR T cell therapy have not been recapitulated in the treatment of solid tumours, and this is believed to be due to poor CAR T cell trafficking and infiltration, the hostile tumour microenvironment and tumour antigen heterogeneity.

In this thesis, we identified the key tumour-intrinsic mechanisms that confer resistance against CAR T cell therapy, and importantly identified for the first time, that loss of *Cbfb* protects tumour cells against T cell-derived TNF. Secondly, we utilised the insights gained to design a rational combination therapy to overcome many of the challenges associated with CAR T cell therapy in solid tumours and demonstrated that smac-mimetics can be used to enhance CAR T cell therapy in this context. Finally, we investigated mechanisms of CD19 antigen loss in B-ALL in order to identify potential genes to target to prevent acquired resistance to CAR T cell therapy, forming the basis for future work.

Importantly, insights gained from the work in this thesis elucidate some of the resistance pathways utilised by tumour cells in order to evade immune

pressure from CAR T cells, highlight the cytotoxic potential of TNF in CAR T cell therapy and provide a novel strategy to enhance their efficacy in solid tumours. Taken together, this work addresses several of the existing limitations of CAR T cell therapy in both B cell leukaemias and against solid malignancies, and the novel combination therapy designed in Chapter 4 is poised to be rapidly translated in to the clinic.

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APPENDIX

1. Chromatin Immunoprecipitation and sequencing analysis associated with Chapter 3

1.1 Materials and methods

MC38 cells were treated with recombinant TNF (10 ng/mL) or left untreated for 2 hours. Cross-linking and immunoprecipitation was conducted as previously described [355]. Cells were harvested and cross-linked with 1% formaldehyde for 20 min at room temperature and quenched with the addition of 1.25 M glycine. Cells were washed three times (ice-cold PBS, 1400RPM, 5 min) and lysed in Ch-IP lysis buffer (20 mM Tris-HCl [pH8], 150 mM NaCl, 2 mM EDTA [pH8], 1% NP-40, 0.3% SDS in H₂O). Lysates were sonicated in a Covaris ultrasonicator to achieve an average estimated DNA fragment size of between 300–500 bp. Immunoprecipitation was performed in Ch-IP dilution buffer (20 mM Tris-HCl [pH8], 150 mM NaCl, 2 mM EDTA, 1% Triton-X, and protease inhibitors in H₂O) for at least 12 hr at 4°C with anti-*Cbfb* antibody (A303-549A, Bethyl Laboratories inc.). An equal volume of protein A and G magnetic beads (Life Technologies) were used to bind the antibody and associated chromatin, as previously described [356], and reverse cross-linking of DNA was performed by DNA purification using QIAquick PCR purification kits (QIAGEN), as per the manufacturer's instructions.

DNA was quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and indexed libraries were prepared using KAPA Hyper Prep Kit for Illumina platforms (Kapa Biosystems) and the SeqCap Adapter Kit (Roche) following vendor's instructions. Libraries were prepared with the Nugen Ovation Ultralow system (v2, using Nugen protocol M01379v3). Library quality and quantity was evaluated with a Bioanalyser D1000 Ch-IP (Agilent Technologies) and size selected between 200bp and 500bp using a Pippin Prep System (Sage Science). Final QC was done using a Tape Station High Sensitivity DNA Analysis Kit (Agilent Technologies). For sequencing analysis of immunoprecipitated DNA with anti-*Cbfb* antibody, libraries were then

pooled and sequenced on a HiSeq1500 (Illumina) and 13 to 18 million single-end 50 bp reads were generated per sample. CASAVA (v1.8.2) was used for demultiplexing. Data analysis was performed as previously described [357]. Fastq files generated by sequencing were aligned to the mouse reference genome (GRCm38/mm10) using bowtie (v2.2.3), and Samtools (v1.3) was used for manipulation of SAM and BAM files, after which MACS (v2.0.10) was used for peak calling. TDF files were generated using IGVTools (v2.3.72), and Ch-IP-Seq tracks were visualized using IGV (v2.3.55), and motif analysis was conducted by HOMER (v4.8.3).

1.2 Ch-IP sequencing suggests Runx1-specific transcriptional activity

In this Ch-IP-Seq experiment, we sought to identify any *Runx*-independent DNA binding of *Cbfb*, or alternatively to identify which *Runx* gene was responsible for the observed phenotype. We therefore treated MC38 cells with recombinant TNF and sought to identify *Cbfb*-specific DNA binding compared to an untreated control population. Peak calling analysis in untreated MC38 cells suggested low *cbfb* DNA binding at a steady state, however peaks associated with the transcription factor *Runx1* was identified. In the TNF-treated MC38 cells, an additional 49 genes were identified, including genes *Jun*, *Cited2* and *Myc*, which have been implicated in NF- κ B and TNF signalling, further highlighting the role of *Cbfb* in TNF signalling on a transcriptional level (Table 3). Given the low number of genes identified, and in both conditions *Runx1* DNA binding peaks were identified, we postulated that in this context, the observed binding peaks were actually as a result of *Runx1*-DNA binding (Figure 44).

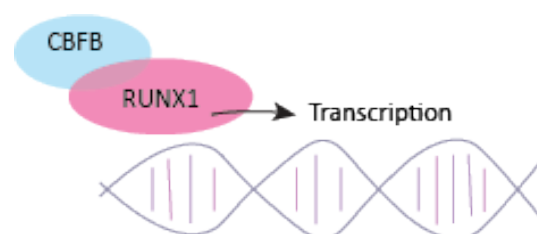
In order to interrogate this further, we conducted Homer Known Motif Analysis to identify the transcription factors associated with the DNA sequences pulled down. Here, we identified that the DNA sequences detected following a *Cbfb*-specific Ch-IP, we associated with *Runx1* DNA binding and transcriptional activity (Figure 44), leading to the conclusion that this phenotype is likely mediated by *Runx1*.

This work was confounded by technical challenges associated with conducting Ch-IP sequencing on co-binding factors, and therefore will need to be repeated in order to validate the results. A Ch-IP sequencing protocol utilising a longer formaldehyde cross-linking step is likely to be beneficial in this case. In addition, in order to entirely exclude the possibility of Cbfb-specific, Runx-independent transcriptional activity, a *Runx1*-specific Ch-IP seq would be useful.

Table 3. Genes associated with *Cbfb* DNA binding identified by Ch-IP seq peak calling

WT Untreated	WT TNF treated		
Vim	Ptma	Ndufs7	Clic5
Dnajc10	Frmd4a	Ckap4	Spdya
Lor	Vim	Phlda1	Fosl1
Dpyd	Dnajc10	Hmga2	Malat
Ctps	Cd44	Mir5100	Neat1
Cxcl1	Cabpb1	Smcr7	Ahnak
Ttc28	Lor	Stx8	
Ubc	Lamtor5	Aurkb	
Fam71f1	Abca4	Ccl2	
Cpa2	Cyr61	Vmp1	
Vmp1	3110043O21Rik	Tex14	
H3f3b	Cdk5rap2	Kansl1	
Cdh18	Nfib	H3f3b	
Scrib	Jun	Odc1	
Runx1	AV051173	Cmpk2	
Pgp	Prdx1	Galc	
Clic5	Cxcl1	Klf6	
Slc6a7	4930467D21Rik	Hist1h1e	
Neat1	Tgfbr3	Hist1h1c	
	Ubc	Cks2	
	Stard13	Etohd2	
	Fam71f1	Plk2	
	Ccdc77	Arhgef3	
	Kdm5a	Ghr	
	Itpr2	Cdh18	
	Cntnap4	Myc	
	Gse1	Asap1	
	Smad3	Scrib	
	Anxa2	Runx1	
	Cited2	Pgp	
	Bc1	Ler3	

A



B

Cbfb Ch-IP seq Homer Known Motif Enrichment			
Transcription Factor	Motif	p-value	Rank
RUNX1		1x10 ⁻²⁴	1
RUNX1		1x10 ⁻¹⁹	2
RUNX-AML		1x10 ⁻¹⁵	4

Figure 44. Proposed Runx1-dependency for the observed phenotype. (A) Schematic of proposed *cbfb*-facilitated Runx1-dependent transcription (B) Homer Known Enrichment Analysis of *Cbfb* Ch-IP sequencing.