Dual-specific Chimeric Antigen Receptor T Cells and an Indirect Vaccine against Pancreatic Cancer

By

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Submitted in total fulfillment of the requirements of the degree of

Doctor of Philosophy

March 2020

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ABSTRACT

Pancreatic cancer is one of the most aggressive malignancies with an overall 5-year survival rate of <7%. Pancreatic cancer is highly resistant to radiotherapy and chemotherapy, and surgery is not feasible in most patients. In this thesis, I developed a new form of treatment for pancreatic cancer, based on immunotherapy.

Adoptive cell transfer (ACT) is a promising form of cancer immunotherapy, which involves the isolation and reinfusion of tumour specific T lymphocytes into patients. While ACT can eliminate substantial burdens of some leukaemia, the ultimate challenge remains the eradication of large solid tumours and metastases for most cancers, including pancreatic cancer.

In this thesis, an enhanced ACT treatment strategy for pancreatic cancer was developed, which was termed 'ACTIV: Adoptive Cell Transfer Incorporating Vaccination'. This treatment included dual-specific T cells that expressed a chimeric antigen receptor (CAR) specific for the tumour antigen Her2, and a TCR specific for the melanocyte protein (pMEL, gp100). These dual specific T cells were termed 'CARaMEL T cells'. CARaMEL T cells were administered together with an injection of a recombinant vaccinia virus vaccine expressing gp100 (VV-gp100). We hypothesized that adoptively transferred CARaMEL T cells would proliferate mediated by their gp100 TCR, in response to the VV-gp100 vaccine, and kill $Her2^+$ tumours through their anti-Her2 CAR.

Functional assays performed *in vitro* indicated that murine CARaMEL T cells mediated antigen-specific cytokine secretion and killing abilities against pancreatic cancer cells, and demonstrated potent proliferative ability in response to gp100 antigen, confirming our hypothesis. In addition, I found that ACTIV therapy inhibited tumour growth and prolonged the survival of mice bearing $Her2^+$ subcutaneous murine pancreatic tumour. However, tumours usually relapsed after ACTIV therapy administration. Therefore, I directed my study to augment the anti-tumour activity of ACTIV therapy by the administration of either a histone deacetylase inhibitor (Panobinostat) or an immune agonist monoclonal antibody specific for CD40.

Panobinostat significantly suppressed the growth of pancreatic cancer cells *in vitro* through apoptosis and cell cycle arrest. Also, Panobinostat significantly increased the growth suppression of pancreatic cancer cells mediated by CARaMEL T cells. In addition, I found that

the combination of ACTIV therapy and Panobinostat significantly reduced the tumour growth and prolonged the survival of mice bearing $Her2^+$ subcutaneous murine pancreatic tumours.

In addition, administration of an agonist CD40 monoclonal antibody with ACTIV therapy significantly reduced the tumour growth and prolonged survival of mice bearing subcutaneous $Her2^+$ pancreatic tumours through a T-cell-dependent immune mechanism.

Finally, I explored the clinical translational potential for ACTIV therapy through the generation of human CARaMEL T cells expressing both a Her2-specific CAR and a gp100-TCR. *In vitro* functional assays indicated that human CARaMEL T cells mediated powerful and antigen-specific killing and cytokine secretion against Her2, together with a strong proliferative ability in response to gp100 antigen. In addition, I found that the administration of both human CARaMEL T cells and an adenovirus vaccine expressing gp100 led to potent anti-tumour activity against subcutaneous human $Her2^+$ pancreatic tumours in immunodeficient mice.

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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08 March 2020

PREFACE

Some of the work in this thesis has been published (Chapter 1 section 1.7 and 1.8).

I would like to acknowledge those who directly contributed to the work presented in this thesis:

• Clare Slaney, a Post-doctoral researcher, Cancer Immunotherapy Laboratory, Peter MacCallum Cancer Centre, for her contribution in Figure 4.2 of this thesis.

• Bianca von Scheidt, Research assistant, Cancer Immunotherapy Laboratory, Peter MacCallum Cancer Centre, for her contribution in the acquisition of data presented in Figure 4.10 in this thesis.

• Advanced Histology and Microscopy (CAHM) at Peter MacCallum Cancer Centre that contributed in paraffin embedding, sectioning and CC3 staining of tumour tissues Figure 4.9.

• All additional significant intellectual and/or technical contributions are duly noted by authorships in publication in Section 7, Chapter 1 of this thesis.

PUBLICATIONS

- B. von Scheidt, M. Wang, A. J. Oliver, J. D. Chan, M. K. Jana, **A. I. Ali**, F. Clow, J. D. Fraser, K. M. Quinn, P. K. Darcy, M. H. Kershaw, and C. Y. Slaney. *Enterotoxins can support CAR T cells against solid tumors.* **Proceedings of the National Academy of Sciences**, 2019, vol. 116, no. 50, pp. 25229-25235, doi: 10.1073/pnas.1904618116.

- **A. I. Ali**, A. J. Oliver, T. Samiei, J. D. Chan, M. H. Kershaw, and C. Y. Slaney. *Genetic Redirection of T cells for the Treatment of Pancreatic Cancer*. **Frontiers in Oncology**, 2019, vol. 9, p. 56.

- Slaney CY, von Scheidt B, Davenport AJ, Beavis PA, Westwood JA, Mardiana S, Tscharke DC, Ellis S, Prince HM, Trapani JA, Johnstone RW, Smyth MJ, Teng MW, **Ali A**, Yu Z, Rosenberg SA, Restifo NP, Neeson PJ, Darcy PK, Kershaw MH. *Dual-specific Chimeric Antigen Receptor T Cells and an Indirect Vaccine Eradicate a Variety of Large Solid Tumors in an Immunocompetent, Self-antigen Setting*. **Clinical Cancer Research**, 2017, 23(10); 2478-2490.

ACKNOWLEDGEMENTS

First and for most, I would like to offer my thanks, gratitude and obedience to Allah the great from whom I receive my help and guidance in my life. There are many people that I would like to acknowledge for their support during my PhD studies.

As my supervisors, A/P. Michael Kershaw, Prof. Phil Darcy, Prof. Ricky Johnstone and Dr. Clare Slaney have my sincerest gratitude. I would like to greatly thank you for your research guidance, unconditional support and mentorship on my PhD journey. I deeply appreciated your patience, welcoming attitude and scientific advice. I believe that PhD studies under your guidance helped me grow and develop a lot. I will always cherish the great opportunity to be a part of remarkable, intellectual and supportive environment that you provided.

Mike, thanks to give me this great opportunity. You taught me lots of lessons in this life and also in science. Thanks for everything you did for me.

Dr. Caroline Owen, thank you for your great and unconditional support in every single step during my PhD studies, you helped me a lot, you made things easier for me and you gave me lots of advice. I really appreciate this.

Dr. Karen Sheppard thank you for guidance and mentorship during my Ph.D. studies, it has been invaluable hearing your advice and experience.

Also, I would like to acknowledge all members of the Cancer Immunotherapy Research Laboratory, Jenny Westwood, Bianca Von Scheidt, Ashleigh Unsworth, Jack Chan, Tinaz Samiei, Amanda Oliver, Paul Beavis, Melissa Henderson, Sherly Mardiana, Junyun Lai, Imran House, Lauren Giuffrida, Kevin Sek, Kirsten Tod and Ryan Cross. Also, I would like to acknowledge Andrew Jefferies and Jason Brady. Indeed, thank you to all mentioned and other members of the Cancer Immunotherapy Research Laboratory. Your cooperation and assistance over the last four years have been immense and invaluable. A special mention to Bianca for easing me into the laboratory and her patience, assistance and willingness to help within the lab and with technical details.

Within Cancer Research, I would like to thank members from the animal facility (Michael Durrant, Stephanie Lewis Lee and Shellee Brown), FACS (Ralph Rossi, Viki Milovac and Sophie Curcio), microscopy and histology (A/Prof. Sarah Ellis, Metta Jana and Marne

Prinsloo) and media kitchen (Graham Heffernan and Lara Sekhon). A special mention to Michael Durrant who helped me many times in intravenous injections any time I asked him and taking care of my mice. Also, a special mention to Viki who was beside me during all my PhD stages and helped me in FACS experiments and supported me spiritually.

To all my friends outside of the Cancer Immunotherapy Research Laboratory (Jovana Gotovac, Valeria Arcucci, Sabrina Caiazzo, Ruofei Liu, Daniela Castiblanco, Shatha Abu-Hammad and Maria Joao Silva). Jovana my best friend in Melbourne, thanks for everything you made for me and hope this friendship will last forever.

Finally and most importantly, I would like to express my deepest thank you and dedication of this thesis to my wonderful family mom, dad, Waseem, Fairuz, Lubna, Kamilya, Mohammed, Shazshaz, Hamoud and all of your partners and nice kids. All of you were very supportive all the time. Thanks for everything. Dad thanks to call me almost every day to check on my progress. My wonderful mom, thanks for all your invaluable support. Also, you Fairuz thanks to take my early morning calls during my way to Peter Mac. Lubna thanks for coming to Australia to support me in my completion seminar and your delicious brownies. Faisal thanks for supporting me during my PhD journey day by day and minute by minute. Thank you for standing by me over the years of my PhD study. My beloved family your patience, unconditional support and love will forever be sincerely cherished.

LIST OF ABBREVIATIONS

°C	Degrees Celsius
3-MCA	3-methyl-cholanthrene
7AAD	7-amino-actinomycin D
Ab	Antibody
ACK	Ammonium-chloride-potassium
ACT	Adoptive cell therapy
ACTIV	Adoptive cell transfer incorporating vaccination
Ad	Adenovirus
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ATF	Activating transcription factor
Bcl-2	B-cell lymphoma 2
BM	Bone marrow
BrdU	Bromodeoxyuridine
CAHM	Centre for Advanced Histology and Microscopy
CAR	Chimeric antigen receptor
CARaMEL T cells	Dual-specific T cells express anti-Her2 CAR and pMEL-TCR
CARTmeso	cells mesothelin-specific CAR T cells
CCL	Chemokine C-C motif ligand
CCR	Chimeric costimulatory receptor
CD	Cluster of differentiation
CDKs	Cyclin-dependent kinases
CEA	Carcinoembryonic antigen
CETN1	Centrin1
c-FLIP	Cellular FLICE-like inhibitory protein
CLL	Chronic lymphocytic leukaemia
CPM	Mean counts per minute
CR	Complete response
CRISPR	Clustered regularly interspaced short palindromic repeats
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
CYT	Cytoplasmic domain
DCs	Dendritic cells
DISC	Death inducing signal complex
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EC	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
Fab	Fragment antigen-binding
FACS	Fluorescence activated cell sorter

FADD	FAS associated death domain
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FcR	Fragment crystallisable receptor
FDA	Food and drug administration
g	Gram
GM-CSF	Granulocyte macrophage colony stimulating factor
gp100	Human glycoprotein 100
GTP	Guanosine triphosphate
GVAX	(GM-CSF) gene-transfected tumour cell vaccine
GVHD	Graft versus host disease
Gy	Gray
H&E	Haematoxylin and eosin
HDAC	Histone deacetylases
HDACi	Inhibitors for histone deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her2	Human epidermal growth factor receptor 2
hgp100	Human glycoprotein 100
HLA	Human leukocyte antigen
HRP	Horseradish-peroxidase
iCAR	Inhibitory chimeric antigen receptor
ICOS	Inducible T cell costimulator
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide
IRES	Internal ribosomal entry site
ITAM	Immunoreceptor tyrosine-based activation motif
IU	International units
KO	Knock out
KPC	$Kras^{G12D}$; $Trp53^{R172H}$ Pdx1-Cre
LTR	Long terminal repeat
LTR	Long-terminal repeat
MCA	Methylcholanthrene
MDSC	Myeloid derived suppressor cell
MFI	Mean fluorescent intensity
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
mM	Millimolar
mmol/L	Millimoles Per Litre
mRNA	Messenger RNA
MSCV	Murine stem cell virus
MSLN	Mesothelin
MUC1	Mucin 1
MW	Molecular Weight
NaBu	Sodium butyrate
NBF	Neutral buffered formalin

NEAA	Non-essential amino acid
Neo	Neomycin
NF-κB	Nuclear factor kappa B
ng	Nanogram
NGFR	Nerve growth factor receptor
NK	Natural killer
NKG2D	Natural killer group 2D
NKT	Natural killer T
nm	Nanometre
ns	Not significant
NSCLC	Non-small cell lung cancer
NT mice	Non-treated mice
NT T cells	Non-transduced T cells
NY-ESO-1	New York oesophageal squamous cell carcinoma-1
OS	Overall survival
PANVAC-F	Fowlpox virus
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein-1
PD-L	Programmed death ligand
PFS	Progression free survival
pg	Picogram
pMEL	Melanocyte protein gp100
PMN	Polymorphonuclear
PMN MDSCs	Polymorphonuclear myeloid-derived suppressor cells
PR	Partial response
PRR	Pattern recognition receptor
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
RBCs	Red Blood cells
REP	Rapid expansion protocol
RIT	Radioimmunotherapy
RNA	Ribonucleic Acid
RPMI	Roswell park memorial institute
RT	Room temperature
SAHA	Suberoylanilide hydroxamic acid
scFv	Single chain variable fragment
SD	Stable disease
SEM	Standard error of the mean
TAA	Tumour-associated antigen
TAM	Tumour-associated macrophage
TCR	T cell receptor
TGF	Tumour growth factor
Th	T helper
TILs	Tumour-infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin domain containing molecule 3
TM	Transmembrane domain
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor

TRAIL	Tumour-necrosis factor-related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
TSA	Trichostatin A
VH	Variable heavy domain of antibody
VL	Variable light domain of antibody
VV	Vaccinia virus
WT1	Wilms' tumour 1
µCi	Microcurie
µg	Microgram
µL	Microlitre
µM	Micromolar

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CHAPTER 1: Literature Review

1.1. Introduction

Pancreatic cancer is one of the leading causes of cancer-related deaths in the world [1, 2] with a propensity for metastatic spread. It has a less than 5% five-year survival rate and is expected to be the second highest cause of deaths from cancer by 2030 [3-5]. Most pancreatic malignancies (>90%) are ductal adenocarcinomas, and are the focus in this thesis [6]. Pancreatic cancer is hard to detect in its early stages, because it develops in patients without early symptoms, leaving the patient with limited therapeutic approaches [5]. In the last decade, significant progress has been made in treatment options, but though the outcome has generally improved patients with the resectable form of the disease, there has been minimal progress in treating metastatic disease [6].

The standard of care for pancreatic cancer involves the use of systemic treatments, such as chemotherapy using gemcitabine, FOLFIRINOX (protocol 5-FU, Folinic Acid, Irinotecan, Oxaliplatin) or nab-paclitaxel [7, 8]. The use of the combined regimen, FOLFIRINOX, improved median survival up to 4 months compared to patients that received gemcitabine alone [9]. In addition, the median overall survival was increased by 1.8 months in patients that received gemcitabine with nab-paclitaxel compared with patients that received gemcitabine alone [10]. However, frequent toxic side effects of these chemotherapy regimens can lead to early termination of treatment [7, 9, 11]

Therefore, there is no therapeutic treatment with long-term benefits for advanced pancreatic adenocarcinoma [12-14], as it has high resistance to conventional chemo- and radio-therapy, and has a highly aggressive and immunosuppressive tumour microenvironment (TME) [13] composed of various immunosuppressive cell types including tumour-associated macrophages, and regulatory T cells (T_{reg}) and a paucity of effector T cells. Also, this TME is characterized by a dense desmoplastic stroma, which modulates the flow of blood and cells, and can hinder drug delivery [15].

The current treatment option that has been proven to work is the complete removal of the pancreas (pancreaticoduodenectomy) or part thereof. Pancreaticoduodenectomy has been demonstrated to extend survival of pancreatic cancer patients. The five-year survival rates of patients who had pancreaticoduodenectomy is 25% for node-negative and 10% for node-positive disease [16]. However, pancreaticoduodenectomy is associated with various digestive

complications [17] and does not suit patients with a metastatic form, which comprise > 80% of the patients [4, 6, 18]. In addition, the majority of patients end up relapsing, which reduces the numbers of long-term survivors. For this reason, there is an urgent need to develop novel and better therapeutic approaches to increase the survival rate and duration which has not changed dramatically in decades [6].

Immunotherapy is one of the most promising approaches in treating cancer, which has been under investigation for many cancer types [19]. Immunotherapy helps the body's immune system find and fight the cancer cells [20]. Immunotherapy is potentially safer than other standard treatments such as chemotherapy with fewer side effects. Specificity of the immune system can be enabled by the exquisite ability of T cells to respond specifically against tumour antigens. These can be classified as tumour-specific antigens, expressed exclusively by cancer cells, or tumour-associated antigens (TAAs), which are over-expressed by cancer cells but could be expressed at lower levels on some normal tissues [21]. A range of immunotherapies are in development for pancreatic cancer, including vaccines, immune checkpoint inhibitors and adoptive cell transfer, which are described in detail in the following pages.

1.2. Vaccines

One of the most clinically advanced form of immunotherapy for pancreatic cancer is the use of cancer vaccines [22]. This approach aims to induce the immune system against tumour cells by generating both cellular and humoral immune responses [19]. There are many approaches to deliver anticancer vaccines such as whole-cell vaccines, peptide vaccines, dendritic cell vaccines, Listeria-based vaccines, and recombinant virus-based vaccines.

1.2.1. Whole-cell vaccines

Vaccine therapy relies on the biological administration of antigens expressed by malignant cells that improve the immune system's ability to fight neoplastic cells [23, 24]. Whole-cell vaccines involve the vaccination of patients with tumour cells. One vaccine can target multiple tumour antigens at once, express epitopes for $CD8^+$ and $CD4^+$ T cells and induce antigen specific T cell immune responses to more than one tumour antigen [25-29]. Cancer cells can express neoantigens that result from genetic mutation during the oncogenic process. Many studies have indicated that T cell responses against neoantigens can be observed in a variety of cancers including melanoma, colorectal cancer, squamous cell carcinoma of the head and neck

and lung cancer [30-37]. Neoantigen-based vaccines are demonstrating promising results in a range of cancers including melanoma and glioblastoma [38-41]. It has been found that allogeneic cell lines are more attractive than autologous cell lines as vaccines because they overcome some difficulties in harvesting cancer cells such as the low number of surgical candidates and long culture time, although their effectiveness relies on the existence of shared antigens [25].

1.2.1.1. Algenpantucel-L Vaccine

Algenpantucel-L vaccine is a whole-cell vaccine that consists of irradiated allogeneic pancreatic cancer cells which are engineered to express the murine enzyme α-(1,3)-galactosyltransferase (αGT) [42, 43]. The αGal epitopes are not expressed in the human body [44] however, anti-αGal antibodies are spontaneously produced in response to enterobacteria invasion and the immune system is not tolerant to it and produce large amounts of antibodies targeting αGal epitope [45]. For that reason, this vaccine utilizes this natural immune response to provide an adjuvant to develop an anti-tumour response against pancreatic cancer [42].

In a phase II, open label, multi-institutional, dose-finding clinical trial, investigating Algenpantucel-L combined with chemotherapy and radiotherapy for 70 resected pancreatic cancer patients, disease-free survival (DFS) of 12 months was 62-86%. A dose effect relationship was observed, with disease-free survival rate of 12 months for those patients receiving 300 million cells being 81% comparing to 51% for those given 100 million cells. Also, 12-month overall survival (OS) rate of all patients was 86% (96% at 300 million cell-dose and 79% at 100 million cell-dose). An increase in anti–α gal antibodies was demonstrated in 90% of patients which lasted over 200 days. Also, anti-mesothelin and anti-carcinoembryonic TAAs antibodies were both elevated [46].

Although phase II results have been encouraging; in May of 2016, Newlink Genetics Corporation released its results for phase three of their clinical trial using algenpantucel-L vaccine in patients with resected pancreatic cancer. In the trial, the patients received 300 million cells every two weeks for six months and once every month for another six months. However, the researchers found that the OS was 29.3 months from the time of randomization, which was significantly lower than the anticipated results. The trial showed that algenpantucel-L vaccine was not a significant advance in pancreatic cancer immunotherapy [47].

1.2.1.2. GM-CSF-adjuvanted vaccine

GM-CSF is an inflammatory cytokine [48] that regulates the survival and differentiation of granulocytes and macrophages [49]. GM-CSF is known to facilitate vaccine efficacy through developing and prolonging cellular and humoral immunity [50]. This potent immune stimulatory cytokine is released by macrophages, NK cells, T cells, B cells, mast cells, endothelial cells and fibroblasts in response an inflammatory stimuli [51]. GM-CSF functions to stimulate stem cells to differentiate into monocytes and granulocytes to participate in a robust immune response and cytolytic activity against cancer cells [52]. It also effectively stimulates the production of dendritic cells from bone marrow, which process and present TAAs delivered by the vaccine [53]. The theory of this type of vaccine is that specific cytokines can prime and initiate tumour-specific immunity [54]. GVAX is an allogenic irradiated whole-cell cancer vaccine genetically modified to secrete GM-CSF and deliver TAAs [55].

A phase I clinical trial of GVAX was conducted to study efficacy and safety of this vaccine on 14 resectable pancreatic cancer patients. Eight weeks after surgical removal of the pancreas, GVAX was administered to patients before and after chemo-radiation. The mean DFS was 13 months. Three of the 14 patients showed delayed type hypersensitivity (DTH) and were disease-free from for at least 25 months. It was demonstrated that 50×10^7 GM-CSF secreting cells was considered as the safest dose with few side effects and an enhanced immune response against the tumour [56].

These promising results led to phase II trials of GVAX together with cyclophosphamide in 50 advanced pancreatic cancer patients who did not respond to gemcitabine. Although there was no statistical significance of 1-year and OS rates between the two groups, the study demonstrated enhanced immune responses against tumours in the combined group where cyclophosphamide was administered to patients before GVAX [54] . The results of this study suggested additional investigation of cyclophosphamide modulated immunotherapy in advanced pancreatic cancer patients.

In another phase II clinical trial conducted on 60 patients with resected pancreatic cancer, patients received 5×10^8 GM-CSF-secreting cells in three lymph node regions eight to 10 weeks after surgery followed by Fluorouracil (5FU)-based chemo-radiation. Disease-free patients received another 2-4 additional treatments, each one month apart. Final booster treatments were administered six months after the 4^{th} vaccine treatment. The median DFS and OS were 17.3 months and 24.8 months respectively. It also showed a relation between

induction of mesothelin-specific $CD8^+$ T-cell responses and higher OS suggesting a prognostic importance [57].

1.2.2. Peptide vaccine

Tumour cells express one or more tumour antigen peptide amino acid sequences, which can be combined with an adjuvant to induce tumour-specific responses by $CD8^+$ cytotoxic T lymphocytes (CTL) [58]. These peptide-based vaccines are simple, cheaper than other vaccines and safe [59]. However, alone they have minimal immunogenic potential and limited ability to induce clinical tumour regressions especially in patients with advanced cancer [60, 61]. However, peptide-based vaccines can be combined with adjuvants and other systemic therapies including immune checkpoint blockade to improve therapy [59].

1.2.2.1. K-Ras vaccine

K-Ras is a guanosine triphosphate (GTP)-binding protein that regulates cell growth and survival [62]. *KRAS* is mutated in more than 90% of pancreatic cancer patients [63, 64]. A K-Ras vaccine was the first peptide-based vaccine that was used in pancreatic cancer patients [64]. The initial studies demonstrated safety and transient efficacy of K-Ras vaccine in humans [65]. However, this efficacy had little clinical benefit and did not induce a consistent immune response against this antigen.

Therefore, researchers tried to enhance the immune response to K-Ras peptide vaccine through combining with GM-CSF as an adjuvant in a phase I/II study. Forty-eight pancreatic cancer patients (10 of the 48 patients underwent surgical resection and 38 were with advanced disease) received intradermal synthetic mutant Ras peptides in combination with GM-CSF. The vaccine was well-tolerated in all patients. A peptide-specific immune response was demonstrated in 58% of patients, and their survival rate increased significantly compared with non-responders (median survival 148 days vs. 61 days, respectively). Of the 10 resected patients, nine were stable with 25.6 months mean OS and 11 of 34 patients with advanced unresectable disease were stable for a median of 10.2 months. The study claimed a significant association between prolonged survival rate and the vaccine specific-immune response suggesting a clinical benefit of Ras vaccine in pancreatic cancer patients even with end-stage disease [66].

Another previous study included 24 resected pancreatic cancer patients with *KRAS* mutations were administrated a vaccine of the *KRAS* mutation corresponding to the patient's tumour on day seven of a 10-day course of intradermal GM-CSF and repeated monthly up to three months.

Nine patients (25%) were immune responders [67]. Median recurrence free survival time and median overall survival time were 8.6 and 20.3 months, respectively.

A recent study investigated the safety and efficacy of TG01 (a mixture of seven RAS peptides) to stimulate the response of RAS mutant-specific T-cells [68]. TG01 and GM-CSF were co-administered in 19 surgically resected pancreatic cancer patients who also received gemcitabine. The patient's immune response was investigated using T-cell proliferation and antigen-specific (TG01) delayed-type hypersensitivity. The TG01/GM-CSF regimen induced an immune response in 84% and was well tolerated with some manageable allergic reactions. The median OS was found to be 33.1 months with OS rate at one and two years were 89.5% and 68.4% respectively.

Overall, K-Ras peptide vaccines have been shown to be safe in pancreatic cancer patients and the results could be promising, however, their clinical efficacy still needs to be verified in larger studies.

1.2.2.2. Cocktail Vaccines

Pancreatic cancer express a number of mutations and different antigens that can induce specific B and T cell, thereby providing a number of antigen targets that could be used simultaneously in a “cocktail” vaccine [69-71]. A phase II clinical trial performed by Miyazawa et al. (2016) [72] investigated a peptide cocktail vaccine OCV-C01, which is derived from a mixture targeting the antigen KIF20A and the angiogenic receptors vascular endothelial growth factor receptor (VEGFR) 1, and VEGFR2. This was combined with gemcitabine in 30 patients with pancreatic ductal carcinoma who had undergone surgical resection. The median DFS was 15.8 months and median OS was not reached but its rate at 18 months was 69.0%. The patients responded relatively well to the treatment with the results being better for patients who tested positive for a cytotoxic T lymphocyte (CTL) response specific for peptide KIF20A. All four patients who underwent complete resection did not experience pancreatic cancer recurrence during the reporting period.

In addition to these antigenic targets, a range of other studies have been performed. Other peptide vaccine types that were combined with chemotherapeutic drugs or with GM-CSF in phase I or II clinical trials in pancreatic cancer patients are telomerase [73, 74], mucin-1 [75, 76], anti-VEGFR [77], antigastrin-17 [78] and heat shock protein–peptide complex vaccine [79]. In summary, a range of studies have demonstrated that peptide vaccines could be useful

in managing pancreatic cancer, especially in resected cases and where treatment options are limited. However, further research is necessary with cancer at various stages to determine the effectiveness in the wider pancreatic cancer patient population.

1.2.3. Dendritic cell vaccine

Dendritic cells (DCs) are considered to be the strongest antigen presenting cell (APC) type, with a unique ability to process and present antigens, in the context of MHC, on their surface to prime antigen-specific T-cell responses [80]. For that reason, DCs have proven very helpful in studies aimed at creating effective cancer treatment strategies. DC vaccines are designed to present TAAs such as peptides to stimulate the immune system, or can be engineered to express specific TAA or pulsed with tumour cell extracts [81-83].

In DCs-based vaccines, DCs are taken from the patient, loaded with TAAs and then re-infused back into the patient. Inside the patient's blood, these DCs circulate and reach the lymph nodes where they present the tumour antigens to immune cells in combination with appropriate co-stimulatory signals [81]. In a phase I trial, DCs were collected from seven patients with advanced pancreatic cancer and loaded with a mucin 1 (MUC1)-peptide. Patients were intradermally injected with $3\text{-}6 \times 10^6$ DCs for three to four doses. No grade three or four toxicities were associated with the vaccine. In two of seven patients, PBMCs showed significant antigen-specific production of interferon (IFN)-γ and Granzyme B [84]. In another phase I/II trial; autologous DCs were pulsed with a mucin-derived peptide in 10 resected pancreatic and two biliary cancer patients (total=12). The vaccine was well tolerated in patients and four patients were alive for > four years without evidence of recurrence [85].

Although responses are usually limited in patients with metastatic disease, results have been better when DC vaccines are combined with other forms of cancer treatment [86]. Another study was performed on three patients with resected pancreatic cancer following neoadjuvant chemo-radiotherapy, used autologous monocyte-derived DCs pulsed with CEA-encoding mRNA. The results showed that all the three patients were disease free for more than 2.5 years from the original diagnosis. The study concluded that immunotherapy using DC-based vaccination is well-tolerated, convenient and may lead to higher survival rates [87].

A recent study evaluated the efficacy of DC vaccines when used with chemotherapy [88]. The researchers used Wilms' tumour 1 (WT1), which is an antigen highly present in pancreatic cancer cells [89], to investigate the safety of WT1 peptide-pulsed DC (WT1-DC) combined

with chemotherapy in resected pancreatic cancer patients. Immunohistochemical analysis for WT-1 was conducted in 34 cases of pancreatic cancer. There were no severe side effects except grade I fever in five patients. The researchers observed cytotoxic T-cells that were WT-1 specific in eight patients, and WT1 and human leukocyte antigen (HLA) class I antigens were positive in all patients. This phase I clinical study proved DC vaccines to be effective in inducing WT-1 specific T-cells in surgically resected pancreatic cancer patients. However, a clinical trial has to be conducted to determine the viability of this treatment strategy [88].

In a more recent study, conducted by Hanada *et al*. [90], six pancreatic cancer patients were selected to undergo multimodal therapy consisting of DCs pulsed with WT1 peptide, chemotherapy, radiotherapy and/or surgery. WT1-specific immune response and DC/WT1-I specific DTH reactions were analyzed to evaluate the prognostic markers of the therapy. The researchers found that multimodal therapy consisting of 3-4 types of treatment lead to longer survival rates. Patients with the survival rate of > 1000 days were shown to have more lymphocytes after the DC/WT1-I than those with <1000 days, although the specificity of those lymphocytes was not determined. Based on the findings, the DC/WT1-I was demonstrated to be beneficial with advanced pancreatic cancer cases. However, one limitation to the study is comparing the relatively small sample number, at different stages of pancreatic cancer receiving different treatments, to a general population of pancreatic cancer patients. For that reason, further studies need to be conducted on the viability and benefits of multimodal therapy on pancreatic cancer patients.

1.2.4. Vaccines with microorganisms

1.2.4.1. Recombinant bacterial vaccine

Listeria monocytogenes is a Gram-negative bacterium responsible for several types of foodborne infections [91]. It can internalize efficiently into host cells. When *Listeria. monocytogenes* is inside the cells in phagosomes, they secrete variety of virulence factors, including Listeriolysin O and phospholipase C enzymes, which transform the phagosome to a larger compartment enhancing its replication and escape into the cytoplasm. After that, L. monocytogenes spreads to other cells with no extracellular phase due to its actin-based motility [92]. The antigens of this bacterium are processed through MHC I resulting in priming and stimulation of specific $CD8^+$ T cells [93], which results in strong immunological responses [93]. A range of bacterial-based vector vaccines, including *Salmonella, Shigella, Lactococcus, Listeria,* and *Mycobacterium,* are used as anticancer or anti-infection vaccine vectors [92, 94].

Immunotherapy involving *Listeria monocytogenes*-based vaccination uses live attenuated *L. monocytogenes* bacteria as a vehicle to deliver TAAs to the APCs to induce an immune response against tumours [94].

CRS-207 is a recombinant Listeria-based live-attenuated vaccine with deleted virulence genes and mesothelin expression. Two phase I clinical trials used CRS-207 and ANZ-100 (another live attenuated Listeria-based vaccine with double deleted virulence genes *actA* and *internalin B* (*InlB*) *but without* mesothelin expression. ANZ-100 can activate and recruit both innate and adaptive effector cells when it is taken up by phagocytic cells in the liver and spleen) in patients with mesothelin-expressing cancers and liver metastases respectively. Administration of either ANZ-100 or CRS-207 was safe and induced immune activation of mesothelin-specific T-cell responses and cytokine/chemokine induction at all doses of CRS-207 whereas ANZ-100 induction of chemokines was dose-dependent. Although the study showed no significant survival benefits, immune responses were observed for both ANZ-100 and CRS-207 [95].

A Phase II clinical trial aimed to evaluate the safety and survival benefits of combined CRS-207 with GVAX and low-dose cyclophosphamide in 90 previously treated metastatic pancreatic adenocarcinoma patients [96]. GVAX administered with cyclophosphamide was used as a potential inhibitor for Treg cells. This approach elicited innate and adaptive immune response and resulted in OS of 6.1 months in patients receiving cyclophosphamide/GVAX and CRS-207 versus 3.9 months in patients received cyclophosphamide/GVAX only. The longer OS rate was associated with an enhanced specific $CD8^+$ immune response against mesothelin.

However, in their recent three-arm, randomized, controlled phase IIb trial (ClinicalTrials.gov ID: NCT02004262) they investigated CyGVAX + CRS-207, CRS-207 alone, and standard chemotherapy. Patients were divided into three groups in 1:1:1 ratio into: cyclophosphamide/GVAX + CRS-207 (arm A), CRS-207 (arm B), or physician's choice of single-agent chemotherapy (arm C). Median OS for arm A, B, and C were 3.7, 5.4 and 4.6 months respectively. They all fell short of the anticipated OS of 6.1 months. Chills, nausea, fatigue and pyrexia were the most reported effects of the treatment. The results did not deviate from that of chemotherapy hence did not hold up as a suitable therapy for pancreatic cancer. Therefore, the combination of cyclophosphamide/GVAX + CRS-207 did not improve the survival rate of pancreatic cancer patients over chemotherapy [97] .

IMM-101 is a suspension of a heat-killed whole cell vaccine of *Mycobacterium obuense*. IMM-101 can activate innate and adaptive effector immune cells that have cytotoxic effects against

tumours. Also, IMM-101 may upregulate Type 1 responses, which are important in pancreatic cancer that has been associated with a reduced cytotoxic Th2 bias [98]. In a multicenter, randomized, open-label phase II clinical trial, 110 patients with advanced pancreatic cancer assigned to receive IMM-101 in combination with gemcitabine (75 patients) or gemcitabine alone (35 patients) as a first line treatment. Both the median OS and the median and progression free survival (PFS) were significantly increased by 29% and 83% respectively in the (IMM-101 and Gemcitabine) compared with gemcitabine alone. Adverse effects including asthenia and abdominal pain occurred in 10.8% and 8.1% of the patients, respectively [99]. Also, in a follow up study to monitor the long-term survival rate, stage IV pancreatic cancer patients showed the highest survival benefit: OS increased up to seven months for patients received IMM-101 (1-year OS 22.4%) and Gemcitabine vs. 4.4 months for patients received gemcitabine alone (1-year OS 11.5%) [100].

1.2.4.2. Viral-based vaccines

A phase I clinical trial was carried out on 10 patients with advanced pancreatic cancer. They were vaccinated with (PANVAC-V) vaccinia virus expressing CEA and MUC-1 peptides (as an antigenic targets for the immune system) in addition to the co-stimulatory molecules B7.1, LFA-3, and ICAM-1. The vaccinations were given concurrently with GM-CSF as an adjuvant and was followed by three booster heterologous vaccinations using fowlpox virus (PANVAC-F) expressing the same antigens. Researchers demonstrated an antibody response to vaccinia virus in all patients and a significant increase in CEA- and/or MUC-1-specific immune responses in five out of eight evaluable patients (62.5%). The median OS for all patients was 6.3 months with significantly higher overall survival rate in the patients who showed a vaccine-specific T cell response (15.1 months) compared with non-responders (3.9 months) [101].

In summary, some clinical trials of different types of vaccines against pancreatic cancer showed some stimulated T cells anti-tumour activity. However, most studies showed little or no clinical benefit, and none of these approaches have yet been implemented as standard care in the clinic [102].

1.3. Immune checkpoint inhibitors

Immune checkpoint receptors are negative immunoregulatory molecules that have an important role in the balance of tumour immunity and inflammatory reactions [103]. Immune checkpoint molecules programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4), whose ligands are programmed death ligand 1 and 2 (PD-L1 and PD-L2)

and cluster of differentiation 80 (CD80) and CD86 respectively. PD-1 and CTLA-4, are inhibitory receptors that down-regulate T-cell activation to promote tolerance to limit damage of healthy tissue [104-106]. PD-L1 is a protein that can be found on some normal and cancer cells. CTLA-4 or PD-1 normally bind with their ligands CD80 and CD86 and PD-L1 respectively on APCs [107-109]. Some cancer cells, including pancreatic cancer, express the immune checkpoint ligand PD-L1, which in turn helps them to suppress immune cells and evade their immune attack [110]. Immune checkpoint inhibitors that have been approved by FDA include anti-CTLA-4 (ipilimumab and tremelimumab), anti-PD-1 (nivolumab, pembrolizumab and pidilizumab), and anti-PD-L1 (atezolizumab (MPDL3280A) and MDX-1105) [111, 112]. Immune checkpoint blockade antibodies showed highly significant benefits in several types of cancer including lung, melanoma and renal cancers [113-115]. Unfortunately, the use of checkpoint inhibitors in pancreatic cancer has shown relatively disappointing clinical benefits [116, 117]. The low clinical benefits are attributed to the low immunogenicity [118, 119] and immunosuppressive tumour microenvironment, together with a prominent desmoplastic reaction (fibrosis) that inhibits immune cell accumulation in the tumours [119, 120].

1.3.1. PD-1/PD-L1

A preclinical study assessed the expression of PD-L1 expression in tumour samples from 51 surgical resected pancreatic cancer patients and evaluated the efficacy of blocking the PD-L1/PD-1 pathway in a mouse tumour transplant model. They found that PD-L1 (but not PD-L2) positive patients had a relatively poorer prognosis than PD-L1 negative patients. They also found that the anti-tumour effects were improved when gemcitabine is combined with PD-1 or PD-L1 blockade [121]. The researchers concluded that PD-L1 could play a crucial role in the prognosis of pancreatic cancer, hence encouraging the development of novel therapies that target the PD-1/PD-L1 pathway.

A phase I clinical trial assessed safety and potential anti-tumour activity of pembrolizumab in 32 patients, including only one pancreatic cancer patient. Pembrolizumab was well tolerated with strong anti-tumour activity in multiple solid tumours. The pancreatic cancer was stabilized as its best response in this patient [122]. Moreover, a phase I clinical trial involved 14 pancreatic cancer patients among 207 different cancer patients; anti-PD-L1 antibody was administered intravenously to evaluate the clinical evidence about the effects of anti-PD-L1 in

advanced cancers. No objective responses found in patients with pancreatic cancer compared to other cancer types [123].

1.3.2. CTLA-4

Several studies were performed on pancreatic cancer patients using anti-CTLA-4 agents. In a phase Ib, multisite, nonrandomized dose escalation trial Aglietta *et al*., assessed the safety and tolerability of gemcitabine with at least one dose of tremelimumab in 34 patients with metastatic pancreatic cancer. This combined treatment was safe and tolerable. The median overall survival was 7.4 months and at the end of treatment, although not designed to determine efficacy, two patients achieved partial response [124].

A phase II clinical trial was conducted by Royal *et al*. [125] to determine the efficacy of Ipilimumab in advanced cases of pancreatic cancer. The subjects were 27 adults with metastatic pancreatic adenocarcinoma, or locally advanced cases, with minimal comorbidities. Grade III or higher of immune-mediated adverse events occurred in three patients while no response was observed in solid tumour evaluation criteria. There were no responders by response evaluation criteria in solid tumour criteria, but one patient experienced a delayed response after initial progressive disease. Therefore, the single agent Ipilimumab was ineffective as it did not reveal any significant improvement in survival or outcome in patients with advanced pancreatic cancer, but it may be effective in combination with other immunotherapies or other treatments.

1.3.3. Combined therapy (vaccines and checkpoint inhibitors)

Combining vaccines and immune checkpoint blockade can provide another therapeutic strategy against pancreatic cancer. For example, a previous preclinical study combined PD-1- or PD-L1-targeted antibody therapy with an immune agonist for pancreatic cancer treatment. This combination enhanced mouse survival compared with PD-1 antibody or GVAX therapy alone [126].

In 2013, 30 patients with advanced pancreatic ductal adenocarcinoma [127] were treated with ipilimumab (Arm 1, n=15) and the other half got ipilimumab + GVAX vaccine (Arm 2, n=15). Two patients in the first group showed evidence of stable disease. Three patients in the second group had evidence of prolonged disease stabilization. The median OS was (3.6 vs. 5.7 months) and one-year OS (7 vs. 27%) for the second Arm. 20% of patients in both arms showed ≥ grade III adverse events. This trial concluded that checkpoint blockade in combination with GVAX

vaccine can afford a clinical benefit, but further studies in a bigger population are required to confirm the findings.

In addition, another study was conducted on 25 metastatic pancreatic cancer patients treated with ipilimumab, targeting CTLA-4, +/- GVAX in addition to another 32 patients treated with nivolumab, targeting PD-1, +/- GVAX and CRS-207. Results showed high diversity in the levels of T cell receptor repertoires in peripheral blood with higher changes in the group who received ipilimumab in combination with GVAX. These changes were consistent with the mechanism of action of checkpoint blockade. Moreover, the patients who received ipilimumab showed significantly longer survival, but not the patients who received nivolumab [128].

To summarize, even though therapy targeting immune checkpoint blockers such as PD-1, PD-L1 and CTAL-4 showed significant enhancement for the treatment of other tumour types, checkpoint inhibitors have failed to demonstrate objective responses or significant anti-tumour activity when given as single agents to pancreatic cancer patients This may be due to an immunosuppressive TME and the poor immunogenicity of pancreatic cancer.

1.4. Targeting the pancreatic tumour microenvironment

Desmoplastic stromal reaction in pancreatic tumours promotes tumour growth and creates a physical barrier for T cell infiltration and drugs [129]. Therapies targeting the desmoplastic stroma and the suppressive TME can broaden the clinical application of immunotherapy and provide an advantage in the management of aggressive pancreatic cancer.

1.4.1. CD40 monoclonal antibodies

CD40 is member of the tumour necrosis factor receptor (TNFR) super family, and it is expressed on the cell surface of immune cells especially B cells, dendritic cells and macrophages. CD154 is the CD40 primary ligand that is present mainly on the surface of activated $CD4^+$ T cells [130, 131]. CD40 ligation activates APCs thereby promoting anti-tumour T-cell responses in addition to cytokines release, macrophage reactivation and infiltration into the tumour stroma. Hence, targeting the CD40-CD154 pathway can reverse an immunosuppressive tumour microenvironment and induce anti-tumour immunity [132-135].

In some tumour models, administration of an αCD40 antibody, including pancreatic cancer, resulted in T cell–dependent [132, 136-139] and T-cell-independent (macrophage-mediated) [132, 138, 140] mechanisms of tumour regression [141]. Preclinical studies showed the role of

CD40 activation in driving anti-tumour immunity, whereby CD40-activated DCs are poised to prime or activate tumour -specific CD4-independent $CD8^+$ T cells [135, 142-148]. Ligation of CD40 on DC, for example, induces production of pro-inflammatory cytokines and upregulated surface expression of molecules that can enhance antigen presentation and $CD8^+$ T cell triggering such as MHC molecules, immunoglobulin superfamily costimulatory molecules, and other TNF superfamily ligands [131, 135]. CD40 agonists can induce direct tumour killing through macrophage-mediated mechanisms [141, 149, 150].

Macrophage-mediated anti-tumour immune mechanisms have been associated with the destruction of pancreatic tumour stroma [132, 151-153] through recalibration of macrophages from M2 (pro-tumour) to M1 (anti-tumour) status [140, 154] via inhibition of the NF-κB pathway [155] or involvement of IFN-γ and CCL2 [156]. Therefore, several CD40 agonist antibodies of varying formulations were found to mimic the signal of CD40 ligand and studies have supported their tolerance and efficacy [131].

Vonderheide *et al.*, conducted a study on patients with advanced solid tumours, aiming to determine safety and the maximum-tolerated dose of CP-870,893 (a fully human CD40 agonist monoclonal antibody), as well as an assessment of its tumour modulation. The patients received intravenous single doses of CP-870,893. Results showed that CP-870,893 was well-tolerated and associated with an anti-tumour response. The most common observed adverse event was cytokine release syndrome, which could be easily managed. Also, other transient laboratory abnormalities were demonstrated after 24-48 hours of antibody administration which including decreased platelets, monocytes, lymphocytes, and elevated serum liver enzymes [157].

Administration of chemotherapy may cooperate with αCD40 antibody by releasing tumour antigens, and thus functioning as a vaccine approach [139, 158]. In a previous phase I clinical trial combined gemcitabine and CP-870,893 in 22 advanced pancreatic cancer patients, the combination was well-tolerated, and four patients reached a partial response. Also, the overall response rate tumour was 19% based on the response evaluation criteria in solid tumours (RECIST 1.0). However, due to the small sample size, researchers could not define the clinical benefit of this combination [159].

In a recent phase Ib trial, where metastatic pancreatic cancer patients received gemcitabine and nab-paclitaxel chemotherapies plus αCD40 antibody (APX005M), with or without PD-1 monoclonal antibody (nivolumab). Among 24 dose-limiting toxicity-evaluable population 58%

of the patients had partial responses, 33% patients had stable disease, 4% had progressive disease, and 4% had no treatment evaluation [160]. A randomized phase II study combining gemcitabine/nab-paclitaxel with or without APX005M and with or without nivolumab as first-line treatment for metastatic pancreatic cancer is in progress [131].

In conclusion, to overcome the immunosuppressive barriers of pancreatic cancer, coordination between different therapeutic approaches is needed. The best results of administration αCD40 monoclonal antibody as a single agent were minimal objective tumour response rates. However, in preclinical pancreatic cancer models the most effective combination treatment of CD40 was in combination with chemotherapy and checkpoint blockade [131, 161], which have also been translated into some clinical trials for patients with metastatic pancreatic cancer [131]. The significant roles of CD40 agonist antibodies are undeniable but advanced phases of clinical trials are warranted to determine the optimal combination for clinical use.

1.4.2. Inhibitors for histone deacetylases (HDACi)

Modifications of gene expression are one of the main characteristic of cancer cells [162]. Histone deacetylases (HDAC) are enzymes that remove acetyl groups from histone and nonhistone proteins to regulate gene expression and repress their transcription [163, 164]. HDAC classes 1, 2, 3 and 7 are overexpressed in pancreatic cancer cells, which have a transcriptional profile increasing cell survival and proliferation [165-169].

Inhibitors for histone deacetylases (HDACi) are a novel therapeutic approach in cancer therapy which is being used in many clinical trials [170]. HDACi can acetylate histones resulting in an open chromatin structure and enhanced gene transcription [162, 171, 172]. Depending on the chemical structure, HDACi can be grouped into different classes including hydroxamic acids such as LBH589 (Panobinostat), suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), and PXD101 (Belinostat); cyclic tetrapeptides, such as Trapoxin and Apicidin; short chain fatty acids, such as sodium butyrate (NaBu) and valproic acid; benzamides, such as MS-275, CI-994 and MGCD0103, and other chemical compounds and synthetic inhibitors [173]. Among these HDACi Panobinostat (LBH589, Novartis Pharmaceuticals) is a potent inhibitor at low nanomolar concentrations across different cancer types [174-179].

A previous preclinical study showed that Panobinostat (LBH589) induced apoptosis in pancreatic cancer cells and significantly reduced tumour growth in a subcutaneous xenograft mouse model as efficiently as gemcitabine [180]. In another pancreatic xenograft model,

Panobinostat inhibited tumour growth compared with Paclitaxel. However, combination therapy resulted in a superior anti-tumour effect than either single agent, leading to 20% tumour regression [181].

Moreover, HDACi have been shown to modulate immunogenicity and improve anti-tumour immune responses [182, 183]. $CD8^+$ T cells have an important role in an effective anti-tumour immunity [184]. Many studies suggested that HDACi can enhance the activation, cytotoxicity, memory function, responsiveness and pro-inflammatory cytokine secretion of $CD8^+$ T cells [185-188]. A previous study showed that Panobinostat enhanced gp100 specific T cell survival and decreased the tumour burden, in a B16 melanoma model, through improving the proliferation, functional status and survival of adoptively transferred $CD8^+$ gp100 specific T cells [187].

Therefore, there is a strong rationale that further studies should be performed using HDACi combined with other approaches against pancreatic cancer.

1.5. Radioimmunotherapy (RIT)

Radioimmunotherapy (RIT) aims to safely provide a high-radiation dose to cancer cells. RIT can efficiently destroy and kill cancer cells [189] through targeting and damaging the DNA of cells [190]. RIT can be administered either locally or systemically according to the stage of tumour (localized, metastatic, or diffused). RIT involves using radionuclides that that are conjugated to monoclonal antibodies targeting TAAs or antigens expressed by cells in TME [191].

1.5.1. ^{90} Y-hPAM4

A phase I dose escalation trial showed that the yttrium-90 labelled humanized clivatuzumab tetraxetan (^{90}Y-hPAM4) antibody (which binds a mucin glycoprotein expressed on pancreatic ductal carcinoma) produced objective responses in patients [192]. The researchers investigated the use of fractionated RIT with low-dose gemcitabine in a phase I trial in patients with advanced pancreatic cancer. Thirty-eight patients with untreated pancreatic cancer were selected as the subjects for the trial (33 with stage IV and five with stage II pancreatic cancer). Each received gemcitabine weekly for four weeks and ^{90} Y-hPAM4 weekly in weeks two, three and four. The cohort was divided into two equal groups, one receiving varying escalating doses

and another group receiving a constant dose of ^{90} Y-hPAM4. Computed Tomography-based Response Evaluation Criteria were used to analyze the effects of the therapy on the tumour. Six patients had a partial response and 16 patients had stabilization as their best response. The median OS was found to be 7.7 months in all 38 subjects, with better efficacy for higher RIT doses. The researchers concluded that low-dose gemcitabine combined with fractionated RIT showed promising activity in advanced pancreatic cancer patients [193].

1.5.2. Centrin1 (CETN1)

Centrin1 (CETN1) is a cancer/testis antigen that has a 25-fold increase in gene expression in 50% of pancreatic cancer patients. A recent study investigated the generation of CETN1 antibodies and their use in RIT and radioimmunoimaging. Their study indicated the safety, efficiency and specificity of ^{213} Bi rradiolabelled anti-CETN1 when used to treat pancreatic cancer *in vitro* and *in vivo* [194]. Therefore, further evaluation of novel antigens in the treatment of pancreatic cancer is justified.

To summarize, RIT is particularly preferred in tumour therapy because it can be tailored to suit the patient's tumour characteristics. The targeted antigens can be highly selective in adhering to the cancer cells, hence protecting neighboring healthy cells. RIT has shown a potential improvement in the OS rate of pancreatic cancer patients.

1.5.3. Abscopal effect after radiotherapy

Radiotherapy is used traditionally as a standard treatment for many cancers. It was considered a local therapy, but distal effects on non-targeted lesions have been observed and are referred to as abscopal effects. These abscopal effects are anti-tumour immune responses affecting the metastatic tumours that are not exposed to the radiation [195, 196]. The mechanism is that the ionizing radiation results in multiple local biologic effects including inflammation and cellular death, which in turn increase the levels of tumour antigens inducing immune responses against them. Therefore, radiotherapy can serve as an *in-situ* vaccine through the induction of immunogenic cell death leading to T cell priming. Furthermore, radiation induces cytokine and chemokine release leading to an inflammatory tumour microenvironment.

A recent study showed that a single dose of stereotactic body radiation therapy augmented the anti-tumour effect of an αCD40 agonist antibody in two pancreatic cancer mouse models, which lead to tumour regression and long-term immunologic memory [197]. Furthermore, another recent study has been conducted on mouse models of pancreatic cancer where they

combined CD40 agonist antibody, radiotherapy and dual immune checkpoint blockades: αCTLA-4 and αPD-1. Radiotherapy and αCD40 managed to overcome tumour resistance to αCTLA-4/αPD-1 immune checkpoint blockades through multiple cellular mechanisms resulting in a long-term immunologic memory and higher survival rates [198]. This study provided proof-of-principle for clinical evaluation of this combined therapy.

A 67-year-old female with advanced metastatic pancreatic cancer was treated with GM-CSF and localized radiotherapy for the primary tumour. There was significant regression of primary tumour and metastasis as well. Moreover, CT scans demonstrated stable disease for several months after treatment, and an abscopal effect of radiation was markedly noticed [199].

On the other hand, Park *et al.* demonstrated that combining PD-1 inhibition with stereotactic body radiotherapy in preclinical melanoma and renal cell carcinoma models resulted in a 66% reduction in non-irradiated metastatic tumour in addition to almost complete regression of the original tumour, whereas PD-1 blockade alone only modestly suppressed primary and secondary tumour growth [200]. Similar promising results have been noticed with the combination of GM-CSF and radiotherapy in treatment of different metastatic solid tumours including breast, small and non-small cell lung, thymic, eccrine, urothelial, ovarian, and cervical cancers [201]. With regard to pancreatic cancer, there are still more than 21 open clinical trials investigating different combination approaches of immunotherapies and radiotherapy [202].

1.6. Oncolytic virotherapy

Since the highly immunosuppressive tumour microenvironment, characteristic of pancreatic adenocarcinoma, has been a great challenge, another promising new therapeutic approach involving oncolytic viruses has recently been evaluated [203]. Oncolytic viruses are genetically engineered to selectively infect, replicate and lyse cancer cells with reduced effects on healthy normal cells around them [203-205]. Various viral backbones can be used to develop specific vectors such as vaccinia viruses (VV), adenoviruses, reoviruses, and herpesviruses [206]. Oncolytic viruses can produce anti-tumour effects by two different mechanisms, tumour cell lysis and immune activation. They are designed to selectively infect tumour cells to replicate inside them causing cell lysis. In addition, cell lysis itself provides antigen release together with pathogen-associated and damage-associated molecular patterns that in turn induce immune response [207, 208].

1.6.1. Adenovirus

Adenovirus is a double-stranded DNA, highly immunogenic virus that depends on coxsackie and adenovirus receptors to enter the cells. It can produce anti-tumour activity by two different mechanisms, tumour cell lysis and immune-activation. Several preclinical studies have showed anti-tumour activity of various oncolytic adenovirus preparations in combination with chemotherapy in pancreatic adenocarcinoma mouse models [209, 210].

Some tumours provide a hostile microenvironment for oncolytic viruses to work effectively [211]. For example, pancreatic cancer typically exhibits a collagen-rich stromal reaction, which limits effective viral access into the tumour [212]. Therefore, complementary treatment by the use of oncolytic adenovirus expressing relaxin (YDC002) is an innovative way of tackling chemotherapy-resistant pancreatic cancer. A recent study aimed to investigate the ability to combine YDC002 with gemcitabine to fight pancreatic cancer [213]. This combination induced potent anticancer effects through degradation of the tumour's extracellular matrix and induction of apoptosis, which can be helpful in treating chemotherapy-resistant pancreatic tumour.

A phase I dose escalation trial administered ONYX-015 (E1b gene-deleted oncolytic adenovirus) intratumoural in 22 patients with unresectable pancreatic cancer every four weeks. The drug was well-tolerated at the highest dose level but neither viral replication nor significant clinical responses were demonstrated. However, all patients showed raised levels of neutralizing antibody against ONYX-015 [214]. In another Phase I/II clinical trial, 21 patients with locally advanced or metastatic pancreatic cancer were administered with gemcitabine and ONYX-015. Two patients demonstrated partial tumour regression while two showed minor responses and six had stable disease. Some of the remaining 11 patients had progressive disease and some were excluded from the trial due to treatment-associated toxicity [215].

1.6.2. Vaccinia virus

Vaccinia virus (VV) is a double-stranded DNA virus that can infect most mammalian cells. Recombinant VVs have shown potential in preclinical studies to be an effective therapeutic technique in the management and treatment of pancreatic cancer. However, clinical trials using oncolytic viruses in pancreatic cancer patients have demonstrated limited clinical responses.

GLV-1h68, a recombinant vaccinia virus, was found to be able to infect and lyse tumour cells with minimal toxicity in both cell cultures and mouse models. Moreover, it led to enhanced and quicker responses when combined with cisplatin or gemcitabine to treat PANC-1 tumours [216]. Also, GLV-1h151, a recombinant vaccinia virus, combined with conventional radiation therapy demonstrated a synergistic cytotoxic effect in different cell lines compared with using either single treatment alone. Cell lysis was attributed to viral replication. The same combination resulted in enhanced tumour regression with no evidence of toxicity in mice. These results promote the combination of GLV-1h151 and radiation as a novel therapeutic approach for pancreatic adenocarcinoma [217].

A recent experimental preclinical trial combined oncolytic adenovirus and VV to enhance vaccine immunogenicity. Induced pluripotent stem cell-derived tumour cells from KPC (LSL-K-ras^{G12D}, LSL-p53$^{R172H/+}$, Pdx-Cre), transgenic mice were infected with oncolytic adenovirus as prime and VV as boost before administration to KPC mice prior to significant spontaneous pancreatic cancer development, thereby serving as a whole cell vaccine. The regimen was well-tolerated and mediated significant immune responses and disease inhibition. The study claimed that this regimen can provide cancer prevention for those who are at risk, which in turn will significantly affect the disease survival statistics [218].

In summary, oncolytic virotherapy is a promising approach with its ability to kill tumour cells, even those that are resistant to chemotherapy. This form of therapy has shown some potential in managing the already difficult pancreatic cancer, and further modifications may be effective in tackling the problem. Since the viruses preferentially target malignant cells, this mode of therapy leaves healthy cells intact. However, the technique is limited by its relatively poor intratumoural penetration, which minimizes its replicative potential [219]. Arming the viruses with immune inducers and cytotoxic transgenes may enhance this approach.

1.7. Adoptive cell transfer (ACT)

Adoptive cell transfer (ACT) is one new promising form of immunotherapy [220]. ACT can be categorised into three groups according to the source and method of isolating T cells: tumour infiltrating lymphocytes (TILs), engineered T cells that express a specific cancer T-cell receptor (TCR), and T cells that express a chimeric antigen receptor (CAR) [221]. In the TIL-based ACT method, TILs are isolated from the tumour tissues of cancer patients, activated *in vitro* using a high concentration of IL-2, and re-infused to the patients [222, 223]. These TILs can be very efficient in killing some types of tumour cells and ACT has emerged as a promising

therapy for metastatic melanoma patients, with objective cancer regression in approximately half of the patients [224]. However, TILs cannot be isolated from most patients with other types of cancer. TILs may also become immune tolerated which means they can be rendered non responsive to cancer cell antigens [225]. To overcome these issues and render T cells reactive against tumours, genetic modification methods were developed. T lymphocytes are harvested from peripheral blood lymphocytes (PBLs) and genetically modified using plasmid transfection, mRNA or via viral vector transduction, to express receptors against tumour-associated antigens (TAA) [225, 226], using either TCR or CAR [220, 225, 227-230]. However, viral vectors remain the most frequently used method, and there are relatively fewer studies using plasmid transfection using Sleeping Beauty or PiggyBac plasmids [231]. After that, these T cells are activated and expanded *in vitro* to large numbers prior to re-infusing into the patient [224, 229, 232, 233].

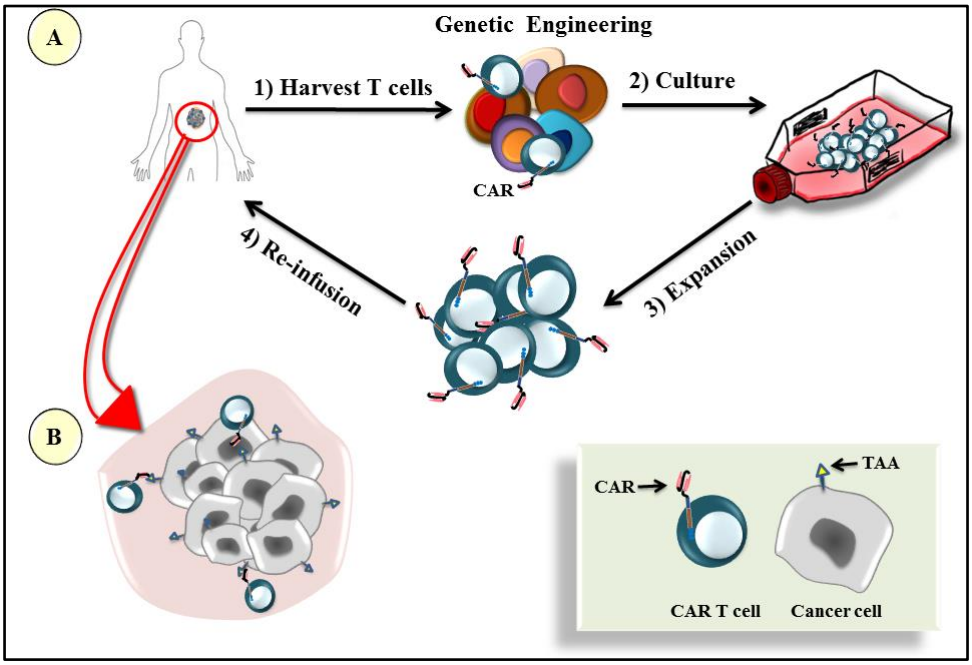

Figure 1.1. Schematic of adoptive autologous CAR T cell transfer

(**A**) **1**. T cells are isolated from the peripheral blood of a cancer patient. **2**. T cells are genetically engineered to express a -TAA-specific CAR **3**. CAR T cells are expanded *in vitro* to reach high numbers of cells. **4**. CAR T cells are re-infused back to the patient. (**B**) CAR T cells migrate, recognize and destroy tumour cells.

TCR engineering is a method to introduce genes encoding a TCR to redirect the specificity of patient-derived peripheral T cells [234, 235]. Overcoming the limitation of the restricted numbers of peripheral tumour-antigen reactive T cells [236]. On the other hand, CARs are synthetic receptors, which are usually comprised of three components. The extracellular

portion is usually a single-chain variable fragment (scFv), derived from a monoclonal antibody, which is capable of binding specific tumour antigens independent of the major histocompatibility complex (MHC). The cytoplasmic tail contains signalling domains derived from activation and costimulatory molecules (for example: CD3, CD28 and 4–1BB) that provide activation and co-stimulation following CAR-antigen interaction. The extracellular and cytoplasmic domains are linked by a spacer, which consists of a hinge region that mediates CAR flexibility and ensures the appropriate sitting of the binding domain during the interaction between the scFv and the antigen. The corresponding portions of CD8 and CD28 are frequently used as the hinge region [225, 230, 237-239].

The first generation of CARs was designed to contain CD3ζ or FcRγ signalling domains but was limited by the lack of costimulatory signalling. The subsequent second generation of CARs were designed to incorporate CD28 or CD137 cytoplasmic co-stimulatory domains. The third generation of CARs contains additional signalling domains (CD137, CD28, and/or OX40) (**Figure 1.2**) [129].

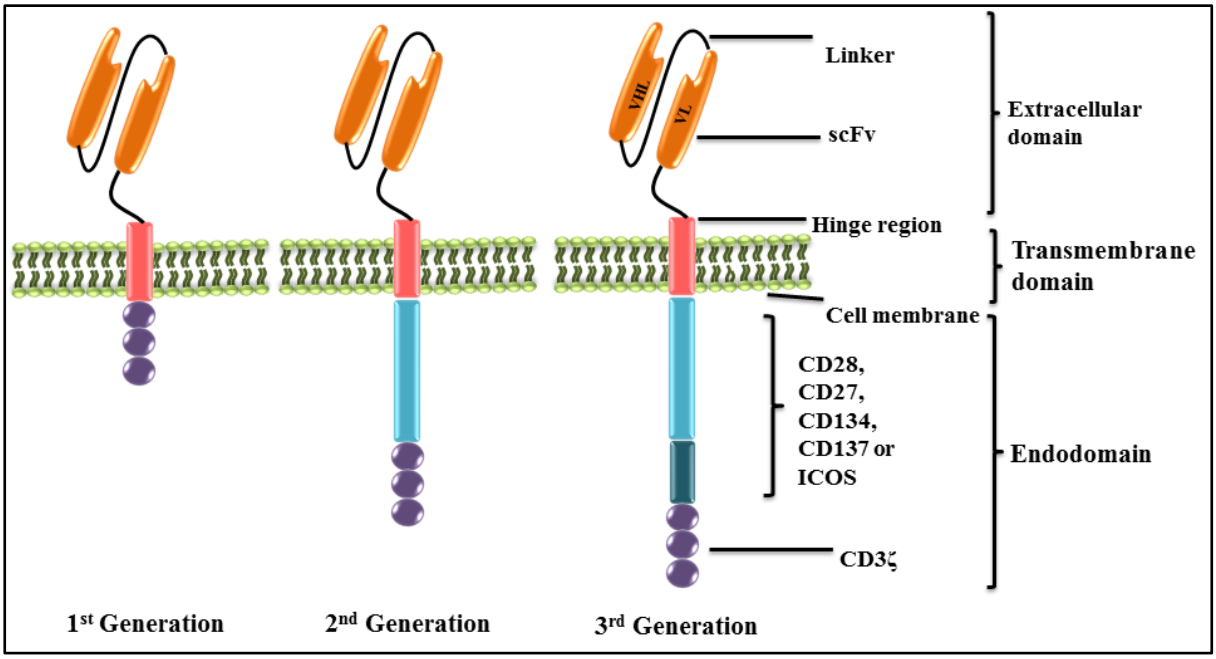

Figure 1.2. Generations of CAR T cell constructs
T cells can be engineered to respond against TAAs expressed on tumour cells by insertion of a CAR. CARs are composed of an extracellular domain that is derived from a monoclonal antibody and linked by a transmembrane spacer to a cytoplasmic signalling domain that contain T cell signalling proteins. The 1st generation CARs contained CD3ζ or FcRγ, while the 2nd generation receptors incorporated CD28 or 4-1BB cytoplasmic co-stimulatory domains fused to CD3ζ. The 3rd generation CARs contain two costimulatory domains connected to CD3ζ. ICOS, inducible T cell co-stimulator; VL, variable light chain; VH, variable heavy chain; scFv, single-chain variable fragment.

These T cells can be very efficient in killing tumour cells and ACT using CAR T cells has shown great promise in clinical studies for the therapy of haematological cancers with clinical response rates up to 90% [240-244]. However, clinical experience using this approach for solid tumours is much more limited [129, 225, 245, 246]. These limited responses may be due to the histological structure and the strong immunosuppressive microenvironment of solid tumours, which may block CAR T cell anti-tumour activity. Other obstacles that limit the success of CAR T cell approach against solid tumours include the lack of appropriate and unique targeted TAAs to avoid on target/off tumour toxicity [247], inefficient trafficking, penetration and persistence of CAR-T cells in tumours and the high antigen heterogeneity of solid tumours [245, 248-255].

A substantial number of clinical trials involving the use of CAR technology have recently been undertaken on pancreatic cancer patients in an attempt to determine the safety, efficacy and the feasibility of CAR T cell therapy approach. Several preclinical and clinical studies targeted different TAAs that are expressed in pancreatic cancer tumours including mesothelin (MSLN), mucin 1 (MUC1), prostate stem cell antigen (PSCA), carcinoembryonic antigen (CEA), human epidermal growth factor receptor 2 (Her2), Claudin 18.2, cluster of differentiation (CD)-133, Epithelial cell adhesion molecule (EpCAM) and CD70 [129]. I reviewed the progress of using CAR T cells in pancreatic cancer in these preclinical and clinical settings, and I discussed the hurdles for utilizing the full potential of CAR T cell therapy in a 2019 published review paper with the title:

(**Genetic Redirection of T Cells for the Treatment of Pancreatic Cancer, PMID: 30809507, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6379296/**), which is presented below as part of this introductory thesis chapter.

frontiers in Oncology

REVIEW
published: 12 February 2019
doi: 10.3389/fonc.2019.00056

Genetic Redirection of T Cells for the Treatment of Pancreatic Cancer

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Conventional treatments for pancreatic cancer are largely ineffective, and the prognosis for the vast majority of patients is poor. Clearly, new treatment options are desperately needed. Immunotherapy offers hope for the development of treatments for pancreatic cancer. A central requirement for the efficacy of this approach is the existence of cancer antigen-specific T cells, but these are often not present or difficult to isolate for most pancreatic tumors. Nevertheless, specific T cells can be generated using genetic modification to express chimeric antigen receptors (CAR), which can enable T cell responses against pancreatic tumor cells. CAR T cells can be produced *ex vivo* and expanded *in vitro* for infusion into patients. Remarkable responses have been documented using CAR T cells against several malignancies, including leukemias and lymphomas. Based on these successes, the extension of CAR T cell therapy for pancreatic cancer holds great promise. However, there are a number of challenges that limit the full potential of CAR T cell therapies for pancreatic cancer, including the highly immunosuppressive tumor microenvironment (TME). In this article, we will review the recent progress in using CAR T cells in pancreatic cancer preclinical and clinical settings, discuss hurdles for utilizing the full potential of CAR T cell therapy and propose research strategies and future perspectives. Research into the use of CAR T cell therapy in pancreatic cancer setting is rapidly gaining momentum and understanding strategies to overcome the current challenges in the pancreatic cancer setting will allow the development of effective CAR T cell therapies, either alone or in combination with other treatments to benefit pancreatic cancer patients.

Keywords: chimeric antigen receptor, pancreatic cancer, tumor microenvironment, pancreatic ductal adenocarcinoma, adoptive cell transfer, immunotherapy

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *16 November 2018*
Accepted: *21 January 2019*
Published: *12 February 2019*

Citation:
Ali AI, Oliver AJ, Samiei T, Chan JD, Kershaw MH and Slaney CY (2019) Genetic Redirection of T Cells for the Treatment of Pancreatic Cancer. Front. Oncol. 9:56. doi: 10.3389/fonc.2019.00056

INTRODUCTION

Pancreatic cancer presents a major challenge in the clinic and is one of the most aggressive tumor types. Pancreatic cancer is the fourth most common cause of cancer death (1, 2) and it is on its way to be the second most common cause of cancer-related deaths by 2030 (3). Patients with pancreatic cancers have a median survival rate of 5 months after diagnosis and the overall 5-years survival rate is <5% (4).

The most common clinical therapeutic approaches against pancreatic cancer include surgical resection, radiotherapy, chemotherapy, and combination of these treatments (5). Surgical resection may lead to longer-term survival, but only a small number of the patients are considered resectable

(6–8) because most patients that present to the clinic are with advanced or metastatic disease (9). In addition, the removal of part or the full pancreas is technically difficult, and even if the operation is successful, the 10-years survival rate is still <10% (10). Radiotherapy is usually not curative on its own and is used to alleviate symptoms. Although in the last two decades, chemotherapy and targeted therapy (such as Erlotinib targeting epidermal growth factor receptor, Sunitinib targeting multiple receptor tyrosine kinases, and Everolimus targeting mTOR kinase) have been used for patients with unresectable locally advanced or metastatic pancreatic cancer (11), these have only generated modest improvements in survival (12, 13). Therefore, the need to develop alternative effective therapies for pancreatic cancer is urgent.

Currently, immunotherapy has been offered as an important cancer treatment for a number of cancer types (14, 15) and recent preclinical and clinical evidence suggest that therapies utilizing the immunity could potentially be effective against this devastating disease (16). However, there has been limited success in the use of checkpoint blockade immunotherapies such as PD1/CTLA4 antibodies or vaccines in the treatment of pancreatic cancer (17).

Adoptive cellular therapies involving an infusion of effector immune cells into patients have generated remarkable responses in some cancers (18) and chimeric antigen receptor (CAR) T cell therapy represents a promising therapeutic modality for some difficult cancers including pancreatic cancers. This review aims to summarize the recent development of cellular therapies and clinical data in CAR T cell trials for pancreatic cancer. As pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas and represents the vast majority of pancreatic cancer deaths, we will emphasize CAR T cell work on PDAC in this article.

CAR T CELLS

CAR T cells present an exciting opportunity for cancer immunotherapy. As a form of adoptive immunotherapy, CAR T cells are generated from patient autologous T cells isolated from peripheral blood. Patient T cells are transduced *ex vivo* to express a CAR specific for a tumor antigen of choice and adoptively transferred into the patient to treat established cancers (19). CARs are composed of an antibody single-chain variable fragment (scFv) conjugated to intracellular signaling domains containing CD3-ζ chain and one or more co-stimulatory domains such as CD28 and CD137 (18, 20–22) (**Figure 1**). The CAR scFv confers the ability to T cells to directly recognize cancer antigens independent of MHC antigen presentation, and CAR specific recognition/binding to tumor antigen drives CAR T cell activation and tumor cell killing (23, 24). The first generation of CARs that was designed to contain CD3ζ or FcRγ signaling domains was limited by the lack of costimulatory signaling. The subsequent second generation of CARs has been designed to incorporate CD28 or CD137 cytoplasmic co-stimulatory domains. The third generation of CARs contains additional signaling domains (CD137, CD28, and/or OX40) (18, 20). The latter generations of CAR T cells are better equipped to overcome the immunosuppressive tumor microenvironment (TME), however, it remains unclear what combination of signaling domains is necessary for maximal anti-tumor response.

The use of CAR T cells for the treatment of B cell malignancies demonstrated significant responses in patients (25, 26). Given the success in clinical trials, the use of CD19-targeted CAR T cell therapies was approved by the FDA in 2017. Approved CAR T cell therapies include tisagenlecleucel (Kymriah) for the treatment of children and adolescents with refractory/relapsed B-cell acute lymphoblastic leukemia (B-ALL), and axicabtagene ciloleucel (Yescarta) for adult relapsed-refractory large B-cell lymphoma patients. However, despite the successes in hematological cancers, clinical trials targeting solid tumors have demonstrated only moderate efficacy. This is largely attributed to the immunosuppressive TME, limited activation and trafficking of CAR T cells to the tumor site, heterogeneous antigen expression/distribution in some solid tumors and availability of validated antibodies that could be utilized in the CAR constructs (27–29).

A range of approaches aimed at enhancing CAR T cell efficacy is currently undergoing investigation. A notable strategy that has demonstrated promising effects *in vivo* is the use of dual-specific T cells. Dual-specific T cells co-express a CAR against a tumor antigen and a TCR against a strong immunogen (30). Through vaccination, dual-specific T cells can engage the cognate immunogen of the chosen TCR presented by antigen presenting cells (APCs) on MHC molecules. A recent study using the "adoptive cell transfer incorporating vaccination" (ACTIV) therapy regimen for dual-specific T cell treatment has demonstrated durable responses in a range of solid tumors *in vivo* (31, 32). Use of the specialized "CARaMEL" dual-specific T cells, expressing a CAR against HER2 and TCR specific for the melanocyte protein gp100 (also known as pMEL), drove dramatic T cell expansion and tumor regression in a number of solid tumor models. Moreover, surviving mice that received ACTIV therapy developed potent immune memory responses against pre-existing tumor cells. These results provide encouraging evidence for the investigation and development of dual-specific T cells for the treatment of difficult cancers including pancreatic cancer.

CAR T CELL THERAPIES IN TREATING PANCREATIC CANCER

In recent years, CAR T cell therapies have been tested in both preclinical and clinical settings for treating pancreatic cancers. However, a focus for the field remains the discovery and validation of pancreatic cancer-specific antigens.

Mesothelin

Mesothelin (MSLN) is a glycoprotein present mainly in mesothelial cells and overexpressed in a variety of human cancers including malignant pleural mesothelioma, ovarian, lung and pancreatic cancers (33). MSLN has been reported to be expressed by >80% of PDACs and its expression correlated with poor

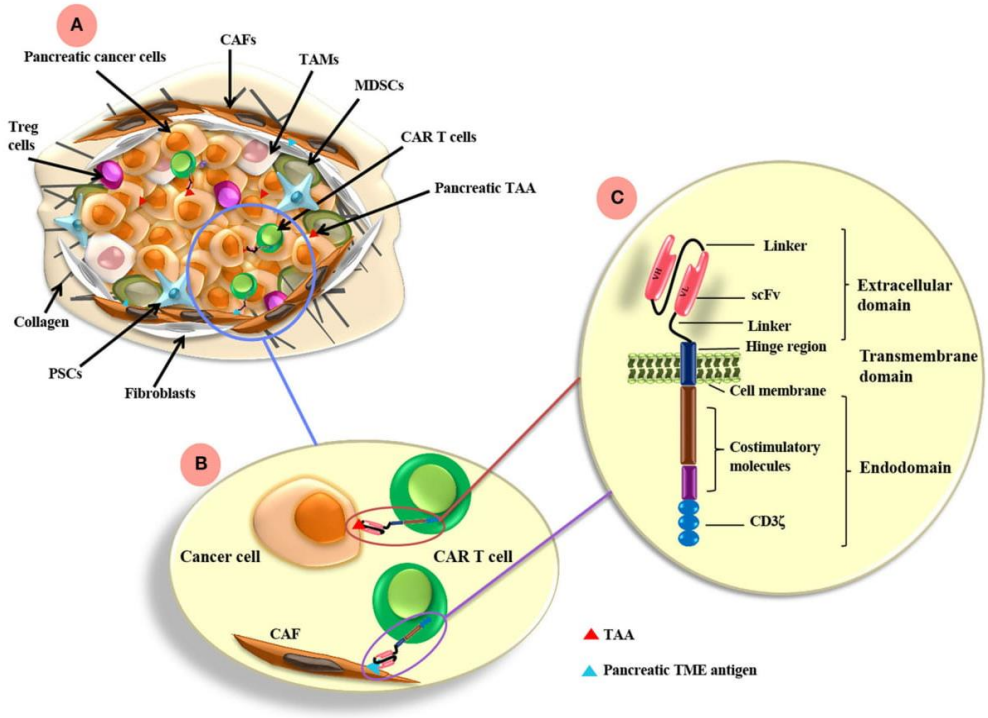

FIGURE 1 | CAR T cell antigen-targeting strategies and pancreatic cancer TME. **(A)** The pancreatic TME consists of tumor cells as well as many immunosuppressive cells, such as CAFs, TAMs, MDSCs, PSCs, and Treg cells. **(B)** CAR T cells can be directed to the TAA expressed on pancreatic cancer cells and/or other antigens targeting the TME components, such as FAP on CAFs. **(C)** CARs are composed of extracellular, transmemebrane and endo-domains. The extracellular domain consists of an antibody variable heavy chain (VH) and a light chain (VL) domain, which are derived from an scFv from an antibody specific for a TAA. A flexible hinge region links the extracellular domain to a transmembrane and endodomain. The endodomain has cytoplasmic signaling regions derived from CD3ζ and costimulatory signaling domains. TAMs, tumor-associated macrophages; CAFs, cancer associated fibroblasts; MDSCs, myeloid-derived suppressor cells; Tregs, regulatory T cells; PSCs, pancreatic stellate cells; FAP, fibroblast activation protein; scFv, single chain variable fragment. TAA, tumor associated antigen; TME, tumor microenvironment.

prognosis (34). Although MSLN is believed to play a role in cell adhesion and positively regulates tumor invasion and growth, its biological function is unclear (34). Because MSLN is expressed only on non-crucial tissues, it is an attractive target for CAR T cell based immunotherapy.

Preclinical studies have demonstrated that CAR T cells against MSLNs could potentially be effective against PDAC. When MSLN-CAR T cells were adoptively transferred intratumorally or intravenously (i.v.) into NSG mice bearing pre-established subcutaneous patient-derived mesothelioma, the tumor burden was greatly reduced in size and some tumors were completely eradicated, demonstrating the potential of targeting this antigen (35). Due to the concerns for potential on-target/off-tumor toxicity, phase I clinical studies used mRNA-based methods to generate CAR T cells that express CARs transiently to limit the duration of toxicity. Given the short-term expression of CARs, multiple injections were required. In a study carried out by Beatty et al (36), a patient with metastatic PDAC was given eight doses of MSLN-CAR T cells by i.v. infusion and two doses via intratumoral injections. The CAR T cells were detected in the extravascular tumor compartments 3 days after the initial i.v. infusion. The patient demonstrated stable disease 3 weeks post MSLN-CAR T cell administration, without overt evidence of toxicity against normal tissues. An anti-tumor effect was observed based on the development of novel humoral immune responses post the cell infusion. In another phase I trial carried out by the same group, six metastatic PDAC patients were treated with mRNA-based MSLN-CAR T cells three times per week for three consecutive weeks. The treatment was well-tolerated and the disease was stabilized in two of the treated patients. Importantly, one patient had total metabolic active volume decreased by 69.2%, although there was no detected effect on the primary tumor (37). Given the promising results, trials of MSLN-CAR T cells engineered by traditional viral transduction methods have also been initiated (**Table 1**).

Prostate Stem Cell Antigen

Prostate stem cell antigen (PSCA) is a glycosylphosphatidylinositol-anchored cell surface protein involved in intracellular signaling, although much of its function

TABLE 1 | Clinical trials involving CAR-based immunotherapy in pancreatic cancer from www.clinicaltrials.gov.

Target antigen	Sponsor/collaborator	Cancer type	Status/estimated completion date	Number of patients	Trial ID	Notes
MSLN	National Cancer Institute, USA	Pancreatic cancer Cervical cancer Ovarian cancer Mesothelioma Lung cancer	Recruiting/December, 2029	136 estimated	NCT01583686	Intravenous infusion of retroviral transduced MSLN-CAR T cells and low dose IL-2 with cyclophosphamide and fludarabine preconditioning.
	University of Pennsylvania, USA	Pancreatic cancer	Active, not recruiting/September, 2021	18 estimated	NCT03323944	1–3 × 10^7/m^2 (Cohort 1) or 1–3 × 10^8/m^2 (Cohort 2 and 3) lentiviral transduced MSLN-CAR T cells with/without cyclophosphamide preconditioning.
	Shanghai Gene Chem Co., Ltd./Shanghai Cancer Hospital, China	Metastatic pancreatic cancer	Unknown/July, 2018	20 estimated	NCT02959151	Intratumor injection or i.v. at one dose of 1.25~4 × 10^7 CAR^+ lentiviral transduced MSLN-CAR T cells/cm^3 tumor bulk.
	University of Pennsylvania, USA	Metastatic PDAC	Completed/March 2017	16 actual	NCT01897415	1–3 × 10^8/m^2 RNA transfected MSLN-CAR T cells i.v. injected three times weekly for up to 3 weeks. No cytokine release syndrome, neurologic symptoms or dose-limiting toxicities reported. One patient has a response in the live, but no activity in the primary tumor (37).
	University of Pennsylvania, USA	Metastatic PDAC Epithelial ovarian cancer Malignant epithelial pleuralmesothelioma	Completed/November, 2015	19 actual	NCT02159716	1–3 × 10^7 and 1–3 × 10^8/m^2 lentiviral transduced MSLN-CAR T cells i.v. injected with or without cyclophosphamide preconditioning. Safe in patients with recurrent serious ovarian cancer. CAR T cells expanded in the blood of all patients. One patient had clearance of pleural effusion (104).
	Chinese PLA General Hospital, China	Malignant mesothelioma Pancreatic cancer Ovarian tumor Triple negative breast cancer Endometrial cancer Other $MSLN^+$ tumors	Unknown/November 2018	20 estimated	NCT02580747	Retroviral transduced MSLN-CAR T cells.
	China Meitan General Hospital/Marino Biotechnology Co., Ltd. China	$Mesothelin^+$ tumors	Recruiting/August 2019	20 estimated	NCT02930993	i.v. infusion with 5 × 10^4-1 × 10^7/kg MSLN-CAR T cells in a three-day split-dose regime. Cyclophosphamide preconditioning.
	University of Pennsylvania, USA	Pancreatic cancer Mesothelioma	Completed/September 2017	18 actual	NCT01355965	Three infusions of 1 × 10^8-1 × 10^9 mRNA MSLN-CAR T cells every other day for 2-cycle of three infusions. The pancreatic patient was given eight doses i.v. infusions and two intratumor injections. Anti-tumor effect observed and no overt toxicities (36).
	University of Pennsylvania/University of California, USA	Pancreatic cancer	Completed/November, 2017	4 actual	NCT02465983	i.v. infusion of 1–3 × 10^7/m^2 or 1 × 10^8/m^2 combined lentiviral transduced MSLN-CAR T cells and CD19-CAR T cells (to deplete B cells and impede the antibody response against MSLN CAR T cells) with cyclophosphamide preconditioning.

(Continued)

TABLE 1 | Continued

Target antigen	Sponsor/collaborator	Cancer type	Status/estimated completion date	Number of patients	Trial ID	Notes
	First Affiliated Hospital of Wenzhou Medical University, China	Pancreatic cancer	Active, not recruiting/October, 2020	10 estimated	NCT03497819	Pancreatic arterial or i.v. infusion of lentiviral transduced MSLN-CAR T cells and CD19 CAR T cells with cyclophosphamide preconditioning.
	Shanghai GeneChem Co., Ltd. China	Pancreatic cancer	Unknown/September 2018	30 estimated	NCT02706782	Transcatheter arterial infusion of 1–10 $\times$ 10^6 MSLN-CAR$^+$ T cells/kg. Cyclophosphamide preconditioning.
MSLN, PSCA, CEA, HER2, MUC1, EGFRvIII and other targets	First Affiliated Hospital of Harbin Medical University/Shanghai Unicar-Therapy Bio-medicine Technology Co.,Ltd. China	Pancreatic cancer	Recruiting/June 2019	10 estimated	NCT03267173	A single dose of 10^7 /kg CAR T cells administered i.v.
	Cancer Research UK, UK	Pancreatic cancer Breast cancer Colorectal cancer Gastric cancer Lung cancer Ovarian cancer Unspecified adult solid tumor	Terminated/April 2010	14 actual	NCT01212887	The pancreatic cancer patient received 10^9-5 $\times$ 10^{10} retroviral transduced CEA CAR T cells i.v. and seven doses IL-2 with cyclophosphamide and fludarabine preconditioning. All patients had adverse events with grade $\leq$2 and lack of prolonged CAR T cell persistence led to the premature termination of the trial. No objective clinical response observed although serum CEA level reduced in the pancreatic cancer patient (47).
CEA	Roger Williams Medical Centre/University of Colorado, USA	Liver metastases Pancreatic cancer	Active, not recruiting/August, 2018	5 actual	NCT02850536	Three hepatic artery infusions of CEA-CAR T cells delivered by the Surefire Infusion System at 1-week intervals. Low dose IL-2 for 4 weeks.
	Southwest Hospital, China	Pancreatic cancer Lung cancer Colorectal cancer Gastric cancer Breast cancer	Recruiting/December, 2019	75 estimated	NCT02349724	CEA-CAR T cells were well-tolerated in high doses with some efficacy reported in the colorectal cancer cohort (105).
	Roger Williams Medical Center/Sirtex Medical, USA	Liver metastases	Active, not recruiting/January 2019	8 actual	NCT02416466	Three doses of CEA-CAR T cells and 1 dose of Selective Internal Radiation Therapy (SIRT) with Yttrium-90 at 2-weeks intervals combined with low dose IL-2 for 6 weeks.
PSCA	Bellicum Pharmaceutical, USA	PDAC Gastric adenocarcinoma Prostate adenocarcinoma	Recruiting/December, 2020	138 estimated	NCT02744287	i.v. infusion of retroviral transduced PSCA CAR T cells (BPX-601) with rimiducid (a homodimerizing molecule that enhances BPX-601 activation).
CD70	National Cancer Institute, USA	Pancreatic cancer Renal cell cancer Breast cancer Melanoma Ovarian cancer	Recruiting/January, 2028	113 estimated	NCT02830724	i.v. infusion of retroviral transduced CAR T cells. The CAR consists of the CD70 receptor CD27. Cyclophosphamide and fludarabine preconditioning with high dose IL-2.

(Continued)

TABLE 1 | Continued

Target antigen	Sponsor/collaborator	Cancer type	Status/estimated completion date	Number of patients	Trial ID	Notes
MUC1	PersonGen BioTherapeutics (Suzhou) Co., Ltd./The First People's Hospital of Hefei; Hefei Binhu Hospital, China	Pancreatic carcinoma Hepatocellular carcinoma Non-small cell lung cancer Triple-negative invasive breast carcinoma	Unknown/October, 2018	20 estimated	NCT02587689	
	PersonGen BioTherapeutics (Suzhou) Co., Ltd./The First People's Hospital of Hefei/Hefei Binhu Hospital, China	Pancreatic carcinoma Hepatocellular carcinoma Non-small cell lung cancer Triple negative invasive breast carcinoma Malignant glioma of brain Colorectal carcinoma Gastric carcinoma	Unknown/July, 2018	10 estimated	NCT02839954	Anti-MUC1 CAR-NK cells.
HER2	Southwest Hospital, China	Pancreatic cancer Breast cancer Ovarian cancer Lung cancer Gastric cancer Colorectal cancer Glioma	Recruiting/September, 2019	60 estimated	NCT02713984	
EpCAM	First Affiliated Hospital of Chengdu Medical College, China	Pancreatic cancer Colon cancer Esophageal carcinoma Prostate cancer Gastric cancer Hepatic carcinoma	Recruiting/December, 2020	60 estimated	NCT03013712	1–10 × 10^6 lentiviral transduced EpCAM-CAR$^+$ T cells/kg. Vascular interventional or endoscopy infusion. Precondition before CAR T cell infusion.
Claudin 18.2	CARsgen Therapeutics, Ltd., China	Pancreatic carcinoma Adenocarcinoma of esophagogastric junction	Not recruiting/December, 2020	48 estimated	NCT03302403	i.v. infusion. Fludarabine and cyclophosphamide preconditioning.
	Changhai Hospital/CARsgen Therapeutics, Ltd., China	PDAC Advanced gastric adenocarcinoma	Recruiting/December 2021	24 estimated	NCT03159819	i.v. infusion of lentiviral transduced CAR T cells with lymphodepletion preconditioning.
CD133	Chinese PLA General Hospital, China	Pancreatic cancer Liver cancer Brain tumor Breast cancer Ovarian tumor Colorectal cancer Acute myeloid and lymphoid leukemias	Unknown/October 2018	20 estimated	NCT02541370	Retroviral transduced CD133-CAR T cells.

remains unclear. PSCA is expressed in the epithelial cells including that of prostate, kidney, skin, stomach, urinary bladder, esophagus and placenta, and also expressed in differentiating cells such as the ones of prostate and gastric epithelial cells. PSCA has also been detected in several cancer types including prostate, urinary bladder and pancreatic cancers (38). Aberrant overexpression of PSCA is detected in nearly 60% of the primary PDACs, while the gene expression is not detected in normal pancreatic duct (39). Therefore, PSCA has been proposed as a specific biomarker for PDAC patients and a promising target for CAR T cell therapies in treating PDAC. An advantage of targeting PSCA is that it is upregulated in pancreatic cancer cells from early stages of malignant transformation (40), including premalignant pancreatic intraepithelial neoplasias. PSCA may therefore serve as a useful target of immunotherapy that could eliminate malignant cells at all stages of PDAC.

Strategies using CARs against PSCA have been tested in preclinical settings. The 1st generation CAR T cells specifically killed $PSCA^+$ pancreatic cancer cell lines without lysing $PSCA^-$ target cell *in vitro* (8). In a more recent study, multiple CAR constructs were compared. Adoptive transfer of these human CAR T cells in this study demonstrated significant antitumor activity in a murine model of human pancreatic cancer. Interestingly, although the third-generation CAR containing CD28 and CD137 costimulatory domains induced greater persistence of CAR T cells *in vivo*, the second generation CAR that does not contain CD137 domain, induced a better antitumor effect, with 40% of mice demonstrating tumor eradication (40). The efficacy between second- and third- generation CARs have been compared by various studies and the discrepancies between these reports indicate that the optimal CAR design needs to be empirically determined for disease and antigen targeted (41, 42). Being encouraged by the preclinical success, a trial using CAR T cells against PSCA has been initiated and is currently recruiting patients (NCT02744287).

Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein belonging to the immunoglobulin (Ig) superfamily and plays a role in cell adhesion. CEA is one of the "oncofetal" antigens, typically produced in the gastrointestinal tissue during fetal development (43, 44). Although CEA is expressed in various healthy epithelia of pulmonary and gastrointestinal tracts, its distribution is often limited to the luminal surface, thus difficult for CAR T cells to access (44). After neoplastic transformation, luminal epithelia cells lose the apical polarity of CEA expression and CEA becomes accessible to immune cells. Some CEA is released to the serum and soluble CEA in the circulation of patients is used as a marker for cancer progression. CEA is highly expressed on the surface of the majority of PDAC cells (45). Together with its restricted expression in normal tissues, CEA is an attractive target for CAR T cell treatment in PDAC. A few versions of anti-CEA CARs have been developed in the past few years, each targeting different CEA epitopes.

CEA-CAR T cells exhibited cytotoxicity against CEA expressing cancer cells *in vitro* and demonstrated anti-tumor effect *in vivo* in a clinically relevant orthotopic CEA^+ murine model. The recipient CEA transgenic mice express CEA in their intestinal and pulmonary tracts. Ten days post intrapancreatic injections of Panc02-CEA^+ cells, the recipient mice received CEA-CAR T cells. Interestingly, the injection of the CAR T cells eradicated tumors without any damage to normal tissues that are CEA^{low} (46).

However, a recent clinical trial using CEA-CAR T cells treating patients with advanced CEA^+ cancers demonstrated acute respiratory toxicity, which resulted in the premature closure of the trial (47). The expression of CEA on lung epithelium was considered to result in the toxicity, which was associated with pre-conditioning. In a recent clinical trial using T cells modified to express an anti-CEA TCR for treating CEA^+ metastatic colorectal cancer, severe autoimmune colitis and pneumonia was observed in all of the three patients and led to the halt of the trial (48). This was likely due to the ability of TCR-redirected T cells to engage CEA presented by MHC.

Mucin 1

Mucins are high-molecular-weight glycoproteins with the presence of a heavily *O*-glycosylated tandem repeat region that is rich in proline, threonine and serine residues. The large gel-forming mucins are an extracellular secretion of goblet cells and their functions include lubrication of the epithelial surfaces and protection from physical and chemical insult. The epithelial membrane-tethered mucins are distinct from the conventional secreted mucins and are transmembrane molecules expressed by most glandular and ductal epithelial cells. It is widely accepted that the transmembrane mucin 1 (MUC1) is overexpressed in multiple epithelial adenocarcinomas, such as that of breast, colon, and pancreatic cancers. Importantly, under normal conditions, MUC1 is heavily glycosylated and expressed on the apical surface of epithelial cells, but in tumor cells MUC1 is aberrantly glycosylated. This modification of the MUC1 antigen reveals epitopes associated with the core protein, which is usually masked by oligosaccharides. Overexpression of aberrantly glycosylated MUC1 is associated with multiple metastatic cancers. In particular, MUC1 is aberrantly expressed in 60% of pancreatic cancers and is correlated with poor prognosis, enhanced metastasis, and chemoresistance (49, 50).

Strategies using CAR T cells targeting aberrantly expressed MUC1 have generated exciting results in preclinical studies. Posey et al. (51) generated a CAR that recognizes the aberrant glycoform Tn (GalNAca1-O-Ser/Thr) antigen on MUC1. These CAR T cells are able to recognize multiple types of tumors *in vitro* and exhibited superior tumor rejection and prolonged survival against disseminated pancreatic cancers in a xenograft model. This elegant study highlighted the potential for protein modifications as a target. As one of the most characteristic features of cancer cells is altered glycosylation, changes in glycosylation may expose a range of different cancer-associated epitopes and can serve as a target for CAR T cells. Given the promising outcomes from preclinical studies, multiple early phase trials have been planned and are active for using MUC1-CAR T cells in treating PDAC (**Table 1**).

CD47

CD47 is a transmembrane protein known to mediate a "do not eat me" signal. Structurally, CD47 contains an extracellular N-terminal hydrophilic Ig superfamily domain and an intracellular hydrophobic domain. Signal regulatory protein alpha (SIRPα) has been identified as the receptor to CD47. The binding of tumor-expressing CD47 to SIRPα on immune cells leads to the activation of the downstream signaling pathway in immune cells and inhibits the immune phagocyte-dependent clearance of tumors. CD47 has been identified in several types of hematological and solid cancers including pancreatic cancer (52, 53). In addition, CD47 is expressed on high levels on cancer stem cells (CSCs), but not on normal cells in the pancreas (52). Therefore, targeting CD47 has been a subject of intense interest in recent years.

CD47-specific CAR T cells were recently developed by ProMab Biotechnologies. These CAR T cells demonstrated high cytotoxicity against a few types of cancer cells including pancreatic cancer cells. Importantly, intratumoral injection of these CAR T cells significantly decreased pancreatic xenograft tumor growth. The same research group also developed humanized CD47-CAR T cells that contain humanized CD47 scFv. These humanized CD47-CAR T cells demonstrated specific killing of $CD47^+$ cancer cells *in vitro* and it will be interesting to assess their efficacy in clinical trials (54).

Tyrosine Kinase Growth Factor Receptors

The tyrosine kinase growth factor receptors are transmembrane proteins that play a key role in medicating intracellular signal transduction cascade for cell proliferation and differentiation. Up-regulation of some of the receptors are mechanisms of cancer development and progression, and the overexpression of a number of these receptors have been identified in many types of cancers (55). Therefore, CARs designed to target tyrosine kinase growth factor receptors have been developed and clinical trials are under way to test the safety and efficacy of many of these targets.

Human epidermal growth factor receptor 2 (HER2, also known as ERBB2) is a transmembrane glycoprotein belonging to the epidermal growth factor receptor (EGFR) family. The binding of HER2 to its ligand induces heterodimerization of the receptors, which mediates the activation of intracellular tyrosine kinase signaling cascades and leads to cell proliferation and differentiation. Overexpression of HER2 induces dimerization of HER2 and initiates signal transduction without ligand binding. Overexpression of HER2 has been reported in multiple cancer types making it an attractive target for CAR T cell treatment (20). However, an early HER2-CAR T cell trial, utilizing a third generation CAR, reported a patient death post the CAR T cell treatment (56). This patient was diagnosed with colon cancer metastatic to the lung and liver. The patient was preconditioned using cyclophosphamide (CY) and flurodarabine. Following intravenous infusion of 10^{10} HER2-CAR cells in 30 min, the patient experienced acute respiratory distress and subsequent death. At autopsy, multiple organs including the lung showed signs of ischemia and injury, and her serum samples post infusion showed significant increased levels of multiple cytokines, including IFN-γ, IL-6, and TNF-α. This report raised safety concerns on targeting HER2, but a more recent study using lower numbers of HER2-CAR T cells, and a second generation CAR in treating 19 patients with $HER2^+$ sarcoma, demonstrated safety (57).

HER2 expression is observed in 20–60% of pancreatic cancer cases and therefore, it is a potential target for CAR T cell treatment (58–61). Recently, a Phase I clinical trial used HER2-CAR T cells to treat two pancreatic cancer patients. Although the trial demonstrated safety, only moderate responses were achieved (62). In contrast, results from preclinical studies have been promising. A recent study used HER2-CAR T cells to treat mice bearing xenografts derived from stage IV PDAC patients and achieved complete remission in both local and disseminated disease settings. In addition, in this study, the authors used an antibody-based switchable CAR system. These switchable CAR T cells bind to a specific peptide that is genetically engrafted onto a tumor-binding Fab molecule. The switch acts as a bridge between the tumor cells and CAR T cells and has a short half-life and thus, limits potential immunogenicity. The comparison between the switchable and conventional CAR T cells demonstrated that the anti-tumor efficacy of these switchable CAR T system was not compromised (63). The discrepancy in efficacy results from clinical and preclinical studies may be due to the different levels of HER2 expression in patients and xenografts, and the immunosuppressive TME in the patients.

Other putative growth factor receptors that could be targeted against pancreatic cancer using CARs include insulin-like growth factor receptor-1 (IGF1R), EGFR, vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and platelet-derived growth factors (PDGFRs) that are expressed at elevated levels in pancreatic cancers and contribute the cancer's malignant phenotype (55). IGF1R is expressed in a variety of cancers and blocking IGF1R expression enhances apoptosis and suppresses metastasis in pancreatic cancer cells (64). IGF1R-CAR cells have demonstrated efficacy in murine models of sarcoma xenograft (65) and could be potentially useful in the treatment of PDAC. EGFR is a surface glycoprotein that belongs to the EGFR family of tyrosine kinase receptors. EGFR is aberrantly activated in a number of epithelial tumors and its overexpression has been detected in up to 90% of pancreatic tumors (66, 67). The value of EGFR-CAR T cells in treating solid cancers has been demonstrated in both preclinical (68) and clinical settings (69) in a few different cancer types but its potential in treating PDAC is yet to be tested.

CD24

CD24 is a mucin-like protein. It was originally discovered as the ligand for P-selectin and involved in signal transduction mediated by the members of the protein tyrosine kinase family. CD24 expression is observed in over 70% of PDAC tumors and in putative PDAC cancer stem cells (CSC). CSC can also express CD44 and epithelial specific antigen (ESA) or CD133. Given the prevalence of early-disseminated metastases, CSCs are believed to play a key role in the pancreatic cancer development

and progression. Among these PDAC CSC protein markers, CD24 has the lowest expression in normal tissue and thus is proposed as a suitable target antigen for immunotherapy (70, 71). A study carried out by Maliar et al. examined the therapeutic efficacy of both HER2-CAR and CD24-CAR in treating PDACs in murine models. In mice bearing subcutaneous human PDAC cell line Capan-1 (positive for both HER2 and CD24), intratumor injection of the CAR T cells targeting either HER2 or CD24 greatly inhibited tumor growth, in some cases, eradicated tumors. In mice bearing orthotopic Capan-1 tumors, i.v. injections of these two different CAR T cells also demonstrated anti-tumor effects to both primary and metastatic tumors at the liver and lymph nodes. Interestingly, the cells that were dissociated from patient pancreatic cancers for xenograft in this study, displayed a heterogeneous expression pattern of antigens, including both HER2 and CD24. The adoptive transfer of HER2-CAR or CD24-CAR to mice bearing these xenografts significantly arrested the tumor growth, but to a different degree, dependent on the CAR-targeted antigen specificity. The results from this study highlight the antigen heterogeneity nature of human pancreatic tumors (72). It remains to be seen if the administration of CAR T cells targeting both antigens will enhance efficacy. This study highlights potential therapeutic limitations using CARs targeting a single tumor antigen in treating complex cancers that are heterogeneous in antigen expression patterns and distributions.

Fibroblast Activation Protein

Fibroblast activation protein (FAP) is a type II integral membrane serine protease. Healthy adult tissues have no detectable FAP expression. However, under certain biological circumstances, such as remodeling, wound healing, and embryogenesis, FAP expression has been observed. FAP is also present in a large proportion of tumor stromal fibroblasts in the majority of epithelial carcinomas including pancreatic cancers (73) and its expression correlates with poor prognosis in pancreatic cancer patients (74). The carcinoma-associated fibroblasts (CAFs) are a central player in tumorigenesis and metastasis and the key characteristics of CAFs is the expression of FAP (75). Due to its high expression in CAFs, FAP has been tested as a CAR target.

Tran et al. investigated the use of FAP-CAR T cells targeting tumor stromal fibroblasts in a number of mouse tumor models and human pancreatic cancer xenografts. Despite *in vitro* activity observed, the injection of the FAP-CAR T cells only elicited limited *in vivo* anti-tumor effect. Unexpected side effects such as cachexia and lethal bone toxicities were observed. The off-target effect was due to the expression of FAP on murine bone marrow stromal cells (BMSCs). In this study, human BMSCs were also identified in expressing FAP and could be recognized by FAP-CAR T cells (76). The finding that FAP is expressed by BMSCs raised safety concerns for therapies targeting FAP.

Interestingly, in separate studies carried out by Wang et al. (77) and Kakarla et al. (78) using FAP-CAR T cells, no toxicity was observed. The reason may be due to the different scFvs used. The scFv used by Wang et al. targets a different FAP epitope and only eliminate FAP^{hi} cells, while sparing FAP^{low} cells including BMSCs.

CLINICAL TRIALS USING CAR T CELLS AGAINST PANCREATIC CANCER

A substantial number of clinical trials involving the use of CAR technology have recently been undertaken on pancreatic cancer patients in an attempt to investigate the potential safety, effectiveness and feasibility of the approach. A variety of tumor associated antigens (TAA) expressed on pancreatic cancer cells have been targeted in various clinical trials to redirect CAR T cells against pancreatic cancer including MSLN, CEA, PSCA, MUC1, and HER2 (**Table 1**).

A range of CAR formats are used in these clinical studies, although chiefly second-generation formats, with either CD28 or CD137 cytoplasmic domains. Transduction methods also vary, including electroporation with CAR-encoding RNA, but viral vectors, either retroviral or lentiviral, are the main method of CAR gene delivery (**Table 1**). Typically, dose escalation is used in these trials, which aim to determine safety, with doses ranging from 1×10^7 to 3×10^8 per m^2. Higher doses of RNA transfected T cells are used, since they present less of a risk due to their limited duration of CAR expression. The majority of studies involve a single dose of CAR T cells; some trials use multiple doses (**Table 1**).

There is substantial variation in the use of preconditioning, although some level of lymphodepletion is proposed for most studies. The combination of CY and fludarabine is often preferred to induce a deep level of lymphodepletion to enhance engraftment of transferred CAR T cells. While most of the studies detailed in **Table 1** involve the transfer of CAR T cells alone, some propose to use additional drugs to enhance CAR T cell activity. Thus, IL-2 is proposed for some studies to provide T cells with a supporting growth factor.

To enhance the effectiveness and the safety of CAR T cell therapy, some clinical trials use reagents that activate CAR T cells. For example, in trial NCT02744287 targeting PSCA, a dimerizer agent, Rimiducid (AP1903) is used. Rimiducid is administered with PSCA-specific CAR T cells that contain an inducible MyD88/CD40 (iMC) costimulatory domain. In another trial (NCT02416466), radiation is delivered using Yttrium-90 microspheres to maximize the tumoricidal effects of CAR T cells and minimize the effects on healthy liver parenchyma in patients with liver metastases.

The majority of clinical trials of CAR T cells in pancreatic cancer are in early stages of recruitment, but some have been completed and initial reports are available. CAR T cells are generally well-tolerated in pancreatic cancer patients, although some toxicity was reported when targeting CEA (47). Despite the early nature of most trials, there are some reports of CAR T cell efficacy. The CARsgen trial reported their early results at the 2018 CAR-TCR Summit in Boston that using an anti-Claudin-18.2 CAR treating pancreatic and gastric cancer patients resulted in some objective responses without overt toxicities (79, 80). However, there are no descriptions of significant responses in most trials to date, some unique challenges presented by pancreatic cancer may have to be overcome to maximize responses.

TUMOR MICROENVIRONMENT AS A UNIQUE CHALLENGE FOR CAR T CELLS IN PANCREATIC CANCER

A major obstacle for immunotherapies, in particular CAR T cell therapies, in solid tumors is the immunosuppressive tumor microenvironment (TME) (75). The TME impacts on the efficacy of CAR T cells both by limiting their infiltration and suppressing their function within the tumor (29, 81).

A unique feature of the pancreatic TME is the desmoplastic stromal reaction, which promotes tumor growth and provides a physical barrier for therapeutic drugs and T cell infiltration. In fact, PDAC is one of the most stroma-rich cancers and in some cases, the stromal components precede pancreatic cancer cells. In normal pancreas, the pancreatic stellate cells (PSCs) are a rare population and function to store retinoids in a form of lipid droplets in the cytosol. During pancreatic cancer progression, the pancreatic stellate cells (PSCs) become activated by tumor-secreted cytokines, lose retinoid droplets and transform into a myofibroblast phenotype. The activated PSCs secrete extracellular matrix (ECM) proteins and deposit collagens to form a dense fibrotic cancer stroma (82). In addition, activated PSCs secrete cytokines (such as IL-6 and IL-11) and chemokines (such as CXCL12, CCL5, CCL2, and CCL17) that recruit immunosuppressive leukocyte subsets (82–85). Indeed, as discussed above, CAR T cells that target the highly expressed CAF protein, FAP, are considered in pancreatic cancer due to the significant role these cells play in tumorigenesis (77, 86, 87) and in addition, FAP is positive in PDAC-derived ECMs but negative in normal PSCs (88). The particularly harsh TME is a major barrier to CAR T cell efficacy in pancreatic cancer and thus additional modulators will be required for durable responses (**Figure 1**).

Approaches that enable CAR T cells to sustain and function in the TMEs have been investigated in a large number of preclinical and clinical studies in a range of malignancies, and the results have revealed various potential strategies against pancreatic cancers. For example, depleting immunosuppressive cell subsets such as Tregs, MDSCs and TAMs has demonstrated enhanced efficacy of CAR T cell therapies (29). These suppressive immune cell types are enriched in pancreatic cancer and are associated with increased tumor growth and poorer prognosis (89–91). Various reagents that have shown to eliminate these suppressive immune cells or modulate their functions have been tested in murine models in recent years, such as IL-2 toxin and anti-CD25 (for Treg depletion), CSF-1R inhibitor and CCR2 toxin (for MDSCs/TAMs) (92–95). A few anti-TAM drugs such as antibodies against CCL2, CSF1R that inhibit the recruitment and survival of TAMs and MDSCs are currently under clinical investigation (96). Strategies of using CARs against these suppressive cells are emerging. For examples, inserting CSF1R-CARs into NK and T cells for killing TAMs have demonstrated promising outcomes *in vitro* (97). In our laboratory, using ACTIV therapy that involved injecting dual-specific CAR T cells and a vaccine, TAMs decreased significantly post the treatment, coinciding with tumor regression (32). In humans, the engraftment of T cells is enhanced with myeloablative preconditioning regimes and thus specific depletion methods could enhance CAR T cell efficacy in pancreatic cancer patients (98, 99).

Another strategy for enhancing CAR T cell efficacy in solid tumors is blocking the inhibitory signals received by the T cells from immunosuppressive populations in the TME. Checkpoint inhibitors, against molecules such as PD-1, CTLA4, TIM-3, and LAG-3 have shown promise as single agents in a number of cancer types (100) but unfortunately, none of these treatment as a single therapy has generated significant clinical benefit in pancreatic cancers. CAR T cells can express high amounts of these checkpoint molecules, which can lead to apoptosis and hypo-function. Results from preclinical studies clearly demonstrated that CAR T cell treatments could benefit from the addition of checkpoint inhibitors (101, 102). Some early clinical data also support the use of a combination of the checkpoint inhibitors with CAR T cell therapy in treating difficult cancers. A study carried out by Chong et al. reported that in a diffuse large B cell lymphoma patient refractory to CART19, after PD-1 blockade, the patient had an expansion of the CAR T cells and clinically significant antitumor response (103).

In addition, a number of strategies have been tested to enhance CAR cells to sustain in a suppressive cytokine milieu. Methods such as introducing the CD137 signaling domain within the CAR intracellular domain to increase mitochondrial biogenesis (104), expressing regulatory subunit I anchoring disruptor (RIAD) peptide to CAR T cells to disrupt protein kinase A (PKA) activation (105), and constitutively expressing CD40L by CAR T cells (106) have all demonstrated potential in enhancing CAR T cell treatment efficacy in solid cancers. A recent elegant study combined MSLN-CAR T cells with an oncolytic adenovirus expressing TNF-α and IL-2 to treat human PDAC xenograft models and a sygeneic mouse tumor model. This strategy significantly enhanced CAR T cell anti-tumor efficacy. The anti-tumor effect was linked to increased tumor-infiltrating lymphocytes and altered TME including altered polarization of macrophages and maturation of dendritic cells (107).

Given the limited options for efficacious pancreatic cancer treatment and the success of CAR T cells in hematological malignancies, in which TME differs in its degree of immunosuppression, overcoming this obstacle in pancreatic cancer will be an important consideration for future research.

CONCLUSIONS AND FUTURE PERSPECTIVES

Pancreatic cancer is the most lethal cancer and new therapies are urgently needed. CAR T cell therapy represents a revolutionary treatment for cancers and has generated remarkable responses in hematological malignancies. The extension of CAR T cell therapy into pancreatic cancer recently started and this field is moving forward rapidly. Due to its unique immunosuppressive TME and antigen complexity and heterogeneity (108), pancreatic cancer presents one of the most difficult cancers for immunotherapies. Understanding the pancreatic cancer TME and how this TME affects CAR T cell efficacy is key in designing effective

CAR T cell treatments. In addition, besides expanding the CAR-antigen landscape, targeting multiple antigens simultaneously (109) and using strategies targeting neoantigens (16) could provide significant opportunities for treating pancreatic cancer.

AUTHOR CONTRIBUTIONS

AA, MK, and CS: conception and design; AA, AO, TS, JC, MK, and CS: write, review, and revision of the manuscript; MK and CS: supervision.

FUNDING

This work was supported by grants from the Peter MacCallum Cancer Center Foundation, the National Health and Medical Research Council (NHMRC) of Australia (1103352 and 1132373), the National Breast Cancer Foundation (NBCF) of Australia (IIRS-18-064) and Susan G. Komen Breast Cancer Foundation (16376637). CS was supported by a Fellowship from the NBCF. AA was supported by a University of Melbourne International Research scholarship, AO by an Australian Postgraduate Award.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

My above published article covered the field up to February 2019. However, since then there have been several updates to the field, for which I present details here. In an update to a phase I study investigating the safety and the feasibility of mesothelin-specific CAR T cells (CART-meso) in 15 patients with different cancer types including pancreatic cancer, patients were administered with a single CART-meso cell infusion with or without a preconditioning lymphodepleting step using cyclophosphamide. CART-meso cells were generally well tolerated but one patient showed grade IV sepsis. 11/15 patients demonstrated stable disease and cyclophosphamide enhanced CART-meso cells expansion but not persistence in the blood after 28 days [256]. Even with this limited activity more studies are still ongoing to enhance the anti-tumour activity of CART-meso cells (ClinicalTrials.gov: NCT0354298and NCT03323944).

Moreover, to enhance the migration and persistence of CAR T cells in tumours, Jin et al. recently used preclinical tumour models, including pancreatic cancer, that naturally express IL-8 or secrete IL-8 following radiation. They found that CAR T cells engineered with the IL-8 receptors chemokine receptor type 1 (CXCR1) or CXCR2 improved both the migration and persistence of these cells in the tumour, which induced complete tumour regression with long-lasting immunologic memory [257]. In another recent study, to enhance the safety of CAR T cells, Raj *et al.* utilized anti-Her2 switchable CAR-T cells against aggressive and disseminated patient-derived xenograft models derived from stage IV pancreatic patients. Switchable CAR-T cell activity is controlled by varying the amount of an intermediating bispecific antibody linking a CAR to TAA. These CAR T cells were very efficient and induced complete remission and afford potentially safer and more effective immunotherapy treatment [247].

The wide expression of the immune checkpoint molecule B7-H3 (CD276) in several solid tumours, lead a recent study to develop CAR T cells that target this molecule. B7-H3 CAR-T cells demonstrated potent ability to control the growth of different solid tumours including pancreatic cancer in orthotopic and metastatic PDX xenograft mouse models without evident toxicity [258]. Interestingly, B7-H3 functions as an immune checkpoint in T cells, so targeting this molecule may combine checkpoint blockade with CAR T cell activity.

1.8. Overall summary and focus of thesis

Pancreatic cancer is predicted to be the second leading cause for cancer-related deaths within the next decade. This challenging disease is characterized by the aggressive immunosuppressive TME. Immunotherapy approaches are increasingly effective in improving the OS of other cancer types. However, in pancreatic cancer, there is an urgent need for deeper understanding of its immunology in order to translate this knowledge into effective therapies. The CAR T cell approach is a promising therapy that can bring hope to fight this silent killer.

A range of approaches aimed at enhancing CAR T cell efficacy are currently undergoing investigation. A notable strategy that has demonstrated promising effects *in vivo* is the use of dual-specific T cells. Dual-specific T cells co-express a CAR against a tumour antigen and a TCR against a strong immunogen. Through vaccination, dual-specific T cells can engage the cognate immunogen of the chosen TCR presented by APCs on MHC molecules, enabling their activation, proliferation and enhancing their persistence. In our laboratory we used a novel regimen: "adoptive cell transfer incorporating vaccination" (ACTIV) therapy using dual-specific T cells to treat a variety of mouse cancers. ACTIV therapy demonstrated durable responses in a range of solid tumours in mice. In that previous study, we generated dual-specific T cells expressing a CAR against Her2 and TCR specific for the melanocyte protein gp100 (also known as pMEL). We termed those dual-specific T cells, "CARaMEL" T cells. The combination of adoptive transfer of CARaMEL T cells and a gp100-specific vaccine drove dramatic T cell expansion and tumour regression in breast tumours, subcutaneous sarcoma, and colon carcinoma present as large liver masses. Moreover, surviving mice that received ACTIV therapy developed potent immune memory responses and were able to reject subsequent challenge with tumour cells [129].

1.9. Research hypotheses and aims

In this thesis I aimed to determine the efficacy of ACTIV therapy against murine and human pancreatic cancers in mouse models Following the next chapter, which presents detailed Materials and Methods, I present three data chapters investigating the hypothesis that: "ACTIV therapy can inhibit tumour growth in subcutaneous mouse models of pancreatic cancer".

First aim (Chapter 3): Dual-specific T cell transfer for pancreatic cancer treatment. In this chapter I evaluated the combination of dual-specific CAR T cells and an indirect vaccine (ACTIV therapy) in murine pancreatic cancer. I studied the function of dual-specific

CARaMEL T cells against two pancreatic cancer cell lines *in vitro* and *in vivo*. Also, I sought mechanistic insight to determine limitations and identify potential ways to improve efficacy.

Second aim (Chapter 4): Enhancing the anti-tumour activity of ACTIV therapy using HDACi and CD40 agonist. In this chapter, I attempted to enhance the anti-tumour activity of ACTIV therapy using HDACi and a CD40 agonist antibody. In addition, I determined the mechanistic contribution of these additions towards anti-tumour activity.

I hypothesized that epigenetic manipulation using histone deacetylase inhibitors (HDACi) can enhance ACTIV therapy by modulating gene expression to sensitize tumour cells to T cell and vaccine activity. I also hypothesized that an agonist CD40 monoclonal antibody may enhance the anti-tumour activity of ACTIV therapy against murine pancreatic tumour through either enhancing T cell-mediated anti-tumour responses [116] and/or increasing macrophage-mediated anti-tumour responses [109].

Third aim (Chapter 5): ACTIV therapy of pancreatic cancer in a humanized xenograft model in mice. In this chapter, I explored the translational potential of ACTIV therapy using human cells. I hypothesized that human dual-specific T cells (CARaMEL T cells) combined with an indirect vaccine would inhibit the growth of human pancreatic cancer cells *in vitro* and *in vivo*, using a tumour xenograft model in immunodeficient mice.

CHAPTER 2: Material and Methods

2.1. Mice

All mice work performed was approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee (AEEC applications E498 and E582). Male and female C57BL/6-CARaMEL, C57BL/6-CARaMEL-Thy 1.1, C57BL/6 wild type, C57BL/6 OT-I, C57BL/6 human-Her2, C57BL/6-pMel-1/CAR-Prf KO, C57BL/6-pMel-1/CAR-IFN-γ KO and NOD SCID mice used in this study were bred on site at the Peter MacCallum Cancer Centre (Melbourne, Vic, Australia).

The immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg (HLA-A/H2-D/B2M) 1Dvs/SzJ (NSG-HLA-A2/HHD) expressing human HLA [259] were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were bred and maintained under pathogen-free conditions at the Peter MacCallum Cancer Centre.

C57BL/6-CARaMEL mice were generated by crossing C57BL/6-CAR (expressing anti-Her2-CD28-CD3ζ CAR) [260] and C57BL/6-pMEL (expressing a TCR specific for human gp100, also known as the premelanocyte antigen, pMEL) [261]. C57BL/6-CARaMEL-Thy 1.1 mice were generated by crossing CARaMEL mice to C57BL/6-Thy 1.1 mice. C57BL/6 human-Her2 mice were generated on a C57BL/6 background and express human Her2 under the control of the whey acidic protein promoter [260]. C57BL/6-pMel-1/CAR-Prf KO mice were generated by crossbreeding C57BL/6-pMel-1/CAR onto perforin knock-out transgenic mice. C57BL/6-pMel-1/CAR-IFN-γ KO mice were generated by crossbreeding C57BL/6-pMel-1/CAR onto IFN- γ knock-out transgenic mice.

All types of mice used in the experiments were used at the age of >8 weeks and gender-matched mice were used within the experiment and they were housed in PC2 specific pathogen-free conditions. Mice were ear-tagged for individual identification. The numbers of the mice were recorded, and mice were randomized to treatment groups after recording the tumour size. A digital caliper was used to measure the two perpendicular axes for tumour size. Mice were humanely culled when tumours reached 150-200 mm^2.

2.2. Cell lines

The murine pancreatic cell lines KPC (83220 PZPR R172H) and Panc02 were kindly provided by Prof Paul Timpson (Garvan Institute of Medical Research, Darlinghurst, NSW, Australia) and Prof Hinrich Abken (University of Cologne, Germany) respectively.

The human cell lines Panc-1, MiaPaCa2, MDA-MB-435, 293A and HeLa-S3 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The human melanoma cell line 623.38 Mel was kindly provided by Dr Steven Rosenberg (National Cancer Institute, Bethesda, Maryland, USA).

The mouse breast cancer cell line E0771 was kindly provided by Dr. Robin Anderson (Peter MacCallum Cancer Centre, Vic, Australia).

The parental tumour cell lines KPC, Panc02, Panc-1, MiaPaCa2, MDA-MB-435 and E0771 were transduced with a retroviral vector, murine stem cell virus (MSCV), to express full length hHer2 antigen, and were referred to as KPC-Her2, Panc02-Her2, Panc-1-Her2, MiaPaCa2-Her2, MDA-MB-435-Her2 and E0771-Her2 respectively as described in **Chapter 2 section 2.5**.

The retroviral vector packaging cell lines (PG13/154-EClIAlB, gp100 TCR) was kindly provided by Dr Steven Rosenberg (National Cancer Institute, Bethesda, Maryland, USA). The PG13 Erb CD28ζ, Her2 CAR packaging line and the empty-vector retroviral vector packaging cell line were generated previously at the Peter MacCallum Cancer Centre [262] from the parental PG13 cell line obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The retroviral packaging HEK293GP cell line used was obtained from ATCC (Manassas, VA, USA).

Primary human T cells were generated from peripheral blood mononuclear cells (PBMCs) of healthy donors kindly provided as fresh buffy coats by (Australian Red Cross Blood Service, Vic, Australia) or from apheresis of healthy donors or a patient with metastatic endometrial cancer after their signed consent forms and approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (Peter MacCallum Cancer Centre project number: 16/17. HREC reference number: HREC/16/PMCC/30).

Human DCs were generated from peripheral blood, provided as fresh buffy coats from healthy donors (Australian Red Cross Blood Service, Vic, Australia).

HLA-A*02^{+} murine DCs were generated from femurs and tibiae from female and male NSG-HLA-A2/HHD mice > eight weeks old.

All tumour cell lines and retroviral vector packaging cell lines were cultured and maintained in DMEM media (Gibco, Life Technologies, Grand Island, New York, USA) containing 10% heat-inactivated fetal bovine serum (FBS), (MultiSer, Thermo Trace, Vic, Australia), 1 mM sodium pyruvate (Gibco), 0.2 mM non-essential amino acids (NEAA) (Gibco), 2 mM glutamine (Gibco), 100 U/mL penicillin/streptomycin (Sigma-Aldrich, St Louis, MO) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in an incubator at 37°C with 10% CO_2.

Murine and human T cells and DCs were cultured and maintained in RPMI media (Gibco, Life Technologies, Grand Island, New York, USA) containing the complete supplements as described above, in an incubator at 37°C with 10% CO_2.

All human and murine cell line cultures were confirmed as mycoplasma free by polymerase chain reaction (PCR) (Cerberus Sciences, Scoresby, Australia).

2.3. Media, buffers and solutions

Table 2.1. List of media, buffers and solutions

Media/Buffer	Ingredients
Supplemented RPMI-1640/10% RPMI	RPMI-1640 media (Life Technologies, Gibco, Grand Island, NY) containing 10% heat-inactivated foetal calf serum (FCS) (MultiSer, Thermo Trace, Vic, Australia), 1 mM sodium pyruvate (Gibco, Thermo Fisher Scientific, MA, USA), 0.2 mM NEAA (Gibco, Thermo Fisher Scientific, MA, USA), 2 mM glutamine (Gibco, Thermo Fisher Scientific, MA, USA), 100 U/mL penicillin/streptomycin (Sigma-Aldrich, St Louis, MO) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
Supplemented DMEM/10% DMEM	DMEM media (Life Technologies, Gibco, Grand Island, NY) containing 10% heat-inactivated foetal calf serum (FCS), 1 mM sodium pyruvate, 0.2 mM NEAA, 2 mM glutamine, 100 U/mL penicillin/streptomycin and 10 mM HEPES.
Fluorescence-activated cell sorting (FACS) buffer	Dulbecco's phosphate-buffered saline PBS $^{-/-}$ supplemented with 2% FCS, 2 mM EDTA, pH 8.
10× Annexin V binding buffer	0.1 M HEPES, 8.18 g NaCl (1.4 M), 0.28 g $CaCl_2$ (0.025 M) made up in 100 mL distilled H_2O, pH 7.4 H_2O was used for further dilutions.
Ammonium-Chloride-Potassium (ACK) lysis Buffer	8.29 g/L NH_4Cl (0.15 M), 1 g/L $KHCO_3$ (1 mM) and 200 mL/L of 0.6 M EDTA made up in 1 L distilled H_2O in final pH 7.4
10 mM Citrate buffer	1.47 g Trisodium citrate dihydrate $Na_3C_6H_5O_7$. (Catalogue number: S1804, Sigma) in 500 mL dH2O, supplemented with Hydrochloric acid HCl to pH 6.
ELISA Blocking Buffer	Dulbecco's phosphate-buffered saline PBS $^{-/-}$ supplemented with 2% FCS.
ELISA wash buffer (PBS/Tween)	PBS with 0.05% Tween.
ELISA substrate solution	One tablet of 3.3'-5.5'-Tetramethylbenzidine dihydrochloride (catalogue number: T3405, Sigma Aldrich, Missouri, USA) in 10 mL phosphate-citrate buffer (2M Na2HPO4, 1 M citric acid, pH 5) to which 2 µL of hydrogen peroxide (0.2 µg/mL, Merck, Australia).
ELISA stop Solution	1 M H_2SO_4 (1/18 dilution of acid; 5.6 mL of H_2SO_4 in 100 mL H_2O).
MACs buffer	PBS pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA.
EasySep human monocyte isolation buffer	PBS containing 2% FBS and 1 mM EDTA.

2.4. Viruses

The recombinant Vaccinia virus (VV-gp100): The vaccinia virus VV-gp100 was previously generated by incorporation of $gp100_{25–33}$ minigene into the *F4L* gene encoding ribonucleotide reductase (RR) [263]. VV-gp100 was kindly provided by Nicholas Restifo (National Cancer Institute, Bethesda, Maryland, USA).

The recombinant adenovirus (Ad2CMV-gp100): is a type II adenovirus and was generated by replacing the El region with the full length human gp100 gene under the control of CMV immediate early enhancer-promoter element. Ad2CMV-gp100 was kindly provided by Dr Steven Rosenberg (National Cancer Institute, Bethesda, Maryland, USA). Adenovirus was produced in the 293A cell line which is a sub-clone of the human embryonic kidney (HEK293) that can help in adenovirus production and amplification. The backbone vector was derived from adenovirus type 2, and it is replication defective due to most of the El-coding **se**quences being replaced with DNA encoding for gpl00 under the transcriptional control of the CMV immediate early enhancer-promoter element [264].

2.4.1. VV-gp100 production and purification

Human cervical carcinoma (HeLa S3) cell line was cultured in T175 flasks (Greiner Bio-One, Düsseldorf, Germany) in supplemented DMEM. The following day, about 80% confluent Hela S3 cells, supernatant was replaced with 5 mL DMEM (without supplements) containing 10^7 PFU VV-gp100. The virus was vortexed, sonicated for one minute at 4°C at high frequency to remove any clumps before it was added to the cells. Cells were then incubated at 37°C, 10% CO_2 for two hours. Next, 15 mL of supplemented DMEM was added to the flasks and incubated for three days. After that, infected Hela S3 cells were collected using a cell scraper and centrifuged at 450 xg for four minutes. The cell pellet from 20 T175 flasks was frozen at -80°C. Cells were thawed and frozen again for three times to lyse the cells. A Dounce tissue homogenizer was used to homogenise the cells (~50 strokes, 10-15 mL per time). Next, cell lysate was centrifuged at 300 ×g for five minutes s to remove any aggregates and frozen again at -80ºC for further viral purification. To isolate and purify VVgp100, the cell lysate was sonicated for one minute at 4°C at high frequency, and then it was layered over a cushion of 5 mL of 36% sucrose in Tris in an ultracentrifuge tube (Beckman #344059). Cell lysate was then centrifuged in a SW 41 Ti Swinging-Bucket Rotor (Beckman Coulter centrifuge) at 32,900 ×g (accelerate Maximum, decelerate 5×) at 4ºC for 80 minutes s. Next, the pellet was resuspended in 700 µL of 1 mM Tris-HCl (pH 9) and frozen at -80°C.

Then, eight tubes of sucrose gradients were prepared for the next day by sequentially layering of the following:

40% × 3 mL (20 ×g dissolved in 50 mL 1 mM Tris pH 9)

36% × 2.2 mL (18 ×g dissolved in 50 mL 1 mM Tris pH 9)

32% × 2.2 mL (16 ×g dissolved in 50 mL 1 mM Tris pH 9)

28% × 2 mL (14 ×g dissolved in 50 mL 1 mM Tris pH 9)

24% × 1 mL (12 ×g dissolved in 50 mL 1 mM Tris pH 9)

Tubes were then left in the dark at 4°C overnight to allow the gradient to diffuse.

On the next day, the cell lysate was sonicated for one minute at high frequency and overlaid on the four tubes of sucrose gradient, which were prepared the day before. The tubes were centrifuged 50 minutes at 26,000 ×g (SW41 Ti) at 4ºC. Then, the middle visible opaque virus band was collected in a 50 mL tube and kept at 4ºC. The viral pellet cells was resuspended in 1 mL 1 mM Tris-HCl (pH 9) and sonicated at the high-speed for one minute. Then, it was layered over the other four sucrose gradient tubes and centrifuged at 26,000 ×g for 50 minutes again. Then, the small middle milky band was combined with the previous band and the volume was brought up to 40 mL in 1 mM Tris-HCl (pH 9). Next, the virus was transferred to four ultracentrifuge tubes and centrifuged at 33,000 ×g using SW41 for 40 minutes. The final product was resuspended in 1.5 mL of 1 mM Tris-HCl (pH 9) and kept at -80°C. The titre of infectious virus particles was determined by plaque assay.

2.4.2. VVgp-100 titration

To titre VV-gp100, 1×10^5 HEK293A cells were cultured in six well plates (Grenier Bio-One, Düsseldorf, Germany) in fully supplemented media and incubated at 37°C and 5% CO_2 overnight. The following day, purified VV-gp100 was diluted (10^{-3} to 10^{-14}) in DMEM media (without supplements). Media from HEK293A cells was removed and cells were infected in duplicate wells with 1 mL of the 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} of sonicated VV-gp100 dilutions. Infected cells were incubated at 37 °C and 5% CO_2 for two hours. Then, 4 mL supplemented DMEM was added to each well and incubated at 37 °C and 5% CO_2 for 48 hours. After that, the media was removed, and 1 mL of ice-cold methanol was added for one minute to fix the cells. Then, methanol was aspirated and 1 mL of 0.1% crystal violet was added to each well for one minute at room temperature. Plates were then washed with tap water and allowed to dry on bench. The titre of VV-gp100 was determined by counting plaques within the wells and multiplying by the dilution factor as the following equation:

$$\mathrm{PFU/mL} = \frac{\text{number of plaques} \times \text{volume of plated virus (mL)}}{\text{dilution of virus}}$$

2.4.3. Ad2CMV-gp100 amplification and testing

Ad2CMV-gp100 was sent to Vector Biolabs (Great Valley Parkway Malvern, PA, USA) to be amplified and produced in large quantities under good laboratory practice conditions. The final adenoviral stocks contained up to 1×10^{10} infectious units/mL. Ad2CMV-gp100 was evaluated *in vitro* by infecting HLA-$A2^+$ PBMCs then testing for recognition by CARaMEL T cells using IFN-γ ELISA.

2.5. Generating $Her2^+$ cancer cell lines

2.5.1. Viral supernatant preparation

The cell line HEK293GP was seeded at 1.4×10^7 in 175 cm^2 flasks (Greiner Bio-One) coated with at 50 µg/mL Poly-D-lysine (catalogue number A3890401, Thermo Fisher Scientific, MA, USA) with 15 mL supplemented DMEM (antibiotic-free). Cells were incubated at 37°C and 5% CO_2 overnight. On the following day, media was replaced with 15 mL fresh supplemented DMEM. DNA lipofectamine complexes were prepared: Solution A: 175 µL of Lipofectamine 2000 (catalogue number 11668030, Thermo Fisher Scientific, MA, USA) in 1750 µL Opti-MEM (catalogue number 31985062, Thermo Fisher Scientific, MA, USA). Solution B: 35 µg of MSCV-Her2 plasmid (addgene plasmid number: 91888, after GFP was removed) and 35 µg MDG/VSV envelope plasmid in 1750 µL Opti-MEM. Solutions were incubated for five minutes at room temperature; then solution B was added into solution A and incubated for 30 minutes at room temperature to allow DNA-lipofectamine complexes to form. To generate retrovirus, the mixed solution was then added drop-wise to the cell monolayer and incubated at 37°C with 5% CO_2 overnight. On the following day, the media was replaced with 30 mL of fresh supplemented DMEM and incubated at 37°C and 5% CO_2 overnight. On the following two days, the supernatant was harvested, filtered through an Acrodisc© 0.45 µm filter (Pall Life Sciences, NY) and frozen at -80°C.

2.5.2. Retroviral transduction of cancer cell lines to express Her2

The cancer cells were seeded at 2×10^5 in six well plates (Grenier Bio-One, Düsseldorf, Germany) and incubated at 37°C with 5% CO_2 overnight. On the following day, the media was

carefully aspirated from the wells and 5 mL of viral supernatant was added with 8 µg/mL polybrene (Hexadimethrine bromide: catalogue number: H9268-5G, Sigma-Aldrich, Missouri, USA). Then plates were sealed and centrifuged at 1200 ×g at room temperature for 90 minutes. Plates were then incubated overnight at 37°C with 5% CO_2. Transduction procedure was repeated twice again on the next two consecutive days. Then, the cells were transferred to 75 cm^2 flasks (Greiner Bio-One). Two days later, cells were analysed for Her2 expression using flow cytometry analysis as described in **Chapter 2 section 2.21.1** Cells were then sorted for high Her2 expression as described in **Chapter 2 section 2.21.3** and 3-5 clones were expanded and used in further *in vitro* and *in vivo* experiments.

2.6. Isolation of murine splenocytes

Spleens were collected from CARaMEL or OT-I mice and crushed in a six well plate with 1.5 mL ACK lysis buffer (**Table 2.1**) for 1 minute at room temperature. Then, cells were filtered through a 70 µm filter with 10 mL of PBS. Cells were centrifuged at 450 ×g for four minutes at 4ºC. Supernatant was aspirated and cells were washed again with 10 mL PBS and resuspended in 1×10^7 cell/200 µL in PBS to be injected in mice or cultured and activated for further *in vitro* assays as described in the next section.

2.7. Isolation of naïve $CD8^+$ murine T cells

Isolation of mouse naïve CARaMEL or OT-I $CD8^+$ T cells in this study was performed using Miltenyi Biotec MACs $CD8\alpha^+$ T cell isolation kit (catalogue number: 130-104-075, Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. First, cell number was determined, and cell pellet was resuspended cell in 40 µL of MACs buffer (**Table 2.1**) per 10^7 total cells. Then, 10 µL of Biotin-Antibody Cocktail per 10^7 total cells was added, mixed well and incubated for five minutes in the refrigerator (6°C). Following this, 30 µL of buffer per 10^7 total cells and 20 µL of anti-Biotin MicroBeads per 10^7 total cells were added, mixed well and incubated for 10 minutes in the refrigerator (2−8°C). For magnetic cell separation step: LS Column was placed in the magnetic field of a MACS separator and the column was prepared by rinsing with 3 mL of buffer. Following incubation, the cell suspension was applied onto the column. The flow-through containing unlabelled cells, representing the

enriched $CD8^+$ T cells, was collected. The column then was washed with a further 3 mL of buffer to collect more unlabelled cells.

2.8. Activation of murine CARaMEL T and OT-I cells

Murine splenocytes were isolated from mice as mentioned in the previous section (**Chapter 2, section 6**). Cells were resuspended in complete RPMI media with supplements at 1x 10^6 cells/mL in 24-well plates (Grenier Bio-One, Düsseldorf, Germany). To activate CARaMEL T cells 0.1 µg/mL of hgp100 $_{(25\text{-}33)}$ (catalogue number: 58686-025, sequence: Lys-Thr-Trp-Gly-Gln-Tyr-Trp-Gln-Val, Mimotopes, Vic, Australia) peptide was added. To activate OT-I T cells 0.1 µg/mL of ovalbumin SIINFEKL peptide (OVA257-264, Auspep, Vict, Australia) was added. In addition, 200 pg/mL of interleukin-7 (IL-7) (catalogue Number: 217-17, Peprotech, Rocky Hill, New Jersey) and 100 U/mL of IL-2 were added. Cells were incubated at 37°C and 5% CO_2 for five days. On day 3, cells were split into additional 24 well plates in supplemented RPMI with IL-7 (200 pg/mL) IL-2 (100 U/mL) to maintain cell densities around 1 × 10^6 cells/mL.

2.9. Retroviral transduction of primary human T cells

Day 0: PBMCs were isolated from 50 mL healthy donor buffy coats which were provided by (Australian Red Cross Blood Service, Australia) in a citrate phosphate dextrose adenine (CPDA) plastic bag or frozen apheresis from patients of the Peter MacCallum Cancer Centre. Buffy coats were diluted in a 1:1 ratio with PBS, and 30 mL aliquots layered onto 15 mL Ficoll-Paque medium (Histopaque, Sigma, USA) in 50 mL tubes, and centrifuged at at room temperature for 30 minutes at 400 ×g with the brake off/soft stop. This procedure caused accumulation of the erythrocytes and the granulocytes at the bottom of the tube, while the lymphocytes and other mononuclear cells remained at the plasma-histopaque interface, which was then collected and transferred to new 50 mL tubes containing 25 mL PBS. The tubes were then centrifuged at 450 ×g at 4ºC for four minutes. This washing step was repeated twice, and viable cell count was performed using 0.4% trypan blue (catalogue number: T10282, Invitrogen). Viable cells were then seeded at 1 × 10^6/mL in supplemented RPMI, and 600 IU/mL of IL-2 and 30 ng/mL of anti-CD3 monoclonal antibody (OKT3, Orthoclone™, Janssen, Australia) were added to the culture to stimulate T cell proliferation.

Day 2: The retroviral producer cell lines (either PG13/154-EClIAlB (gp100 TCR) or PG13 Erb CD28ζ, (Her2 CAR) cell lines) were seeded at 1.1 × 10^7 in 20 mL of supplemented RPMI in

175 cm^2 flasks (Greiner Bio-One). Cells were incubated at 37°C and 5% CO_2 overnight. Also, RetroNectin™ (Retronectin; Takara Shuzo Co. Ltd, Otsu, Japan) was diluted to a concentration of 10 µg/mL in PBS. Non-tissue-culture-treated 6-well plates (Multiwell, Becton Dickinson, San Jose, CA) were coated with 1.5 mL of Retronectin solution in each well and kept at 4°C overnight.

Day 3: The viral supernatant from the producer cell lines was filter through a 0.45 µm filter (Pall Corporation, Cheltenham, Vic, Australia). Retronectin solution was aspirated and the plates were rinsed for three times with 2 mL of PBS, and 4 mL of viral supernatant was preloaded into each well of the Retronectin-coated 6-well plates. Plates with viral supernatant were incubated then for four hours at 37ºC. Then, 2.5×10^6 T cells were harvested and resuspended in 5 mL of filtered retroviral supernatant. After that, these T cells were added onto the prepared Retronectin-coated six well plates with the retroviral supernatant. Then, plates were sealed and centrifuged at 1000 ×g at room temperature for 90 minutes. After centrifugation, plates were incubated overnight at 37°C with 5% CO_2. On the following day, the same transduction procedure was repeated again.

To generate gp100 TCR T cells, the supernatant of PG13/154-EClIAlB, gp100 TCR packaging line was used. To generate anti-Her2 CAR T cells the supernatant of PG13 Erb CD28ζ, Her2 CAR packaging line was used. For dual CARaMEL T cells, a 1:1 ratio of PG13/154-EClIAlB, gp100 TCR and PG13 Erb CD28ζ, Her2 CAR packaging line supernatants were used for transduction.

Cells were then maintained at 1×10^6/mL in complete media supplemented with IL-2 600 IU/mL. On day 5, G418, Geneticin (catalogue number 10131027, Thermo Fisher Scientific, Waltham, MA, USA) was added to the CAR containing cell culture to select the cells using 750 µg/mL. Expression of CAR and TCR on T cells was measured eight days post-transduction by flow cytometry.

2.10. Rapid expansion protocol (REP)

On day 10 transduced T cells were sorted for the positive receptors: Gp100 TCR T cells for single positive TCR; Anti-Her2 CAR T cells were sorted for single positive CAR and dual-specific CARaMEL T cells were sorted according to their double positive receptors (**Chapter 2 section 21.3**). This sorting of T cells yields approximately (2.4×10^5-1.5×10^6) cells and

were expanded in 100 mL supplemented RPMI with 600 IU/mL IL-2 with (30 ng/mL) OKT-3 (anti-CD3) antibody and (50 Gy) irradiated allogeneic PBMCs feeder cells (2×10^8), (Precision X-Ray PXi, North Branford, New Haven County, Connecticut, USA) and incubated in an upright 175 cm^2 tissue culture flask at 37°C in 5% CO_2.

After four days, 50 mL of culture supernatant was removed by aspiration and media was replaced with 50 mL supplemented RPMI containing 6000 IU/mL IL-2. After one week and every day thereafter, cells were split into additional flasks to maintain cell densities around 1 $\times 10^6$ cells/mL. After two weeks of REP cells were ready and expression of CAR and TCR on T cells was measured by flow cytometry and cells were used for further *in vitro* and *in vivo* experiments.

2.11. Cryopreservation of human PBMCs

After counting the cell suspension, cells were centrifuged at 450 ×g for four min at room temperature. Then, the supernatant was removed, and cells were gently resuspended in freezing medium (90% FBS and 10% DMSO) at 1 x 10^7 cells/mL. Next, 1 mL of cell suspension was dispensed in 2 mL CRYO.S vials (catalogue number: 126263, Greiner Bio-One) and the vials were placed in into Mr. Frosty containers (Nalgene, UK) and then placed in a -80 ˚C freezer. After 1-3 days at –80 ˚C, vials were transferred into standard freezer boxes in the vapour phase of a liquid nitrogen container.

2.12. Generation of immature and mature monocyte-derived human dendritic cells

Monocytes were isolated from healthy donor frozen PBMCs using EasySep® negative selection monocyte isolation kits (catalogue number: 19359, StemCell Technologies, Vic, Australia). First, frozen PBMCs were incubated with DNase I Solution (catalogue number: 07900, StemCell Technologies, Vic, Australia) at a concentration of 100 µg/mL at room temperature for 20 minutes prior to labelling and separation. Then cells were filtered to remove any aggregated suspensions through a cell strainer. Next, cell concentration of samples was prepared 5×10^7 cells/mL in 2 mL of EasySep human monocyte isolation buffer (**Table 2.1**). Then, cells were added to a 5 mL polystyrene round-bottom tube (catalogue number: 38007, StemCell Technologies, Vic Australia). After that, 50 µL/mL of isolation cocktail and 50 µL/mL of platelet removal cocktail were added. Then, cells were mixed and incubated for five minutes at room temperature. Following incubation, magnetic particles were vortexed till they

were evenly dispersed, and 50 µL/mL was added, mixed and incubated for five minutes at room temperature. Then, 2.5 mL of buffer was added and mixed by gently pipetting up and down three times. Then the tube was replaced into the magnet (catalogue number: 18000, StemCell Technologies, Vic, Australia) and incubated for 2.5 minutes at room temperature. Next, the magnet and tube were inverted in one continuous motion to pour the enriched cell suspension into a new tube. Isolated cells were then counted and used for co-culture assays and purity was > 97% as determined by CD14 surface staining measured by flow cytometry.

Cells were then cultured in supplemented RPMI containing 50 ng/mL of granulocyte-macrophage colony-stimulating factor GM-CSF (catalogue Number: 300-03, Peprotech, Rocky Hill, New Jersey) and 100 ng/mL of recombinant human IL-4 (IL-4) (catalogue number: 200-04, Peprotech, Rocky Hill, New Jersey) at a cell density of 1×10^6 cells/mL and incubated for six days in non-tissue-culture-treated 6-well plates (Multiwell, Becton Dickinson, San Jose, CA). After three days, 75% of media was replaced with fresh supplemented RPMI containing (50 ng/mL) GM-SCF and (100 ng/mL) IL-4. All non-adherent or loosely adherent cells were saved by centrifuging culture supernatant 10 minutes at 200 ×g and adding the pellet to the fresh culture medium. After six days, the immature monocyte-derived human dendritic cells were ready for use. Mature dendritic cells were generated from immature monocyte derived dendritic cells by adding 50 ng/mL tumour necrosis factor alpha (TNF-α) (catalogue Number: 300-01A, Peprotech, Rocky Hill, New Jersey) to the supplemented RPMI which contain (50 ng/mL) GM-SCF and (100 ng/mL) IL-4 and incubated for two more days.

2.13. Generation of HLA-A*02$^+$ murine DCs

Femurs and tibiae of female and male NSG-HLA-A2/HHD mice (>8 weeks old) were detached and isolated from the surrounding tissues by rubbing with Kim wipes. Then both ends were cut using a scissors and the marrow was flushed with PBS through a syringe fitted with a 0.45 mm diameter needle onto a 70 µm filter on a 50 mL tube. Clusters within the marrow suspension were disintegrated by pushing cells through the strainer using the plunger of a 5 mL syringe. Then, the strainer was rinsed with PBS and cells were centrifuged at 450 ×g for four minutes at room temperature. Cells were resuspended in 1 mL ACK lysis buffer (**Table 2.1**) and incubated for one minute at room temperature. Cells then were washed with 10 mL supplemented RPMI and centrifuged at 450 ×g for four minutes at room temperature. Again cells were washed with 20 mL supplemented RPMI and cells were resuspended in

supplemented RPMI with (50 ng/mL) recombinant murine GM-CSF (catalogue Number: 315-03, Peprotech, Rocky Hill, New Jersey) in a six well non-tissue culture plate (5 mL/well) and incubated at 37°C with 5% CO_2. Every three days cells were split with fresh supplemented RPMI containing (50 ng/mL) GM-CSF. DCs in suspension were harvested after seven to 12 days culture.

2.14. Tumour inoculation and treatment

2.14.1. Murine tumour inoculation and treatment

Panc02-Her2 or KPC-Her2 cells (1×10^6 cells/mouse) in 200 µL PBS were injected subcutaneously into C57BL/6 human-Her2 transgenic mice and allowed to grow for nine days. When tumour size reached about 35-50 mm^2, treatment was initiated. ACTIV therapy consisted of four components. First, mice received 4 gray (Gy) total body radiation for lymphodepleting preconditioning using X-RAD320 (Precision X-Ray PXi, North Branford, New Haven County, Connecticut, USA). Then, mice received intravenous injections of (1×10^7/200 µL) mouse CARaMEL splenocytes in PBS, and after two hours, mice received (2×10^7 PFU/200 µL) VV-gp100 in PBS intravenously. Then, the mice were injected intraperitoneally with (1×10^5 IU/injection) recombinant human interleukin (IL-2) (Biological Resources Branch, National Cancer Institute, Frederick, MD and Jiangsu Kingsley Pharmaceutical, China). IL-2 was administered twice in the following day and once on day two of treatment. A tumour-bearing control group was left non-treated (NT) for comparison. In the experiments involving Panobinostat 15 mg/kg Panobinostat (Novartis Pharmaceutical Inc, Shanghai, China) was administered intraperitoneally into mice. For *in vivo* experiments, Panobinostat was dissolved by 5% dextrose to make a 1.53 mg/mL stock solution and sonicated on high frequency till it was completely dissolved.

In the experiments involving αCD40 antibody, mice were administered intraperitoneally with 10 µg/injection of anti-CD40 monoclonal antibody (clone: FGK4.5, IgG2a, catalogue number: BE0016-2, BioXCell, New Hampshire, USA). This concentration was used to reduce toxicity concerns that had been described previously [265, 266]. Alternatively, control groups of mice were administered with vehicle or 10 µg/injection of isotype control IgG2a antibody (clone: 2A3, catalogue number: BE0089, BioXCell, New Hampshire, USA). Control groups were included in each experiment as indicated in each figure.

2.14.2. Neutralizing and depletion assays

Mice were intraperitoneally injected with 200 µg/mouse of anti-IFN-γ, anti-CXCR3, anti-IFNAR-1, anti-IFNAR-1, anti-F4/80, anti-IL-12 or IgG2a on days (-1, 0, 5, 12, 19) of therapy or 1 mg/mouse of anti-TNF-α antibody on day -1 and then daily for 1 week (**Table 2.2**).

Table 2.2. List of neutralizing and depletion antibodies

Antibody	**Clone**	**Company**	**Administration**
Anti-IFN-γ	XMG1.2	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 200 µg/mouse on days (-1, 0, 5, 12, 19) of therapy.
Anti-TNF-α	TN3-19.12	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 1 mg/mouse on days -1 of therapy and then daily for 1 week
Anti-CXCR3	CXCR3–173	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 200 µg/mouse on days (-1, 0, 5, 12, 19) of therapy.
Anti-F4/80	CI: A3-1	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 200 µg/mouse on days (-1, 0, 5, 12, 19) of therapy.
Anti-IL-12 p75	R2-9A5	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 200 µg/mouse on days (-1, 0, 5, 12, 19) of therapy.
Anti-IFNAR-1	MAR1-5A3	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 200 µg/mouse on days (-1, 0, 5, 12, 19) of therapy.
IgG2a	2A3	BioXCell, New Hampshire, USA	Mice were intraperitoneally injected with 200 µg/mouse on days (-1, 0, 5, 12, 19) of therapy.

2.14.3. Human tumour inoculation and treatment

Panc1-Her2 cancer cells (1×10^6 cells/mouse) in 200 µL PBS were injected subcutaneously into NSG mice and allowed to grow for nine days. On day 10 following tumour injections, mice received the following: (2.5 Gy) total body radiation, intravenous injections of (2×10^7) human CARaMEL T cells and intravenous injection of either

- (8×10^6) HLA-A*02$^+$ human DCs pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide for two hours at room temperature and washed 3× with PBS
- or (1×10^7) HLA-A*02$^+$ mouse DCs pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide for two hours at room temperature and washed 3× with PBS
- or (8.5×10^8 VP) Ad2-CMV-gp100 after sonication at high frequency for one minute.

Mice also received intraperitoneal injection of four doses (1×10^5 IU/injection) IL-2, one injection on the same day of treatment and then every 12 hours. On day 12: the mice treated with DCs received another intravenous injection of human CARaMEL T cells, mouse or human DCs and four doses of intraperitoneal injections of IL-2 as indicated in each figure. Also, the Ad2-CMV-gp100-treated mice groups received another intravenous injection of human CARaMEL T cells and four doses of intraperitoneal injections of IL-2 on day 12 after tumour inoculation. Control groups were included in each experiment as indicated in each figure.

2.15. Tumour cell and T cell co-culture assay

Co-culture assays were performed by coating the wells of round bottom 96-well plates (Greiner Bio One, Frickenhausen, Germany) with 200 µL/well of the following diluted antibodies at 1:1000 in PBS antibodies to be used as positive controls:

- For mouse and human experiments: anti-c-Myc Tag monoclonal antibody (clone: 9B11, #2276S, Cell Signalling Technology).
- For mouse experiments: 0.05 ng/µL anti-CD3e (clone: 145-2C11, catalogue number: 553058, BD Pharmingen, Uppsala, Sweden) and 0.05 ng/µL anti-CD28 (clone: 37.51, catalogue number: 553295, BD Pharmingen, Uppsala, Sweden)
- For human experiments: 0.05 ng/µL anti-CD3 monoclonal antibody (Orthoclone OKT3®, Ortho-Biotech Products, LP, Bridgewater, NJ, USA).

The plates were incubated for two hours at 37°C and followed by washing twice with PBS. For wells containing tumour cells targets, 1×10^5 cells/well of each tumour cell line were added in 100 µL of supplemented RPMI media.

For wells with gp100 peptide pulsed cells; cells were pulsed with 0.1 µM hgp100 $_{(154\text{-}162)}$ peptide (Lys-Thr-Trp-Gly-Gln-Tyr-Trp-Gln-Val, Auspep, Tullamarine, Victoria, Australia) or murine gp100 $_{25\text{-}33}$ peptide for one hour at room temperature. The cells were then washed three times with 10 mL of PBS to remove unbound peptide. For effector T cells preparation, cells were collected in 50 mL tube (Greiner Bio One, Frickenhausen, Germany) and centrifuged at 450 ×g for four minutes. Then, T cells were resuspended in supplemented RPMI media at 2×10^5 cells/well in 100 µL in each T cells containing well. The final volume was topped up with RPMI media to make a final volume of 200 µL. For Panobinostat experiments, 20 µL of Panobinostat with specific concentrations was added to the specified wells. In *in vitro* experiments, Panobinostat was dissolved in Dimethyl Sulfoxide (DMSO) (catalogue number: 20-139, Millipore, Temecula, CA, USA) at a 10 mmol/L stock solution. For further dilutions; Panobinostat was diluted in supplemented DMEM. Then cells were incubated at 37°C and 5% CO_2 for 16-72 hours before performing any functional assays.

2.16. IFN-γ quantification using Enzyme-linked immunosorbent assay (ELISA)

The amount of IFN-γ cytokine released by T cells was determined using ELISA following incubation with antigen$^+$ or antigen-target cells. Flat bottom ELISA 96-well plates (Maxisorp, Nalgene Nunc International, Denmark) were coated with purified rat anti-mouse IFN-γ (1 mg/mL, clone: R4-6A2, catalogue number: 551216, BD Pharmigen) or mouse anti-human IFN-γ (1 mg/mL, clone: NIB42, catalogue number: 551221, BD Pharmigen) diluted (1:500) in PBS and incubated at 4^{o}C overnight covered with Clingwrap to prevent any evaporation. On the following day, the plates were washed eight times with ELISA wash buffer (**Table 2.1**) and flicked to discard solutions followed by patting the plate on a paper towel. To inhibit any non-specific binding, 200 µL FACS buffer (**Table 2.1**) was added into the wells and incubated for one hour at room temperature. The plates then were washed eight times using ELISA wash buffer. Then, 100 µL sample supernatants neat or diluted in FACS buffer (1:10 or 1:50) and standards were added to the wells. To create standard curves, standards were diluted as follows: mouse IFN-γ standards (catalogue number: 554587, BD Biosciences) (10,000 pg/mL - 156.25

pg/mL) or human IFN-γ standards (catalogue number: 558456, BD Biosciences) (10,000 pg/mL - 156.25 pg/mL). Standards were run in duplicates, but samples were run in triplicate.

Then, the plate was incubated at room temperature for two hours, after it was sealed with aluminium foil. After that, the plate was washed eight times with ELISA wash buffer, and 100 µL of biotinylated detection mouse anti-IFN-γ antibody (0.5 mg/mL, clone: XMG1.2, catalogue number: 554410, BD Pharmigen) or human anti-IFN-γ antibody (0.5 mg/mL, clone: 4S.B3, catalogue number: 554550, BD Pharmigen) was added after it was diluted in FACS buffer (1:500) and incubated at room temperature for one hour. Next, the plate was washed eight times with ELISA wash buffer and 100 µL of diluted (1:500) streptavidin horseradish-peroxidase (HRP) (catalogue number: RPN1231V, GE Healthcare) in FACS buffer was added. The plate was wrapped in foil and incubated for half an hour at room temperature. Again the plate was washed eight times with ELISA wash buffer and 100 µL substrate solution (**Table 2.1**) was added to all wells of the plate and covered with aluminium foil to be incubated (10-15 minutes) at room temperature till the colour was developed. To stop the reaction, 50 µL sulphuric acid (1.8 M H_2SO_4) was added to each well. The absorbance was analysed using a Benchmark microplate reader at 405 nm (Bio-Rad, Hercules, CA, USA) and Softmax Pro software (Molecular Devices, Sunnydale, CA, USA). During all incubations, the plates were wrapped in aluminium foil. Concentrations of IFN-γ in the samples were then calculated based on the standard curve absorbance values.

2.17. Chromium release assay

The killing ability of T cells was evaluated using a ^{51}Chromium release assay. Target cells were harvested and washed three times with PBS. Then, 5×10^6 target cells were transferred into a 10 mL tube and resuspended in ^{51}Chromium (Perkin Elmer, Waltham, MA, USA) of 75 µCi/1 $\times 10^6$ cells for incubation at 37°C with 5% CO_2 for 45 minutes. After that, cells were washed three times with RPMI (no supplements) to discard any excess radioactive chromium. Then, cancer cells were seeded at 1×10^4 cells/well in a 96-well V-bottom plate (Maxisorp, Nalgene Nunc International, Denmark). Effector CARaMEL T cells were added at various effector: target (E: T) ratios (40:1, 20:1, 5:1 and 1:1). All wells were topped up with supplemented RPMI to 200 µL final volume. Control wells were included: spontaneous chromium release was obtained in wells having target cells in complete RPMI media only and total chromium release wells contained target cells with 100 µL sodium dodecyl sulphate (SDS). Cultures were

incubated at 37°C for overnight. On the following day, plates were centrifuged at 450 ×g for four minutes. Then, the amount of radioactivity release from the lysed target cells was determined using 100 µL of supernatant in 1.2 mL microtiter tubes (Quality Scientific Plastics, USA) and radioactivity measured in an automatic gamma counter Wallac Wizard 1470 (Amersham Australia, now General Electric Healthcare). The percentage of lysis was calculated using the following formula:

$$\%\ \text{specific}\ {}^{51}\text{Cr release} = 100 \times \frac{(\text{experimental counts/minute} - \text{spontaneous counts/minute})}{(\text{total counts/minute} - \text{spontaneous counts/minute})]}$$

2.18. ^{3}H thymidine incorporation assay

To determine the proliferation activity of T cells (2×10^5) were co-cultured with (1×10^5) irradiated (50 Gy) cancer cells (X-RAD320, Precision X-Ray PXi, North Branford, New Haven County, Connecticut, USA) in supplemented RPMI medium in a final volume of 200 µL in triplicate wells of a 96-well flat-bottom microtiter plates (Greiner Bio One, Frickenhausen, Germany). Also, 50 Gy-irradiated splenocytes from Her2 mice were pulsed with 1 µM gp100 peptide and seeded at a concentration of 1×10^6/100µL. Cells were incubated at 37°C in a 5% CO_2-humidified incubator in supplemented RPMI medium in a final volume of 200 µL. Cells were then cultured with exogenous cytokines mouse T cells: IL-2 (100 IU/mL) and IL-7 (2 ng/mL); human T cells: IL-2 (600 IU/mL).

After 48 hours, 1 µCi (0.037 MBq) tritiated [^{3}H]-thymidine (1.92 TBq/mmol) (catalogue number: NET027X001MC, Perkin-Elmer, Waltham, MA, USA) in a volume of 10 µL of supplemented RPMI was added to the culture. Then, cells were incubated at 37°C for ~ 12 hours. Cells were harvested using a 96-well harvester (TomTec, Orange, CA) and lysed with water onto glass matrix filters (catalogue number 6005422, Easy Tub-c Self Aligning Filter RG, Packard). Filter papers containing the incorporated thymidine were left to dry, then placed in scintillation fluid (OptiPhase HiSafe 3 Perkin Elmer, catalogue number 1200-437). Radioactivity was quantified in a Tri-Carb 1500 liquid scintillation analyser (Packard Instrument, Groningen, Netherlands). Results are expressed as mean counts per minute (CPM) of triplicate determinations.

2.19. Alamar blue (cell viability) assay

Tumour cells were seeded (1×10^5 cells/well) with 2 mL supplemented DMEM in 12 well plates. On the next day, tumour cells were pre-treated with the Panobinostat concentrations as indicated in each figure in 500 µL volume at 37ºC for three hours. Then, cells were washed three times with PBS and 1 mL of the concentrations of VV-gp100 as indicated in each figure was added after virus was sonicated at a high frequency for one minute. Then, wells were topped-up with 1 mL of supplemented DMEM. Seventy-two hours post-infection; 10% of cell culture volume of Resazurin solution was added to the plate and relative fluorescent units were measured after two hours at 550/590 nm excitation/emission using a POLARstar Optima (BMG Labtech, Ortenberg, Germany). The Resazurin solution is a blue dye that is reduced to the fluorescent pink resorufin compound according to the metabolic activity of a cell population, which can indicate the viability and the cell number [267].

2.20. Analysis of immune subsets in tumours

Tumour-bearing mice were euthanized, and tumours were harvested and digested in 2 mL digestion solution (supplemented DMEM containing 30 units/mL DNase type II (Sigma Aldrich, Missouri, USA), 1 mg/mL collagenase IV (Worthington Biochemical, Lakewood, NJ) and 100 µg/mL hyaluronidase type V (Sigma Aldrich, Missouri, USA) and incubated for 20 minutes in a shaking incubator at 110 rotations per minute at 37ºC.

Then, cells were filtered through an Acrodisc© 70 µm filter and washed twice with FACS buffer then resuspended in Fc receptor block. Then cells were stained with specific antibodies for flow cytometry analysis as per **Section 21.1**, **Chapter 2** of this thesis. For experiments that have a re-stimulation step, ionomycin (1 µg/mL) and PMA (10 ng/mL) were added to samples together with GolgiStop (1:500 dilution, BD Biosciences, San Jose, California, USA) and GolgiPlug (1:1000 dilution, BD Biosciences, San Jose, California, USA). The samples were then incubated for four hours at 37°C prior to staining with specific antibodies.

2.21. Flow cytometric analysis

2.21.1. Cell-surface staining of cells

Cell suspensions in a round-bottom 96-well plate were centrifuged for four minutes at 450 ×g and the plate was flicked to discard the supernatant. Next, 50 µL of human (catalogue number

564219, BD Pharmingen) or mouse Fc receptor block (1:100 dilution of 2.4G2 antibody) in FACS buffer (**Table 2.1**) was added to each sample for 10 minutes on ice to inhibit potential non-specific antibody staining. After incubation, the plate was centrifuged and resuspended. The cells were then stained with specific antibody cocktails, diluted in FACS buffer, in the dark for 30 minutes at 4°C in 50 µL of extracellular antibody staining cocktails. Next, the cells were washed twice with FACS buffer and resuspended in 200-400 µL of FACS buffer to be analysed. In cases where Fluorogold was used for viability staining, cells were resuspended in 200-400 µL of FACS buffer containing 2 µM Fluorogold. Antibodies used in the flow cytometry experiments are listed in **Table 2.3**.

Annexin V/7AAD apoptosis assay: cells were washed with (1 ×) Annexin V binding buffer (**Table 2.1**) and stained with Annexin V and 7AAD for 20 minutes at room temperature in the dark. Then 400 µL of (1 ×) Annexin V binding buffer was added to each tube and analysed by flow cytometry within one hour.

2.21.2. Intracellular staining of cells

Cells needing intracellular staining were first stained extracellularly as described in the previous section and fixed and permeabilized with BD Cytofix/Cytoperm™ buffer according to the manufacturer's instructions (catalogue number: 554655, BD Biosciences). Next, anti-IFN-γ fluorochrome-conjugated antibody (**Table 2.3**) was added to cells for and incubated for 30 minutes. Then, the cells were washed with permeabilization buffer twice and resuspended in 200 µL of permeabilization buffer prior to analysis.

BrdU incorporation analysis was performed according to the to the manufacturer's instructions of BrdU Flow Kits (catalogue number: 552598, BD Biosciences). After fixation and permeabilization, DNase (300 µg/mL) was added to wells and incubated at 37°C for 60 minutes, followed by staining with anti-BrdU antibody.

2.21.3. Cell Sorting

Mouse or human tumour cell lines were harvested washed and resuspended in FACS buffer at 30×10^6 cells/mL. Tumour cells were sorted for Her2 expression on the BD FACSAria Fusion 5 machine (BD Biosciences, San Jose, California, USA). Human CARaMEL T cells were stained first with a primary monoclonal antibody anti-Myc, then washed with FACS buffer twice and stained with secondary PE-conjugated antibody (**Table 2.3**). After that, cells were

washed twice with FACS buffer and stained with mouse TCRβ-FITC conjugated antibody and other cocktails (**Table 2.3**). Then human CARaMEL T cells were sorted either for single or double expression of anti-Her2 CAR and mouse TCRβ.

2.21.4. Gating strategies

Samples were gated according to single cells then morphology and size in forward and side scatters. Then live gating was applied on samples. For additional analysis, colours were compensated on the machines. 10,000-25,000 events were recorded in each sample for analysis.

2.21.5. Flow cytometry Analysis

Analysis of stained samples was accomplished using FACSymphony (BD Biosciences, San Jose, California, USA), FACS FORTESSA (Becton Dickinson and Company) or FACS Canto II (Becton Dickinson) and LSRII (BD Biosciences, San Jose, California, USA). Data were analysed using the FlowJo 10.2 software (Tree Star Inc., Ashland, OR).

Table 2.3. List of flow cytometry antibodies

Specificity	Fluorochrome	Clone	Company	Dilution
Annexin V	APC		BD Bioscience	1:100
anti-human CCR7	BV711	G043H7	Biolegend	1:400
anti-human CD14	PE	Clone MφP9	BD Biosciences	1:200
anti-human CD1a	PE-Cy7	HI149	Biolegend	1:200
anti-human CD3	APC H7	SK7	BD Horizon	1:200
anti-human CD3	Pacific Blue	UCHT1	BD Pharmingen	1:200
anti-human CD4	BV510	OKT4	BioLegend	1:200
anti-human CD4	APC	RPA-T4	BD Pharmingen	1:200
anti-human CD45	BV510	HI3	Biolegend	1:200
anti-human CD45RA	FITC	HI00	BD Pharmingen	20 µL/test
anti-human CD80	PE	2D10	Biolegend	1:100
anti-human CD83	FITC	HB15e	Biolegend	1:100
anti-human CD86	AF 647	IT2.2	Biolegend	1:100
anti-human CD8a	PE-Cy	RPA-T8	BD Pharmingen	
anti-human Her2	APC	24D4	Biolegend	1:200
anti-human HLA-A2	FITC	BB7.2	BD Bioscience	1:100
anti-human LAG-3	AF647	11C3C65	Biolegend	1:50
anti-human PD-1	BV785	Eh12.2H7	Biolegend	1:100
anti-human TIM-3	PerCP-eF710	F38-2E2	eBioscience	1:200
anti-mouse CD11c	e450	N418	eBioscience	1:100
anti-mouse CD19	BV785	6D5	Biolegend	1:100
anti-mouse CD3	BV605	17A2	Biolegend	1:50
anti-mouse CD4	e450	GK1.5	BD Biosciences	1:200
anti-mouse CD40	PE	3/23	BD Pharmingen	1:300
anti-mouse CD45.2	APC-eFluor780	104	eBioscience	1:100
anti-mouse CD80	Biotin	16-10A1	BD Pharmingen	1:100
anti-mouse CD86	PE	GL1	BD Pharmingen	1:100
anti-mouse CD86	FITC	GL1	eBioscience	1:100
anti-mouse CD8a	BV711	53-6.7	BioLegend	1:400
anti-mouse CD8a	PE-Cy7	53-6.3	BD Biosciences	1:400
anti-mouse CD90 (Thy-1)	FITC	OX-7	BD Biosciences	1:200
anti-mouse CD90 (Thy-1)	PE	OX-7	BioLegend	1:50
anti-mouse F4/80	BV421	T45-2342	BD Biosciences	1:400
anti-mouse IFN-γ	APC	XMG1.2	BD Biosciences	1:100
anti-mouse Ly6C	Pe-Cy7	HK1.4	BioLegend	1:800
anti-mouse Ly6G	BV605	RB6-8C5	Biolegend	1:200
anti-mouse MHCI (H2Db)	PE	KH95	Cell signalling	1:200

anti-mouse MHCII	APC	M5/114.15.2	eBioscience	1:1000
anti-mouse NK1.1	Pe-Cy7	PK136	eBioscience	1:400
anti-mouse TCRβ	APC-eFluor 780	H57-597	eBioscience	1:200
anti-mouse TCRβ	FITC	H57-597	eBioscience	1:200
anti-mouse/human CD11b	BV711	M1/70	BioLegend	1:400
c-Myc Tag (CAR)		9B11	Cell Signalling Technology	1:50
F(ab')2-goat anti-mouse IgG (H+L) secondary antibody	PE	Polyclonal	eBioscience	1:200
IgG1 isotype	PE	Neu 24.7	BD Biosciences	
IgG1 isotype	PE	MOPC-21	Biolegend	
IgG1 isotype	APC	MOPC-21	Biolegend	
IgG1 isotype	APC/Cy7	MOPC-21	Biolegend	
IgG1 isotype	FITC	MOPC-21	Biolegend	
IgG1 isotype	PE/Cy5	MOPC-21	Biolegend	
IgG1 isotype	PE/Cy7	MOPC-21	Biolegend	
IgG2a isotype	APC	eBR2a	BD Biosciences	
IgG2a isotype	FITC	eBR2a	BD Biosciences	
IgG2a isotype	PE	eBM2a	Thermofisher	
Streptavidin	PE		eBioscience	1:200
Viability	Fixable Yellow		Thermo Fisher	1:400
Viability	FluoroGold		Biotium	1:1000
Viability	7AAD		BD Bioscience	1:20

2.22. Immunohistochemistry (IHC)

2.22.1. Staining process

CD8 staining: Tumours were harvested from mice and fixed in 10% neutral buffered formalin for 24 hours prior to being embedded in paraffin. Tissue sections were cut 4 μm in thickness and put on slides. For staining: slides were dewaxed in xylene and re-hydrated with a series of 100%, 90% and 70% alcohol and a final wash with water. Then, slides were washed again with tap water and they were immersed in antigen retrieval buffer (sodium citrate buffer (10 mM, pH 6)) and incubated in a pressure cooker under high pressure at 125°C for 2.5 minutes.

Sections were then set aside to cool to room temperature. Then, slides were washed with PBS. Then, sections were blocked for 30 minutes with blocking buffer containing 10% goat serum in PBS + 1%BSA at room temperature to inhibit any non-specific binding. Slides were then washed with PBS/Tween and stained with the primary antibody diluted in blocking buffer overnight at 4°C in a humidified box. On the following day, slides were washed twice in PBS/Tween for five minutes on a gentle shaker. Then, to inactivate endogenous peroxidases: slides were immersed in methanol and 1% H_2O_2 for 30 minutes at room temperature. Slides were washed twice in PBS/Tween for five minutes on a gentle shaker. Then, 50 μL of anti-rat ImmPress-HRP was added to each section and incubated for one hour at room temperature in a humidified box. Slides were washed twice in PBS/Tween for five minutes on a gentle shaker. Then, sections were developed with 100 μL of DAB chromogen kit (SK-4100, Vector Laboratories, Inc. Burlingame, CA, U.S.A) and washed in tap water. Slides were then counterstained with haematoxylin, followed by dehydration and coverslip mounting.

Cleaved-caspase 3 (CC3) staining: tumour section slides were sent to the Centre for Advanced Histology and Microscopy (CAHM) at the Peter MacCallum Cancer Centre. The techniques and steps for CC3 staining were the similar to those as above. Tissues were processed through histolene solution and a series of alcohol incubations prior to staining with the antibodies and haematoxylin solutions.

Table 2.4. List of IHC antibodies

Primary antibody	Clone number	Supplier	Isotype control	ImmPress-HRP	Antigen retrieval buffer
CD8 5 µg/mL	4SM15 Rat	eBioscience	Rat, IgG2b (Invitrogen)	ImmPress anti-rat IgG HRP	10 mM Citrate buffer (pH 6)
Cleaved Caspase 3 (CC3)	5A1E	Cell Signalling Technology	Rabbit IgG, polyclonal (Abcam)	ImmPress anti-rabbit IgG HRP	10 mM Citrate buffer (pH 6)

2.17.2. Microscopy image capture

Images of tumour sections were taken using the Olympus VS120 microscope (Olympus, Tokyo, Japan) and VS-ASW software (Olympus Tokyo, Japan). Images were analysed with HALO (Indica Labs, Corrales, USA). Positive staining was quantified, and the percentage of positive cells was calculated using the following formula:

$$\text{Positive staining} = \frac{\text{number of positively stained cells}}{\text{tissue area (mm}^2\text{)}}$$

2.23. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 7; California, USA). Data analyses were conducted using unpaired Student's *t* test to compare two data sets, or using two-way ANOVA to compare more than two sets of data as indicated in each figure. Statistical analyses for tumour size were performed using two-way ANOVA and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves. Data are presented in this thesis as mean ± standard error of the mean (SEM) and a *P* value of <0.05 was considered significant.

CHAPTER 3: Adoptive Transfer of Dual-Specific T Cells for the Treatment of Pancreatic Cancer

3.1. Introduction

Pancreatic cancer is an aggressive malignancy and it is estimated to become the second most frequent cause of death by cancer by 2030, second only to lung cancer [3, 268]. Pancreatic cancer has a devastating prognosis and extremely poor survival rate, with up to 80% of patients dying within the first year of diagnosis and a 5-year overall survival rate of only 7% [3-5, 269, 270].

Current therapies for pancreatic cancer include conventional chemotherapeutic agents like gemcitabine, FOLFIRINOX (protocol 5-FU, Folinic Acid, Irinotecan, Oxaliplatin) and nab-paclitaxel, which improve median survival by only 2–4 months and have frequent toxic side effects [7, 9, 11]. The best curative option for pancreatic cancer is surgical resection; however, surgery is possible in only a few patients as the majority of them present in the clinic with established distant metastases. In addition, even with complete surgical resection, the majority of patients relapse with distant metastasis [4, 18, 271]. Moreover, other targeted agents that inhibit molecular pathways involved in proliferation, angiogenesis and metastasis have failed to afford any significant clinical benefit [272].

The failures in treatment of pancreatic cancer is due to many reasons including the inherent genetic instability of pancreatic cancer cells [273], the remarkable desmoplastic reaction with dense stroma and the immunosuppressive tumour microenvironment (TME), which is characterized by significant myeloid inflammation and poor infiltration of effector T cells [274-276].

Therefore, development of effective and novel therapeutic options for this highly aggressive and lethal tumour is urgently needed. Immunotherapy has demonstrated promising results in various tumour types [123, 277-281] and pancreatic cancer can be a potential target. Adoptive cell transfer (ACT) is a promising form of cancer immunotherapy which involves the isolation and reinfusion of tumour specific T lymphocytes into patients [129, 229]. T cells can be genetically engineered to express tumour-specific chimeric antigen receptors (CARs) [282-284]. CAR T-cell therapy has emerged as a powerful therapeutic and has achieved significant progress in malignant haematological diseases with response rates up to 90% in a subset of B cell cancers such as B-ALL [240-244].

CAR T-cell therapy is currently being explored in solid malignancies, but clinical trials have only resulted in low response rates. Several clinical trials involving CAR-based immunotherapy have been initiated in pancreatic cancer patients targeting different TAA including CEA, MUC1, MSLN, Her2 and PSCA. Unfortunately, most of these clinical trial results and initial reports showed no clinical benefit or significant responses in patients [129, 245, 246, 285]. This limited success of CAR T-cell therapy in solid tumours was due to several factors including poor T cell trafficking into tumours, the lack of T cell persistence and an immunosuppressive TME which can inhibit T cell activity [245, 249-255, 286].

For effective anti-tumour potential of CAR T cells in solid tumours, several requirements must be satisfied. T cells must persist, expand and infiltrate tumour tissues. Previous studies in our laboratory demonstrated that adoptive cell transfer incorporating vaccination (ACTIV) met these requirements and led to eradication of several types of tumours in mice. ACTIV therapy involves transfer of dual-specific T cells expressing a TCR specific for the melanoma-associated antigen pMEL (gp100) and a CAR specific for the solid tumour antigen Her2. The TCR provides appropriate signals for activation, proliferation and persistence of dual-specific T cells and the CAR provides the anti-tumour activity. We termed these dual-specific cells 'CARaMEL T cells'. In ACTIV therapy, CARaMEL T cells are administered together with an injection of recombinant vaccinia virus expressing pMEL 'VV-gp100' (**Figure 3.1**). ACTIV therapy was effective in eradicating large Her2-positive tumours in immunocompetent mice expressing human Her2. Tumour types included breast tumours, subcutaneous sarcoma, and colon carcinoma present as large liver masses [287].

In this chapter I set out to determine the efficacy of ACTIV therapy against mouse pancreatic cancers. I initially investigated some characteristics of the tumour cells, followed by an analysis of the dual-specific CARaMEL T cells and their function against the pancreatic cancer cells *in vitro*. I then determined the efficacy of ACTIV treatment against subcutaneous pancreatic cancer *in vivo,* including determining the contribution from various components of the therapy regimen. Finally, I sought mechanistic insight into therapy to determine limitations and identify potential ways to improve efficacy.

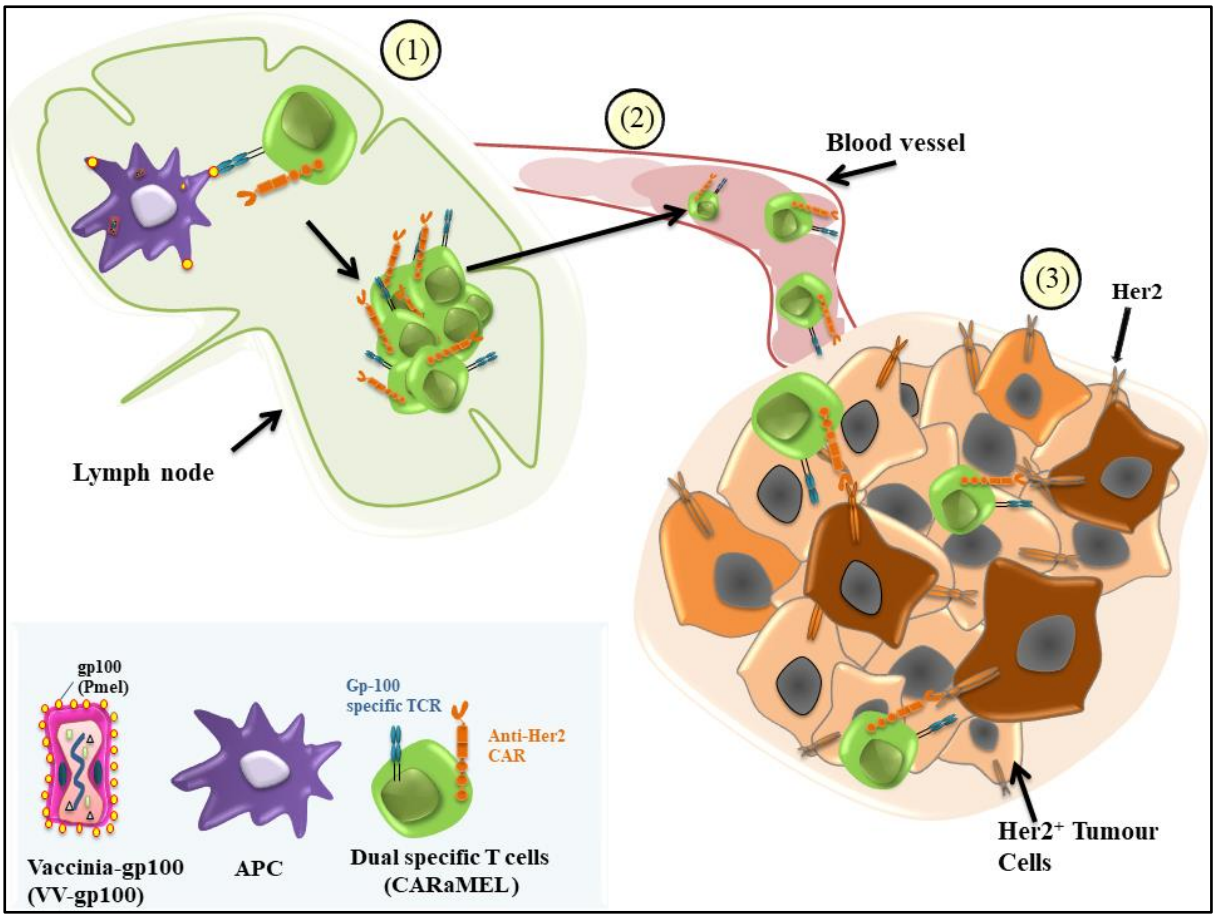


Figure 3.1. Schematic of ACTIV therapy and dual-specific T cells

(**1**) Dual-specific "CARaMEL" T cells expressing both gp100-specific TCR and Her2 specific CAR and VV-gp100 vaccine are administrated into C57BL/6 human-Her2 transgenic mice bearing $Her2^{+}$ tumours. Gp100 antigen of the VV-gp100 vaccine is presented on antigen presenting cells (APCs) in lymphoid tissues of the treated mouse which results in activation of CARaMEL T cells through the gp100 TCR and leads to CARaMEL T cell expansion (**2**) and trafficking to $Her2^{+}$ tumours (**3**) CARaMEL T cells kill tumours through Her2-specifc CAR and VV-gp100 vaccine may also lyse tumour cells after infection.

3.2. Results

3.2.1. The generation of two Her2$^+$ murine pancreatic cancer cell lines

In order to assess the efficacy of ACTIV therapy in pancreatic cancers, I generated Her2$^+$ murine pancreatic cancer models. Two Her2$^+$ murine pancreatic cell lines were generated using a retroviral vector containing Her2 driven by the LTR promoter, as described in the **Material and Methods Section 2.5.** The parental cell lines were the Panc02 line that was derived from pancreatic adenocarcinoma tumours induced by 3-methyl-cholanthrene (3-MCA) of a C57BL/6 mouse [288], and the KPC line (83220 PZPR R172H) that was derived from a spontaneous pancreatic tumour developed in a Kras^{G12D}; Trp53^{R172H} Pd × 1-Cre (KPC) mouse [289]. Her2 expression on both transduced cell lines was confirmed using flow cytometry and compared similarly to the Her2$^+$ positive control breast cancer cell line, E0771 (**Figure 3.2 A and B**). These two cell lines expressing human Her2 served as targets to investigate CAR T cell activity in pancreatic cancer model systems. In addition, they were used to examine CAR T cell activity *in vitro* and *in vivo* in human Her2 transgenic mice, as described below.

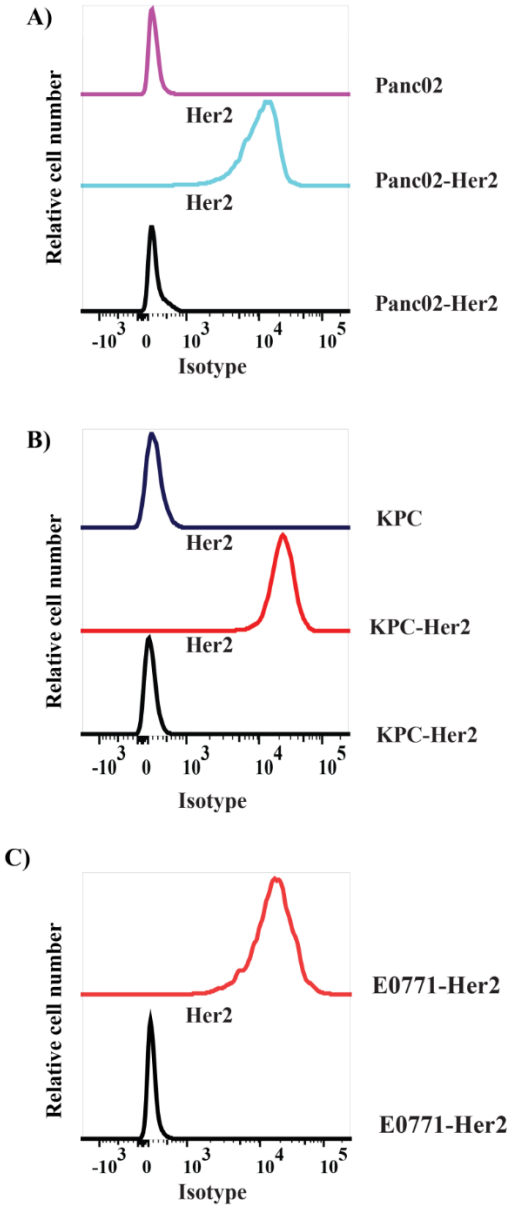


Figure 3.2. Her2 expression on the Panc02-Her2 and KPC-Her2 cells

Tumour cells were stained with anti-Her2 antibody or isotype control after they were generated and sorted for Her2 expression. Flow cytometry was performed to assess the expression of Her2 on (**A**) Panc02-Her2, (**B**) KPC-Her2 and **(C)** E0771-Her2 cells (positive control). Data are presented as histograms. Flow cytometric plots are gated on live cells.

3.2.2. Characterisation of CARaMEL T cells

Dual-specific CARaMEL T cells are a crucial component of ACTIV therapy. Expression of both the anti-Her2 CAR and the anti-gp100 TCR are important for their activity, since the TCR enables expansion in response to a gp100 vaccine and the CAR enables activity against $Her2^+$ tumour cells. As a source of dual-specific CAR T cells, I used splenocytes from transgenic mice expressing an anti-Her2 CAR and a gp100-specific TCR (CARaMEL T cells). These splenocytes are a convenient source of dual-specific T cells expressing consistent levels of both CAR and TCR, without the variability often encountered using dual-specific T cells generated using retroviral transduction. I therefore sought to determine the phenotype of T cells in donor spleens, and in particular validate the expression of the anti-Her2 CAR and anti-gp100 TCR on the surface of T cells. Following staining of splenocytes with specific antibodies and flow cytometric analysis, I found that approximately 30% of the $CD45^+$ cells were $CD3^+$ T cells (**Figure 3.3 A**) and >90% of these T cells expressed both anti-Her2 CAR ($cMyc^+$) and gp100 Vβ13 TCR (**Figure 3.3 B**). Approximately 77% of CAR T cells are $CD8^+$, 10% are $CD4^+$ T cells (**Figure 3.3 C**).

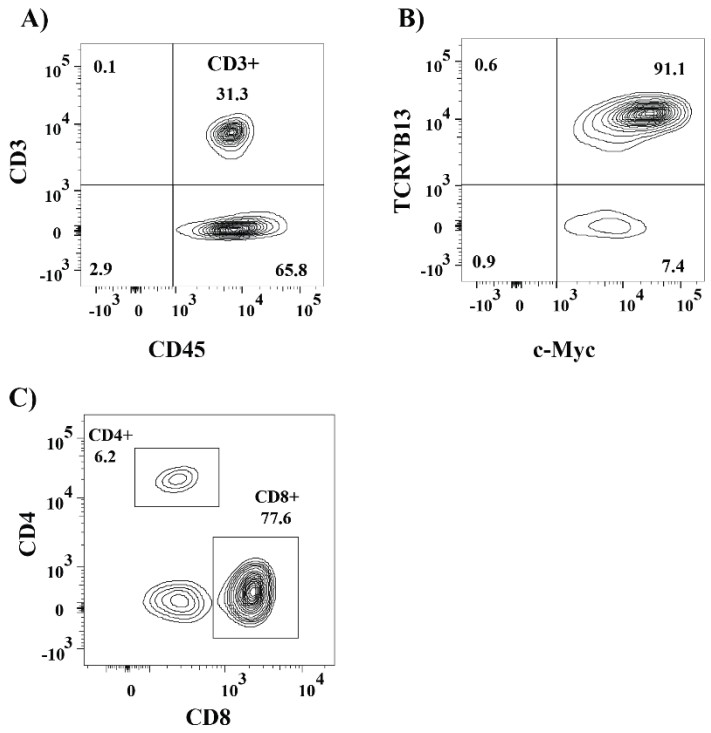


Figure 3.3. Phenotypic analyses of the CARaMEL T cells

CARaMEL splenocytes were harvested from CARaMEL mice, stained with the listed antibodies and flow cytometric analysis was performed to determine (**A**) Percentage of $CD45.2^+$ that were $CD3^+$ T cells. (**B**) Percentage of anti-Her2 CAR and TCRVβ13 of $CD3^+$ T cells. (**C**) $CD4^+$ and $CD8^+$ of $CD3^+$ T cells. Flow cytometric plots are gated on live cells. Data are representative of two independent experiments.

3.2.3. Murine CARaMEL T cells respond to both Her2 and gp100

Having phenotyped murine CARaMEL T cells, I next evaluated the functionality of the CARaMEL T cells for antigen-specific reactivity against $Her2^+$ pancreatic cancer cells.

Interferon-gamma (IFN-γ) is an important cytokine that is secreted by activated T cells which mediates pro-inflammatory activity on immune cells and has important roles in potentiating anti-tumour immunity [290-292]. IFN-γ was used in this project as a representative cytokine to assess cytokine secretion from CARaMEL T cells. CARaMEL splenocytes were incubated with $hgp100_{(25-33)}$ at 1 µM for five days to generate activated CARaMEL T cells. Subsequently, the activated CARaMEL T cells were co-cultured with the Panc02-Her2 and KPC-Her2 cell lines for 16 hours. The parental $Her2^-$ cell lines were used as negative controls. IFN-γ was measured by ELISA according to manufacturers' instructions. CARaMEL T cells secreted IFN-γ specifically in the presence of the Her2 antigen expressed on KPC-Her2 cells and Panc02-Her2 cells, while no IFN-γ was secreted in the absence of Her2 on the parental cells (**Figure 3.4**). In addition, the ability of CARaMEL T cells to respond against gp100 through their TCR was demonstrated when CARaMEL T cells were incubated with splenocytes pulsed with $hgp100_{(25-33)}$ peptide or with the gp100-expressing vaccinia virus (VV-gp100). In contrast, transgenic ovalbumin-specific OT-I cells, which served as control T cells, secreted IFN-γ only in the presence of the ovalbumin peptide, SIINFEKL, and did not respond to Her2 or gp100 stimulation due to the lack of the corresponding receptors.

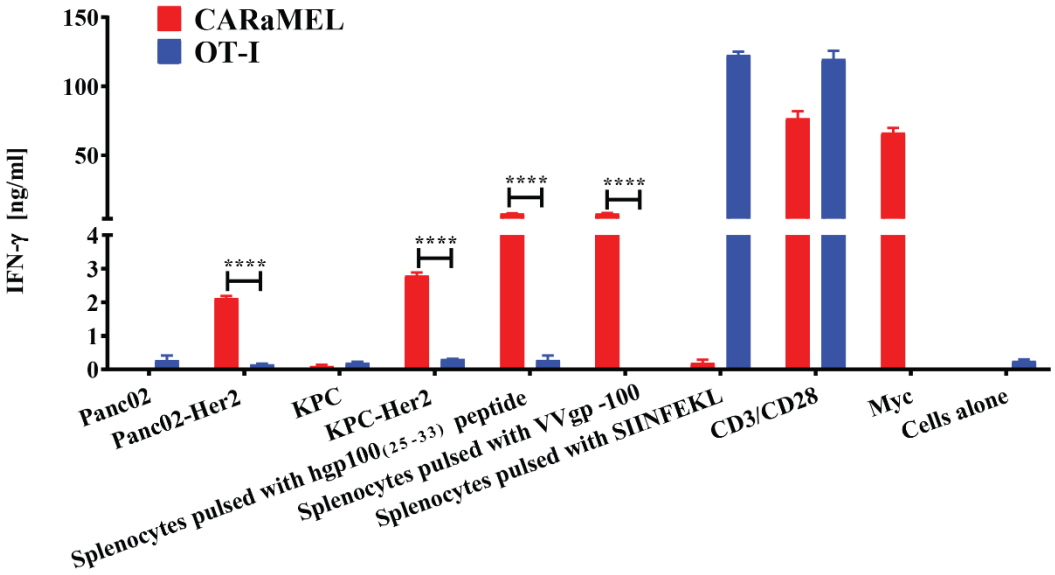


Figure 3.4. Murine CARaMEL T cells secrete IFN-γ in response to Her2 and gp100 peptide

CARaMEL or OT-I T cells were activated for five days in culture with either hgp100 $_{(25\text{-}33)}$ or SIINFEKL peptide, respectively. IFN-γ secretion was assessed in activated (2×10^5) CARaMEL T cells or OT-I T cells co-cultured overnight with ($1x\ 10^5$) parental or Her2 expressing KPC or Panc02 cells. In addition, T cells were incubated with splenocytes pulsed with either VV-gp100 or (1μM) hgp100 $_{(25\text{-}33)}$ peptide. Controls included T cells alone as a negative control, or immobilised αCD3/αCD28 as a positive TCR control or immobilised Myc-antibody as a positive CAR control. IFN-γ levels in the culture supernatants were determined using ELISA. Data are presented as mean ± SEM of three independent experiments, each performed in triplicate. Statistical analyses were performed using the Student *t*-test (**** $p \leq 0.0001$).

3.2.4. CARaMEL T cells lyse Her2-expressing tumour cells

After confirming CARaMEL T cells secreted IFN-γ in response to Her2$^+$ cancer cells, I carried out a chromium release assay to confirm that these CARaMEL cells are able to induce murine pancreatic cancer cell lysis. Murine CARaMEL T cells were co-cultured overnight with ^{51}Cr labelled tumour cells lines: the parental Panc02 and KPC cells and Panc02-Her2 and KPC-Her2 cells at various effector: target ratios. CARaMEL T cells specifically lysed the Her2$^+$ cells, but not the parental Her2-negative Panc02 or KPC cells (**Figure 3.5 A and B**). In contrast to CARaMEL T cells, the negative control OT-I T cells did not lyse any cancer cells given their lack of expression of the anti-Her2 CAR (**Figure 3.5 C and D**).

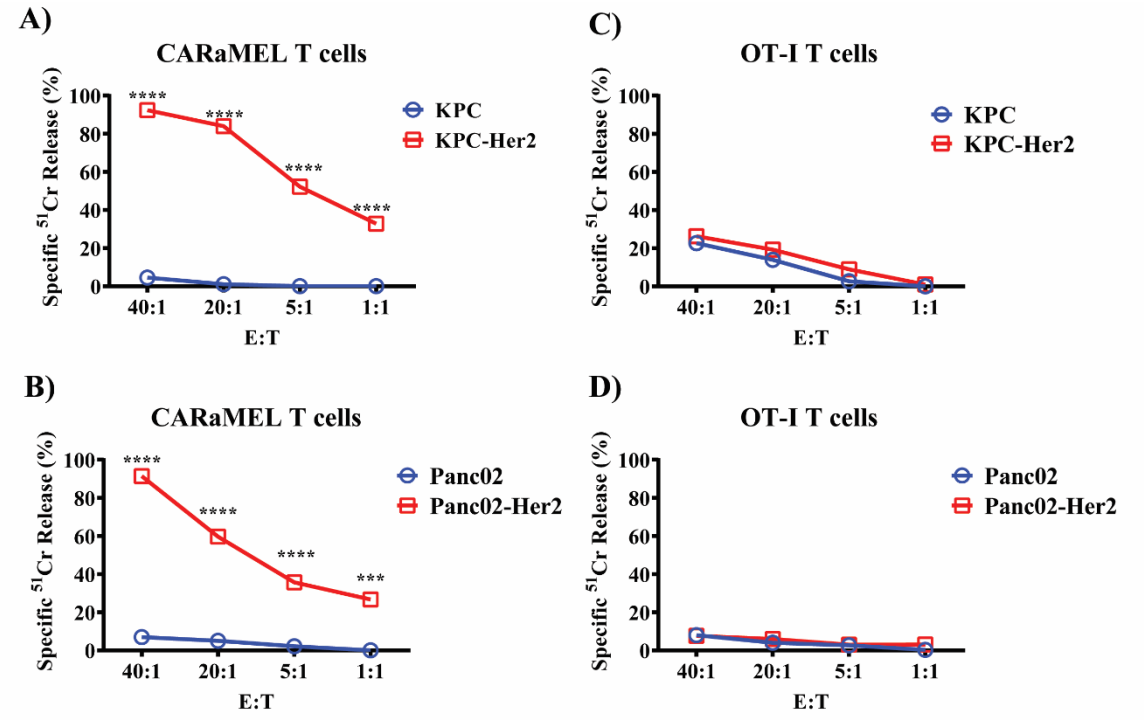


Figure 3.5. CARaMEL T cells specifically kill Her2$^+$ tumour cells *in vitro*

Panc02, Panc02-Her2, KPC or KPC-Her2 were labelled with ^{51}Chromium before being co-cultured with control (OT-I) or CARaMEL T cells, at the indicated effector: target (E: T) ratio. Radioactivity was measured from the supernatants to determine percentage specific tumour cell lysis, following incubation overnight at the indicated T cell (effector) to tumour cell ratios. (**A and B**) CARaMEL T cells against target cells (**C and D**) OT-I T cells against target cells. Data are presented as mean ± SEM of triplicate samples; data here are representative of three independent experiments. Statistical analyses were performed using the Student *t*-test (*** $p \leq 0.001$, **** $p \leq 0.0001$).

3.2.5. Murine CARaMEL T cells proliferate through gp100 TCR stimulation

Since antigen-induced expansion of T cells is necessary in the therapeutic application of CAR T cells against cancers, I tested the relative ability of the CARaMEL T cells to proliferate in the presence of gp100 peptide and Her2 antigen. Proliferation of murine CARaMEL T cells was measured by performing a ^{3}H thymidine incorporation assay (**Figure 3.6**)**.** Naïve $CD8^{+}$ CARaMEL T or OT-I T cells were enriched using the Miltenyi MACs: mouse $CD8a^{+}$ T cell isolation kit according to manufacturers' instructions (**Figure 3.6 A**). These T cells were co-cultured with cancer cell lines to provide the Her2 antigen or splenocytes pulsed $hgp100_{(25\text{-}33)}$ or VV-gp100 to provide the TCR antigen for 16 hours. As shown in **Figure 3.6 B**, CARaMEL T cells proliferated to a greater extent when the TCR stimuli were provided compared to the Her2 antigen. The control OT-I cells did not respond to either Her2 or gp100 peptide. This result supported our hypothesis that ACTIV therapy would enable expansion of CARaMEL T cells and their activation is through the gp100-TCR.

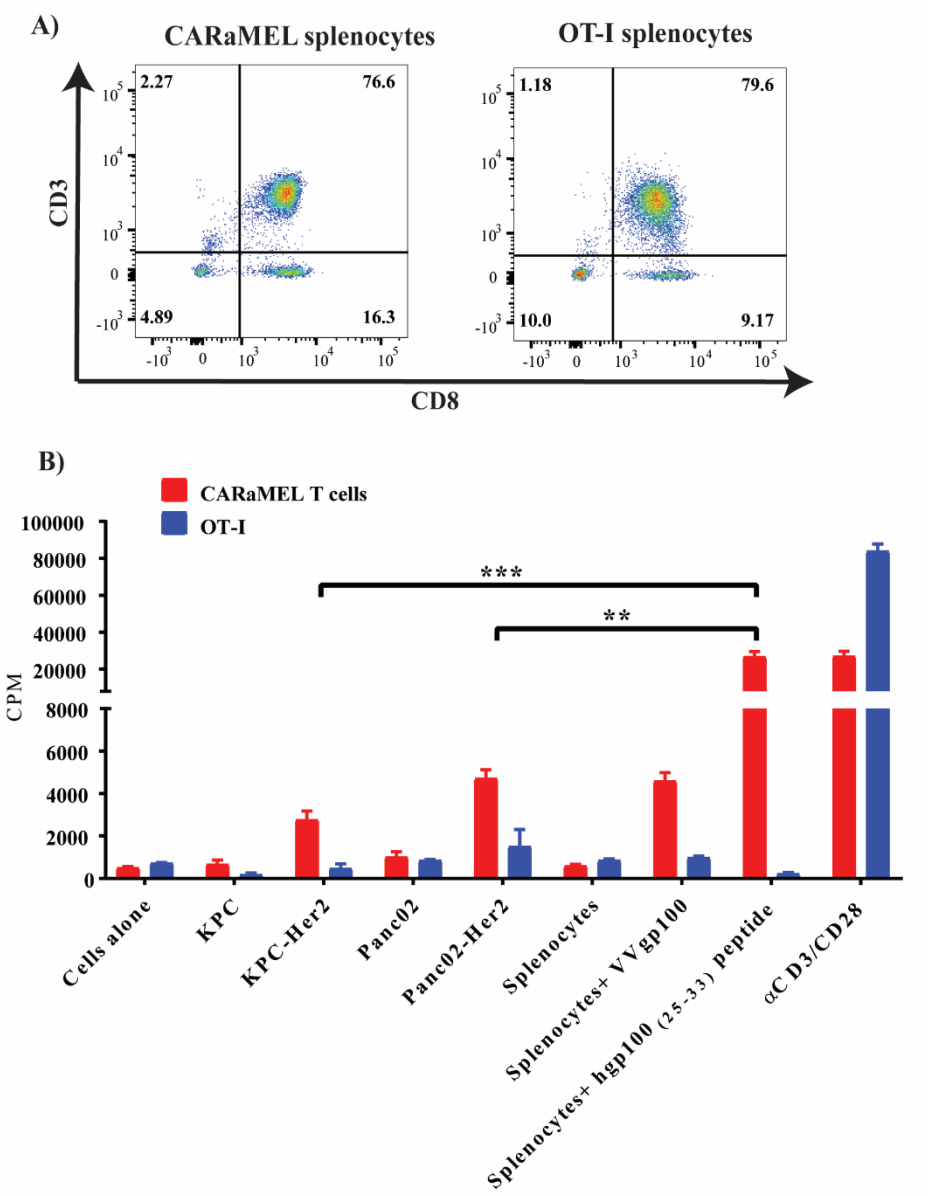


Figure 3.6. CD8$^+$ CARaMEL T cells proliferated to a greater extent when the stimulation was provided through TCR compared with Her2-CAR

Mean proliferation ([^{3}H] thymidine incorporation) is shown for CD8$^+$ splenocytes isolated from CARaMEL or OT-I mice. (**A**) Phenotype of T cells from spleen of CARaMEL T cells and OT-I following isolation using a Miltenyi MACs: mouse CD8a$^+$ T Cell isolation kit. (**B**) Proliferation of CD8$^+$ cells was determined by ^{3}H-thymidine incorporation following co-culture with irradiated cancer cell lines or irradiated splenocytes pulsed with or without VV-gp100 or (1 µM) hgp100 $_{(25\text{-}33)}$ peptide. Controls included T cells alone as a negative control, or T cells incubated with immobilised αCD3/αCD28-stimulated T cells as a positive control. Data are expressed as the mean counts per minute (CPM) of triplicate cultures ± SEM; data representative of three independent experiments. Statistical analyses were performed using the Student *t* -test (** $p \leq 0.01$, *** $p \leq 0.001$).

3.2.6. ACTIV therapy inhibited KPC-Her2 and Panc02-Her2 subcutaneous tumour growth in mice and prolonged survival

To determine the efficacy of ACTIV therapy against pancreatic tumours *in vivo*, I injected the Panc02-Her2 or KPC-Her2 cancer cells subcutaneously in C57BL/6-Her2 mice. C57BL/6-Her2 mice are immunocompetent transgenic mice, which express human Her2 in breast and brain [260, 293]. In this experiment I allowed the tumours to grow for nine days to reach 35-50 mm^2 in size. ACTIV therapy was initiated, which included the following: a lymphodepletion preconditioning procedure of total body irradiation of (4 Gy) to deplete endogenous leukocytes to provide greater levels of growth factors to support engraftment of adoptively transferred T cells [238, 294], followed by intravenous injections with murine CARaMEL splenocytes (1×10^7 cells/mouse). After two hours I injected VV-gp100 intravenously (2×10^7 PFU/mouse). Mice also received intraperitoneal injection of four doses of recombinant human IL2 (IL-2) (1×10^5 IU/injection) with one injection on the same day of ACTIV therapy and then every 12 hours (**Figure 3.7**). A cohort of mice were left untreated as controls.

As shown in (**Figure 3.8**), Panc02-Her2 (**Figure 3.8 A and B**) and KPC-Her2 tumours (**Figure 3.8 C and D**) responded to ACTIV therapy and the tumour growth was significantly inhibited compared to non-treated groups (NT). The tumour inhibition led to a long-term survival benefit. However, both types of tumours usually relapsed after ACTIV therapy. Therefore, I decided to test various approaches to enhance ACTIV therapy which will be described in **Chapter 4**.

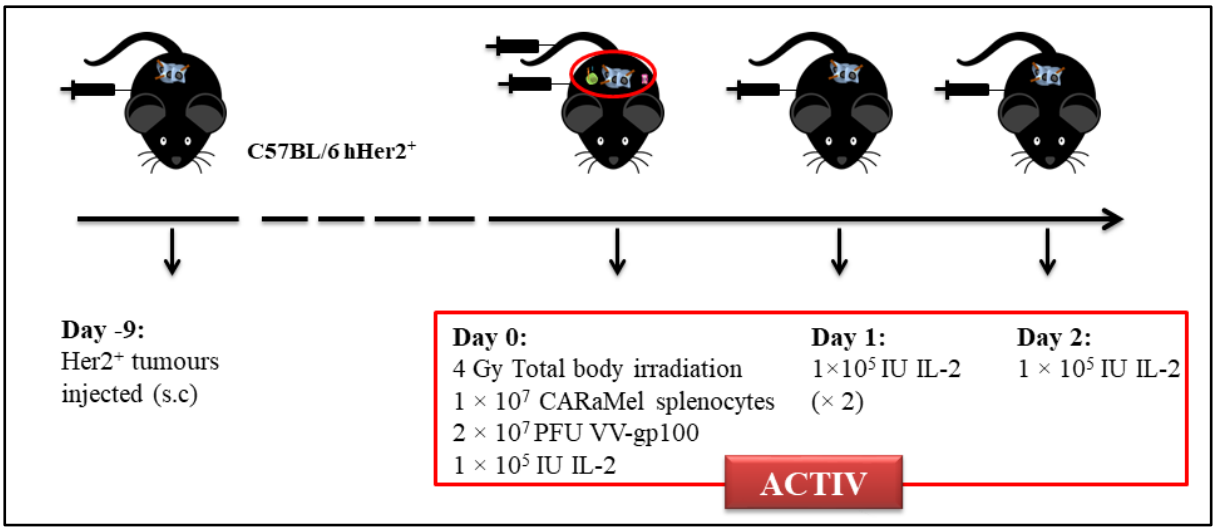


Figure 3.7. Schematic of ACTIV therapy

Her2-expressing cancer cells were injected subcutaneously into C57BL/6 human-Her2 transgenic mice and allowed to grow for nine days. When the tumour size reached about 35-50 mm^2, ACTIV therapy was initiated. First, mice received (4 Gy) total body radiation. Then, mice received intravenous injections of (1×10^7) CARaMEL splenocytes and after two hours mice received (2×10^7) PFU VV-gp100. Mice also received intraperitoneal injection of four doses of IL-2 (1×10^5 IU/injection) with one injection on the same day of ACTIV therapy and then every 12 hours for a total of two days.

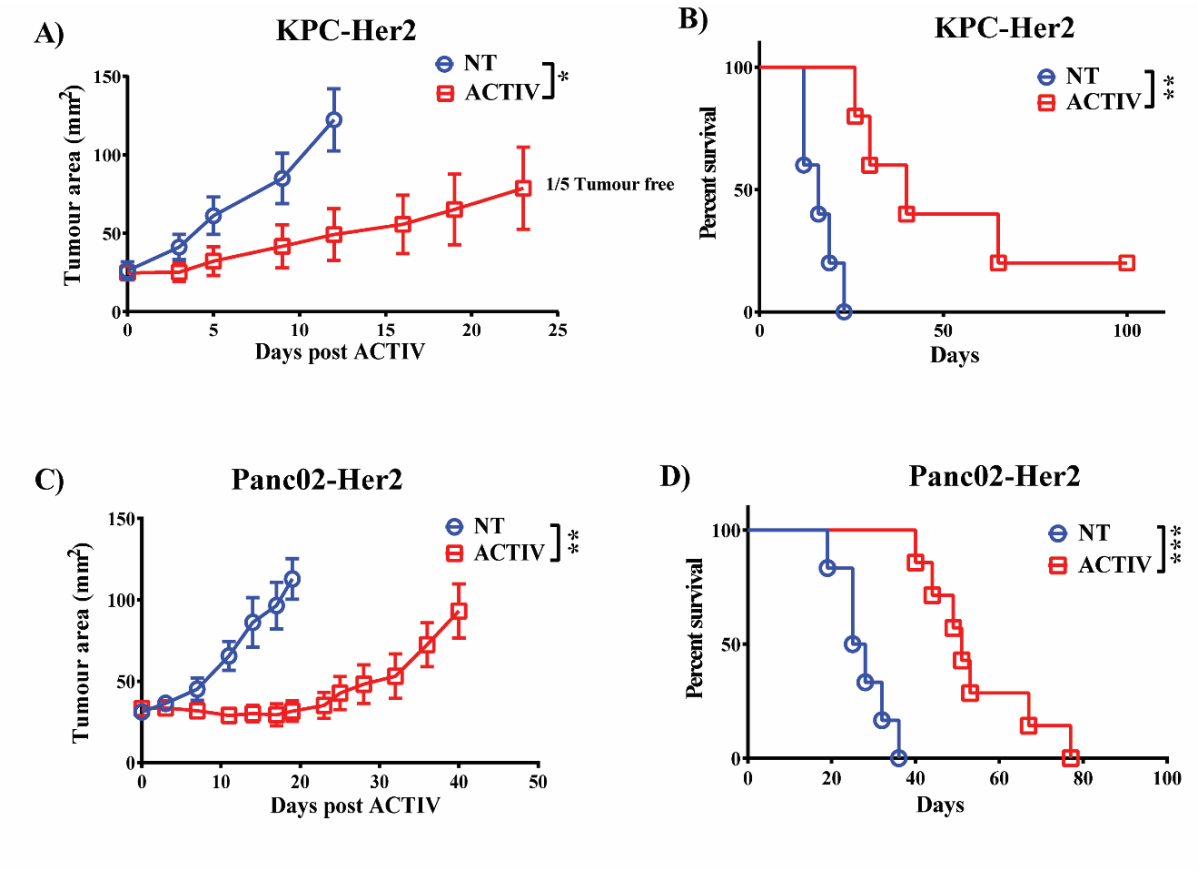


Figure 3.8. ACTIV therapy inhibited KPC-Her2 and Panc02-Her2 subcutaneous tumour growth in mice and prolonged survival

C57BL/6 human-Her2 transgenic mice were subcutaneously injected with (**A and B**) KPC-Her2 tumour cells or (**C and D**) Panc02-Her2 (1×10^6 cells/mouse). The tumours were allowed to grow for nine days. On day 9, the tumour-bearing mice were treated with ACTIV therapy. As controls, a cohort of mice were left non-treated (NT). Tumour areas were measured at the indicated times, and data are the mean ± SEM of 6-8 mice/group, representative of three independent experiments. Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2. (**A and C**) Tumour size panel. (**B and D**) Mice survival panel. (n = six mice/group), pooled from two independent experiments. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.2.7. VV-gp100 was instrumental in ACTIV therapy through activation of CARaMEL T cells but not through oncolysis

In order to gain further understanding into the mechanism of ACTIV therapy, I sought to determine the contribution of each ACTIV component, which were previously shown to be crucial for the efficacy of ACTIV therapy against several other tumour types [287]. I treated mice with the full regimen that included the four components (total body irradiation, CARaMEL splenocytes, VV-gp100 and IL-2) or variations lacking individual components. Tumours in mice treated with ACTIV therapy lacking irradiation or IL-2 showed a trend toward reduced efficacy compared to full ACTIV therapy, but this is not statistically significant (**Figure 3.9 A**). In contrast, tumours progressed quickly when VV-gp100 was omitted from the therapy (**Figure 3.9 A**) and mice had a significant decrease in survival rate compared to the ACTIV treated group (**Figure 3.9 B**). In addition, the group receiving ACTIV therapy lacking CARaMEL T cells had a significant decrease in survival rate compared to mice receiving the full ACTIV regimen (**Figure 3.9 B**).

These observations led us to determine whether VV-gp100 functioned through activation of CARaMEL T cells or direct lysis of tumour cells as VV-gp100 has the potential to infect and lyse tumour cells directly [295]. To answer this question, Panc02-Her2 cells were incubated with various concentrations of VV-gp100 and incubated for 72 hours (**Figure 3.10**). I observed that, even at high virus to cell ratios, VV-gp100 failed to lyse Panc02-Her2 cells **(Figure 3.10 A-C),** despite its ability to lyse the susceptible control cell line, 293A (**Figure 3.10 E-G).** These results indicated that Panc02-Her2 cells were resistant to direct lysis by VV-gp100, potentially due to the absence of the receptor for viral entry [296] and suggested that the main role of VV-gp100 was in the activation and expansion of CARaMEL T cells.

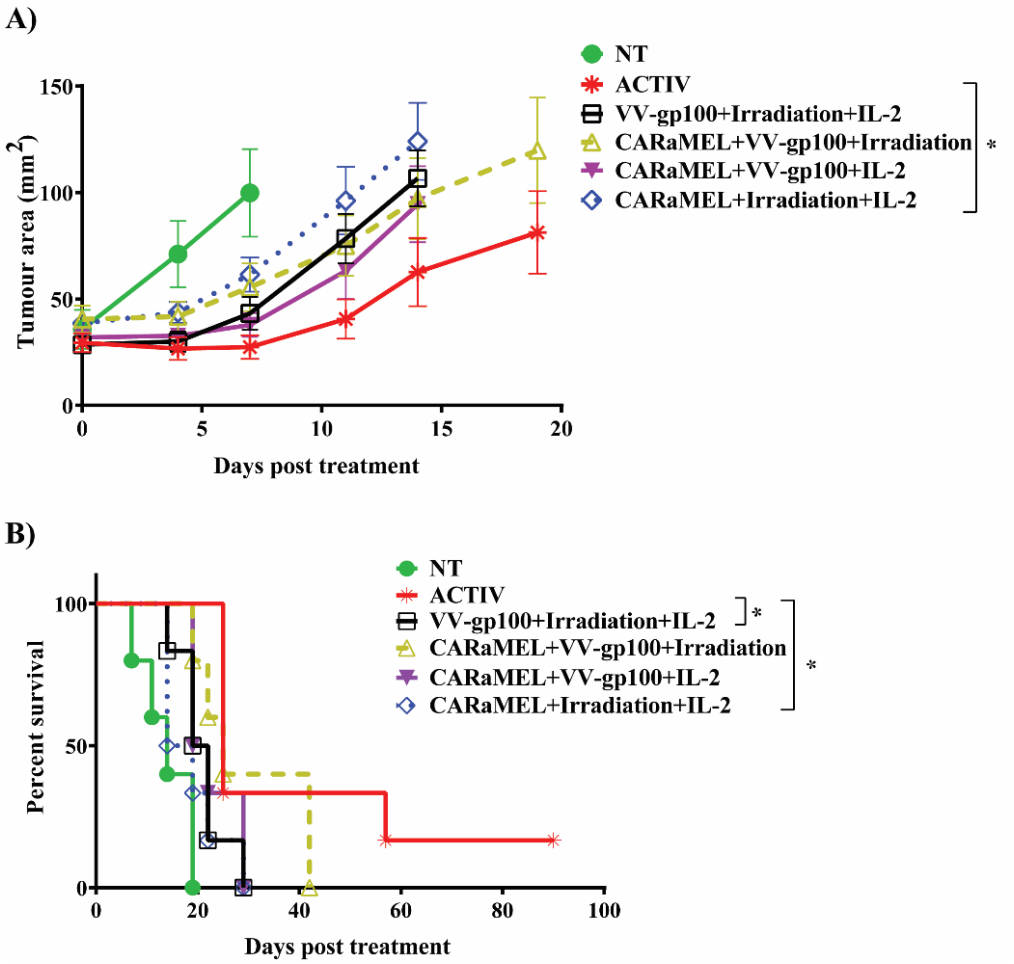


Figure 3.9. Full ACTIV regimen is needed for the best anti-tumour activity

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy or therapy lacking vaccinia virus, irradiation, CARaMEL T cells or IL-2. As a control, a cohort of mice were left untreated. (**A**) Tumour area was measured at the indicated times, and data are mean ± SEM of six mice/group, representative of two independent experiments. (**B**) Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (* $p \leq 0.05$).

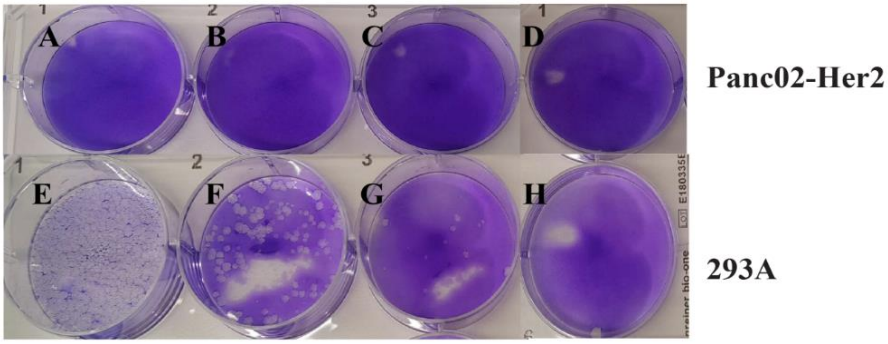


Figure 3.10. Panc02-Her cells are resistant to VV-gp100 infection

Panc02-Her and 293A cells were seeded in six well plates at 0.25 million cells/5 ml of DMEM and incubated overnight. On the following day, wells were infected with VV-gp100 (**A, E**) 1.2×10^{9} PFU/mL, (**B, F**) 1.2×10^{8} PFU/mL, (**C, G**) 1.2×10^{7} PFU/mL. (**D, H**) Control cells 293A were not infected with the virus. Seventy-two hours post-infection, cells were fixed with methanol and stained with 0.1% crystal violet to reveal plaques.

3.2.8. Immunization with VV-gp100 resulted in CARaMEL T cell expansion and accumulation in tumours

To confirm the hypothesis that VV-gp100 could activate/expand CARaMEL T cells, I treated mice bearing Panc02-Her2 tumours with ACTIV therapy or therapy lacking vaccinia virus **(Figure 3.11)**. Spleens and tumours were collected at various time points and flow cytometric analysis was performed to determine the expansion of CARaMEL T cells in spleens and tumours. CARaMEL T cells significantly expanded in the spleens of mice that received CARaMEL T cells and VV-gp100 compared to the group of mice that received CARaMEL T cells alone. The frequency of CARaMEL T cells in spleens of mice that received CARaMEL T cells and VV-gp100 therapy gradually decreased over the next days (**Figure 3.11 A**). CARaMEL T cells infiltrated Panc02-Her2 tumours in mice that received both CARaMEL T cells and VV-gp100, which peaked on day five (**Figure 3.11 B**). In addition, a significant proportion of CARaMEL T cells produced intracellular IFN-γ, demonstrating the ability of VV-gp100 to generate CARaMEL T cells with functional potential (**Figure 3.11 C**). The observation that CARaMEL T cells were detected at a significantly lower frequency in the group of mice that received CARaMEL splenocytes alone indicated the important role of VV-gp100 in the expansion of CARaMEL T cells.

Next, I used IHC to confirm these findings and to determine $CD8^+$ T cell distribution throughout the tumours. Flow cytometric analysis showed that $Thy1.1^+$ $CD8^+$ CARaMEL T cells comprised about 80% of the T cells (**Figure 3.12 A**). Therefore, in this experiment I stained CD8 T cells with an anti-CD8 antibody and not Thy1.1 which can reflect the distribution of endogenous and adoptively transfused T cells. As shown in **Figure 3.12** the tumour sections of mice receiving ACTIV therapy contained an even distribution (**Figure 3.12 A**), and significantly higher numbers of $CD8^+$ T cells (**Figure 3.12 B**), compared with the group of mice that received CARaMEL T cells only.

Taken together, these results suggested that a major contribution of VV-gp100 to ACTIV therapy of Panc02-Her2 tumours was in the expansion of CARaMEL T cells, while direct lysis of tumour cells played little role. In **Chapter 4** of this thesis I pursue this issue further by investigating the effect of epigenetic manipulation that has been previously been demonstrated to enhance the oncolytic potential of vaccinia virus.

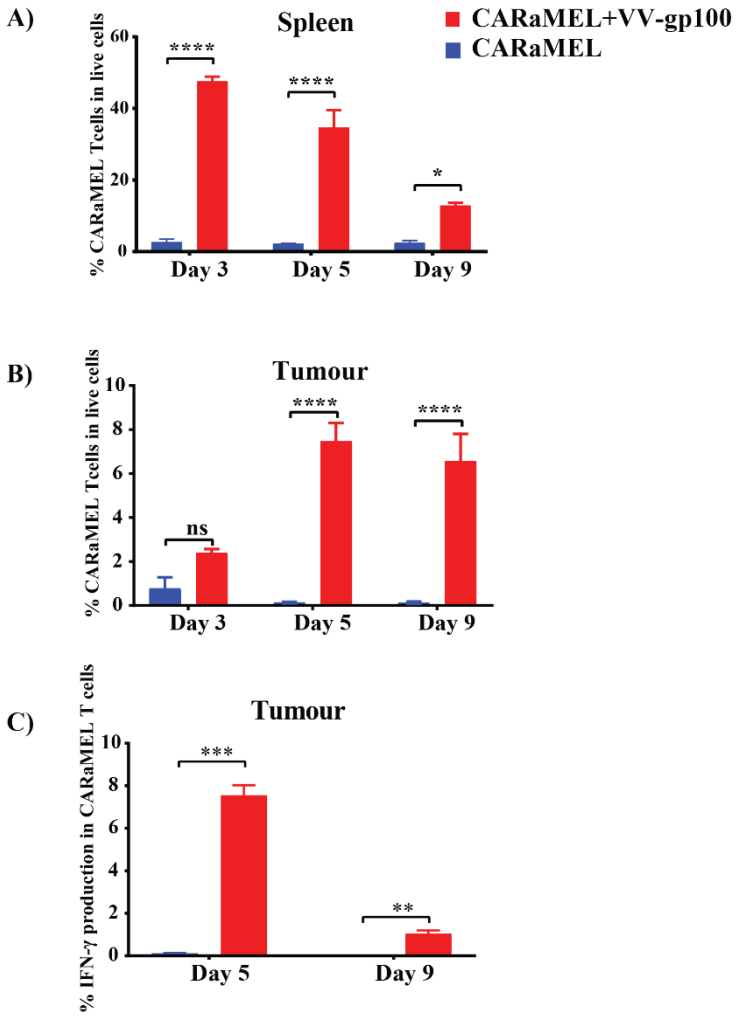


Figure 3.11. Immunization with VV-gp100 resulted in CARaMEL T cell expansion and their accumulation in tumours

Flow cytometric analysis of Panc02-Her2 tumours (**A**) and spleens (**B**) subjected to ACTIV therapy or therapy lacking vaccinia virus. Tumours and spleens were collected at the indicated time points and flow cytometric analysis was performed to determine the percentage of $CD8^+/Thy1.1^+$ T cells in live cells (n = 3 per group; representative of two experiments). (**C**) Percentage of IFN-γ producing cells in $Thy1.1^{+/}CD8^+$ T cells from tumours of mice receiving either ACTIV therapy or ACTIV lacking VV-gp100. T cells were stained with an anti-IFN-γ antibody following incubation with golgistop and golgiplug for four hours prior to being harvested for analysis by flow cytometry. Data are representative of two independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t* –test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p \leq 0.0001$, ns= not significant).

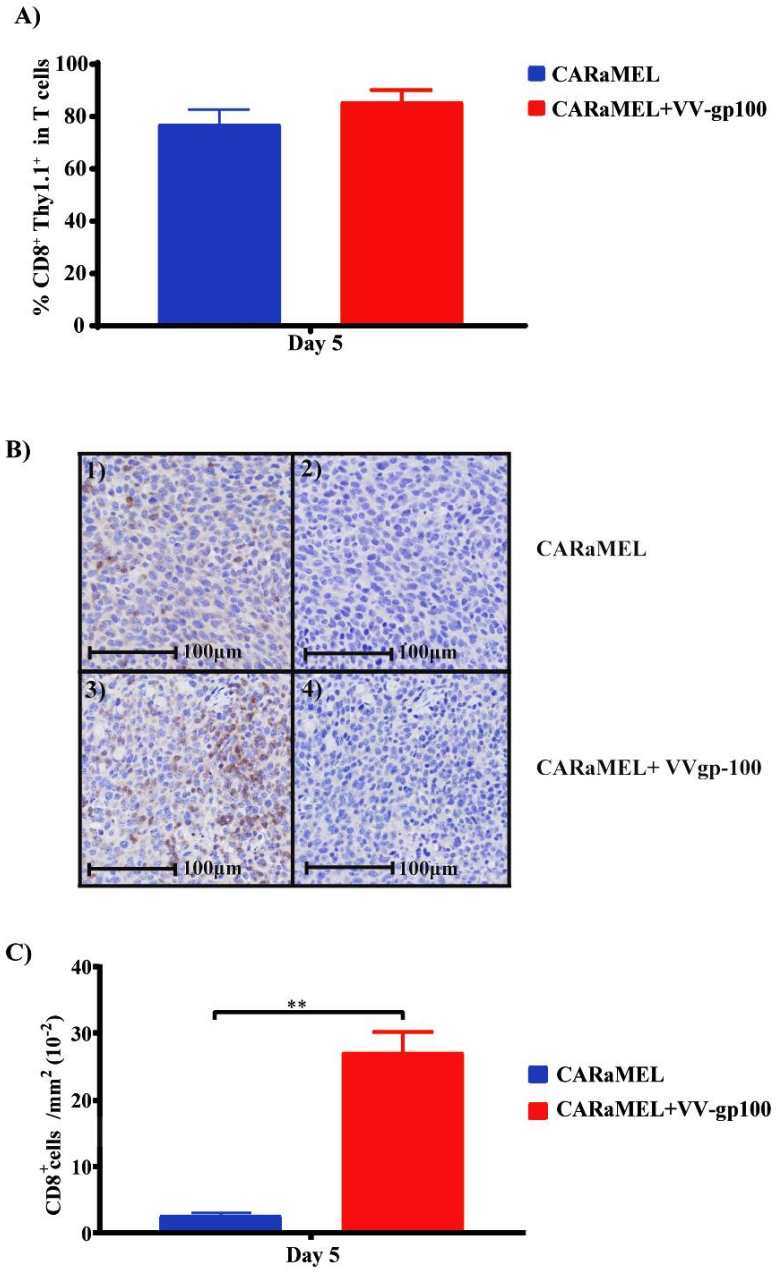


Figure 3.12. ACTIV therapy induces CD8$^+$ T cell accumulation in tumours

(**A**) Flow cytometric analysis of Panc02-Her2 tumours subjected to ACTIV therapy or therapy lacking vaccinia virus. Tumours and spleens were collected on day five post treatment and flow cytometric analysis was performed to determine the percentage of CD8$^+$/Thy1.1$^+$ T cells present from total T cells (n = 3 per group; representative of two experiments). (**B**) Immunohistochemical staining of Panc02-Her2 tumours subjected to ACTIV therapy or therapy lacking vaccinia virus on day 5. Representative sections of tumours from mice treated with ACTIV therapy lacking VV-gp100 (top) or ACTIV therapy (bottom) stained with anti-CD8 (**B1 and B3**) or matched isotype control antibody (**B2 and B4**) captured at 40 × (brown; scale bar = 100 µm; tumours from three mice per group, three sections per mouse. (**C**) Whole IHC section of CD8$^+$ cell counts from three random sections of three different tumours (9 data points) per group enumerated using HALO software. Data from one experiment shown as mean ± SEM of triplicate samples from three individual tumours. Statistical analyses were performed using the Student *t* –test (** $p \leq 0.01$).

3.2.9. CARaMEL T cell proliferation and distribution in ACTIV-treated tumours

To gain insight into why these murine pancreatic tumours did not fully respond to ACTIV therapy, I investigated the cellular compositions of the tumour before and after treatment. I treated a group of mice with ACTIV therapy and left another tumour-bearing group untreated (NT) for comparison. The tumours were collected at three- and seven-days post ACTIV treatment and enzymatically digested into single cell suspensions prior to the staining with antibodies and flow cytometric analysis. The spleens of ACTIV treated mice were also collected to examine CARaMEL T cell expansion. The flow cytometry gating strategy is shown in **Figure 3.13.**

To determine the composition of different leukocytes, I stained for cell-specific markers. The markers used to differentiate each population were as follows: NK cells ($CD45^+$, $NK1.1^+$), total T cells ($CD45^+$, TCR^+), CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$), $CD8^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD8^+$), $CD4^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD4^+$), endogenous $CD8^+$ T cells ($CD45^+$, TCR^+, $Thy1.1^-$, $CD8^+$), endogenous $CD4^+$ T cells ($CD45^+$, TCR^+, $Thy1.1^-$, $CD4^+$), macrophages ($CD45^+$, $MHCII^+$, $CD11b^+$, $Ly6C^-$, $F4/80^{+/hi}$), DC subsets ($CD45^+$, $MHCII^+$, $CD11c^+$, $Ly6C^-$, $F4/80^{+/low}$), monocytic MDSCs ($CD45^+$, $CD11b^+$, $Ly6C^{hi}$, $Ly6G^-$, $MHCII^-$), monocytes ($CD45^+$, $CD11b^+$, $Ly6C^{intr}$, $Ly6G^-$, $MHCII^{low}$), neutrophils/PMN MDSCs ($CD45^+$, $MHCII^{-/low}$, $CD11b^+$, $Ly6G^+$, $Ly6C^{low}$).

Analysis by flow cytometry revealed that only approximately 5% of live cells in NT tumours were T cells which were composed of an equal mix of $CD4^+$ helper T and $CD8^+$ cells (**Figure 3.14 A4, A5 and A6**). In contrast, a large proportion of the cells in the ACTIV treated tumours were cytotoxic CARaMEL T cells. As shown in **Figure 3.14 A6**, an average of 7% of live cells in ACTIV treated tumours were cytotoxic $CD8^+$ T cells on day 3, and the percentage approximately doubled on day 7. In fact, the majority of these cells in tumours were the adoptively transferred $Thy1.1^+$ CARAMEL T cells by day seven (**Figure 3.14 A7**). In the spleen (**Figure 3.14 A8**), an average of 40% of the splenocytes were CARaMEL T cells on day three and this percentage dropped to approximately 20% on day 7, suggesting that CARaMEL T cells proliferated initially in the spleen and then migrated to the tumour site.

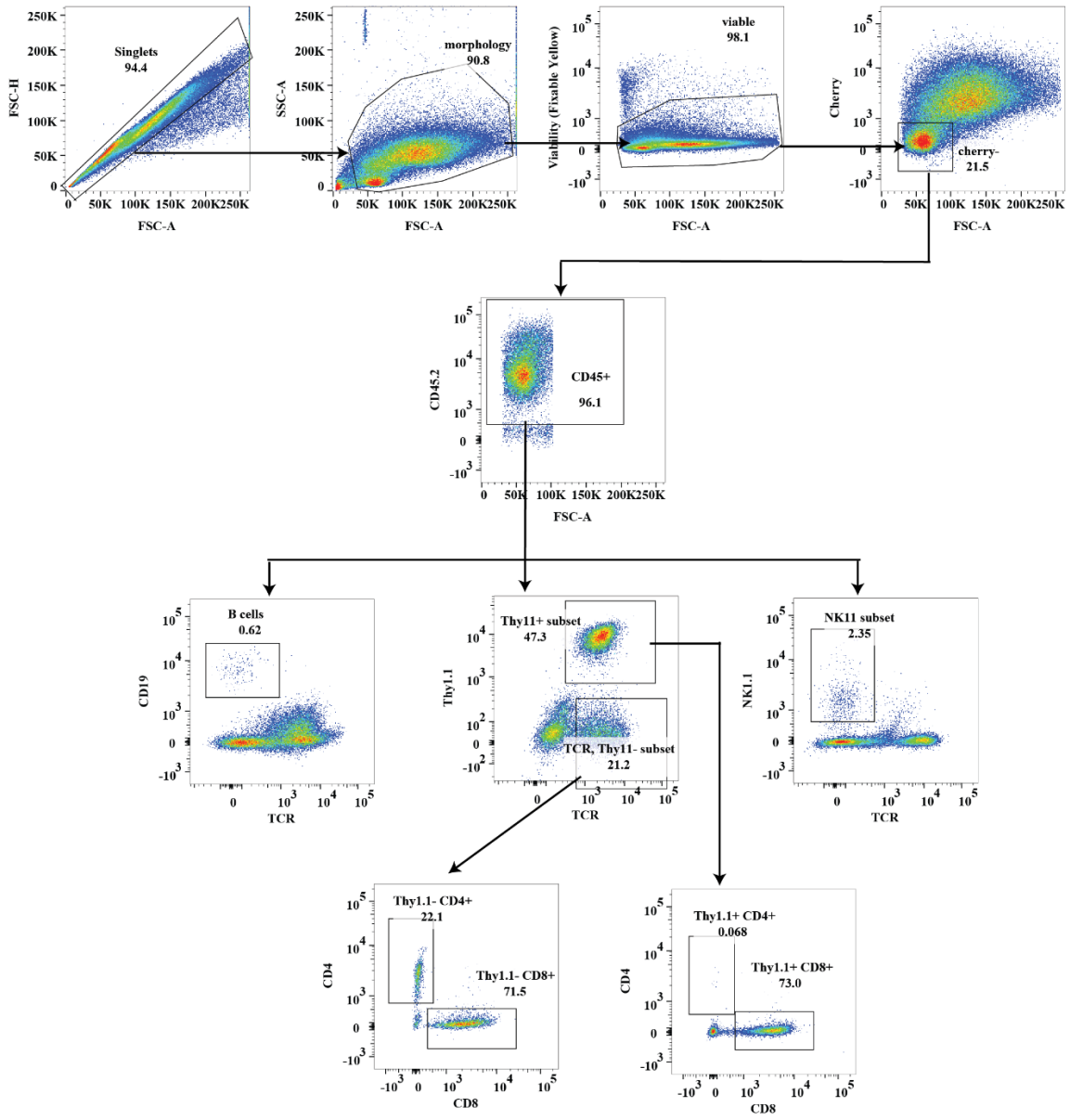
Although substantial numbers of CARaMEL T cells infiltrated tumours, it was possible that the CARaMEL cells were functionally blocked in the tumours. The frequency of tumour-infiltrating leukocytes with the potential to suppress CARaMEL T cell function was determined using flow cytometry. Among the myeloid cell population, I observed a marked decrease in the

percentage of macrophages in the tumours of ACTIV treated mice on both days three and seven compared with non-treated mice (**Figure 3.14 B1**). Also, monocytic myeloid-derived suppressor cells (MDSCs) and neutrophils/polymorphonuclear (PMN)-MDSCs were significantly decreased in ACTIV treated mice on day three compared with untreated mice (**Figure 3.14 B3 and B4**). This decrease may have been due to the preconditioning irradiation used in the ACTIV therapy regimen.

Despite their reduced numbers, macrophages, monocytic- and neutrophils/PMN-MDSCs comprised a substantial proportion of the immune cells within tumours, which can create a highly immunosuppressive TME that inhibits T cell cytotoxicity, and which promotes tumour progression through a variety of mechanisms. These mechanisms include direct inhibitory functions on T cell activation and function [297, 298], depletion of amino acids required for T cell activation and proliferation [299, 300] and other immunosuppressive mechanisms [301-306]. However, it is not clear at this stage whether myeloid cells in ACTIV-treated tumours possessed an immunosuppressive phenotype, and this aspect awaits further investigation.

These results collectively suggest that CARaMEL T cells expanded in the spleens of recipient mice and migrated to Panc0-Her2 tumours, but CARaMEL T cells could have been functionally blocked in the tumours by immunosuppressive molecules or by other tumour-infiltrating cells.

A)


Singlets
94.4
FSC-H
FSC-A
morphology
90.8
SSC-A
viable
98.1
Viability (Fixable Yellow)
Cherry
cherry-
21.5
CD45.2
CD45+
96.1
B cells
0.62
CD19
TCR
Thy11+ subset
47.3
Thy1.1
TCR, Thy11- subset
21.2
NK11 subset
2.35
NK1.1
Thy1.1- CD4+
22.1
Thy1.1- CD8+
71.5
CD4
CD8
Thy1.1+ CD4+
0.068
Thy1.1+ CD8+
73.0

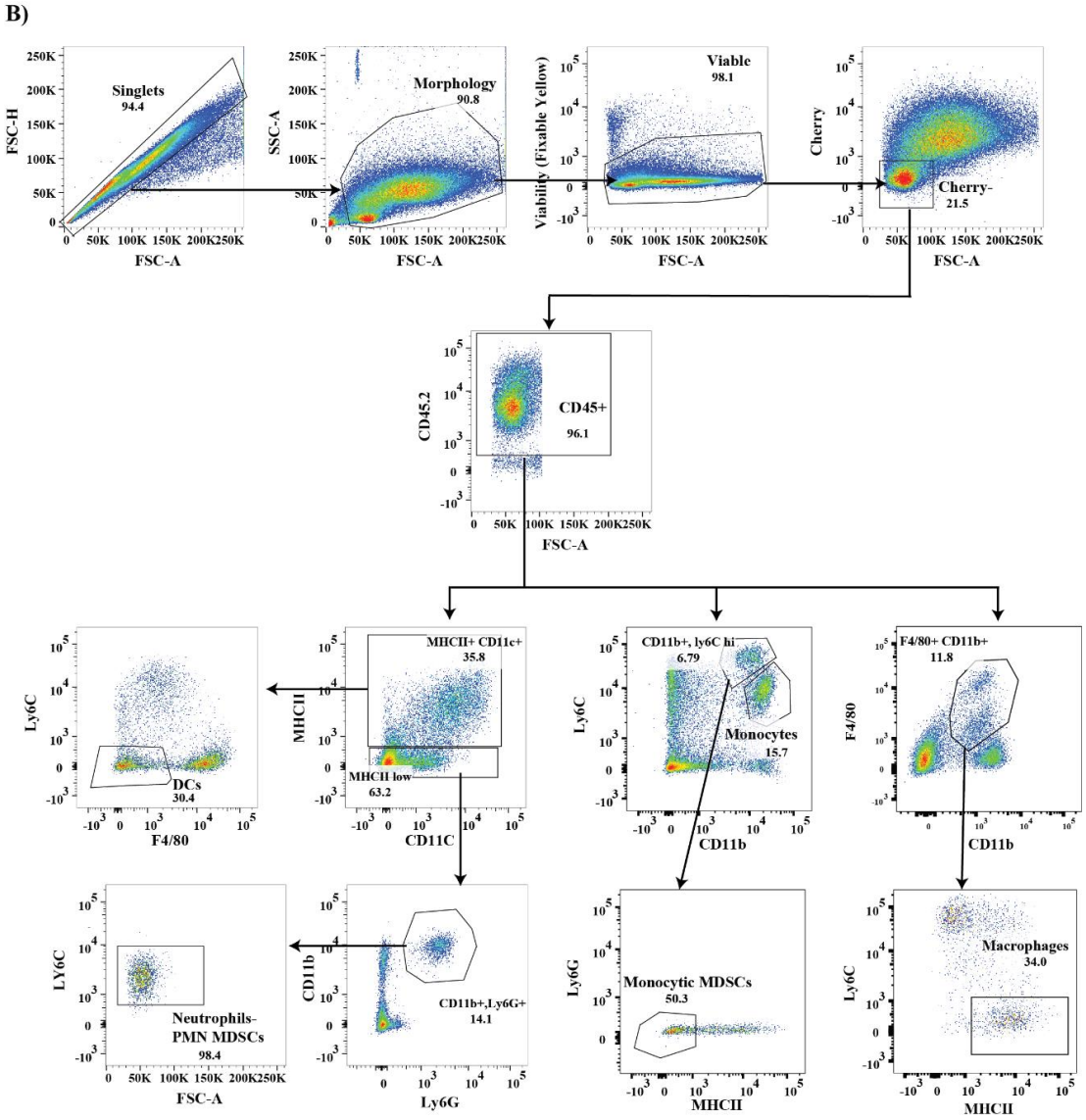
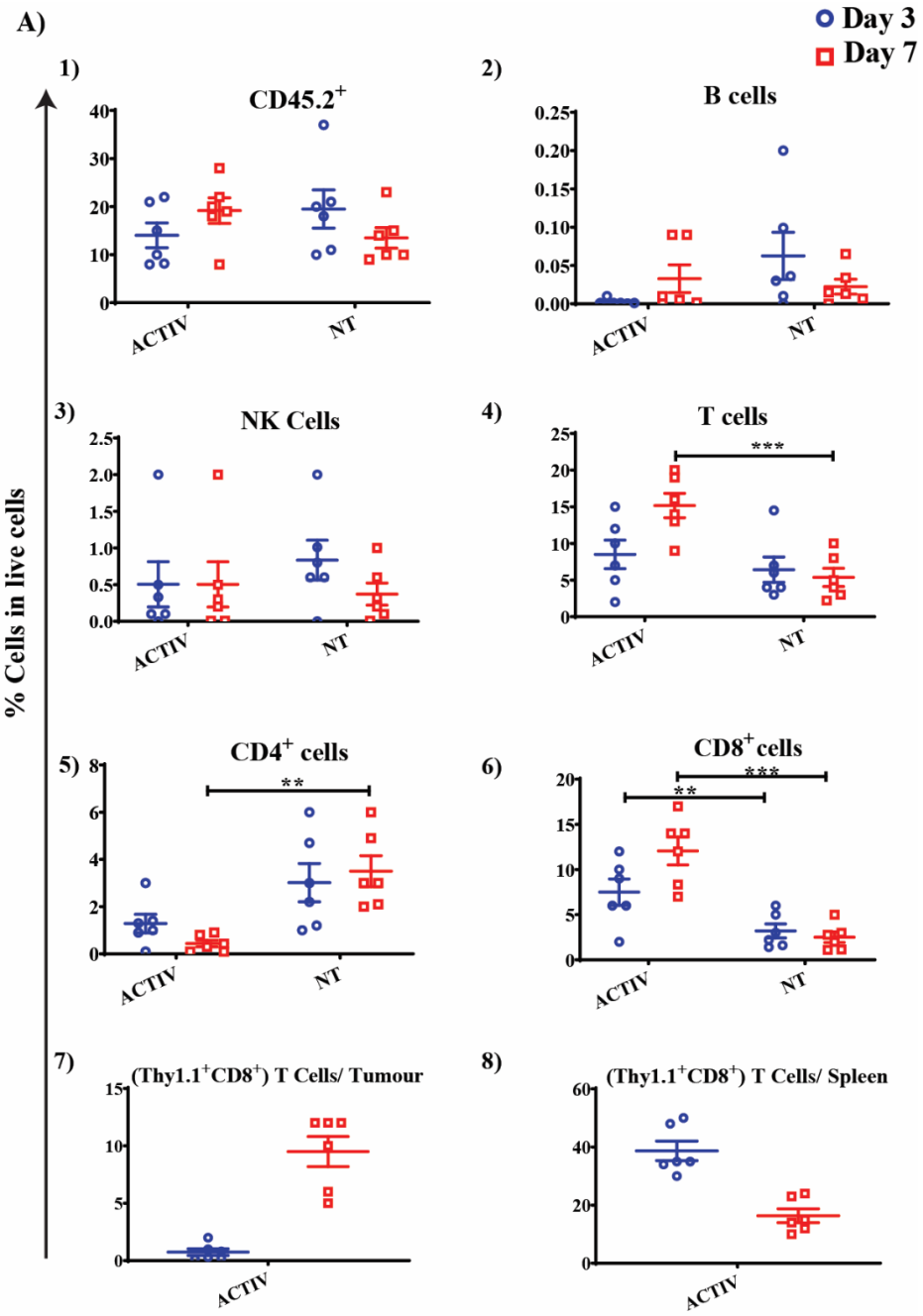


Figure 3.13. Gating strategies for leukocyte populations in Panc02-Her2 tumours

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy or no treatment (NT). Tumours were harvested on day three and seven post ACTIV therapy and were processed into single cell suspensions prior to analysis by flow cytometry. Diagrams display the gating strategy used to evaluate different lymphocyte populations. The markers used to differentiate each were as follows: (**A**) Lymphocyte populations: B cells ($CD45^+$, $CD19^+$), NK cells ($CD45^+$, $NK1.1^+$), total T cells ($CD45^+$, TCR^+), CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$), $CD8^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD8^+$), $CD4^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD4^+$), endogenous $CD8^+$ T cells ($CD45^+$, TCR^+, $Thy1.1^-$, $CD8^+$), endogenous $CD4^+$ T cells ($CD45^+$, TCR^+, $Thy1.1^-$, $CD4^+$). (**B**) Myeloid lineage populations: macrophages ($CD45^+$, $MHCII^+$, $CD11b^+$, $Ly6C^-$, $F4/80^{+/hi}$), DC subsets ($CD45^+$, $MHCII^+$, $CD11c^+$, $Ly6C^-$, $F4/80^{+/low}$), monocytic MDSCs ($CD45^+$, $CD11b^+$, $Ly6C^{hi}$, $Ly6G^-$, $MHCII^-$), monocytes ($CD45^+$, $CD11b^+$, $Ly6C^{intr}$, $Ly6G^-$, $MHCII^{low}$), neutrophils/PMN MDSCs ($CD45^+$, $MHCII^{-/low}$, $CD11b^+$, $Ly6G^+$, $Ly6C^{low}$). Diagrams are representative of two independent experiments.


A)
Day 3
Day 7
% Cells in live cells
1)
CD45.2+
2)
B cells
3)
NK Cells
4)
T cells
***
5)
CD4+ cells
**
6)
CD8+cells
***
**
7)
(Thy1.1+CD8+) T Cells/ Tumour
8)
(Thy1.1+CD8+) T Cells/ Spleen
ACTIV
NT

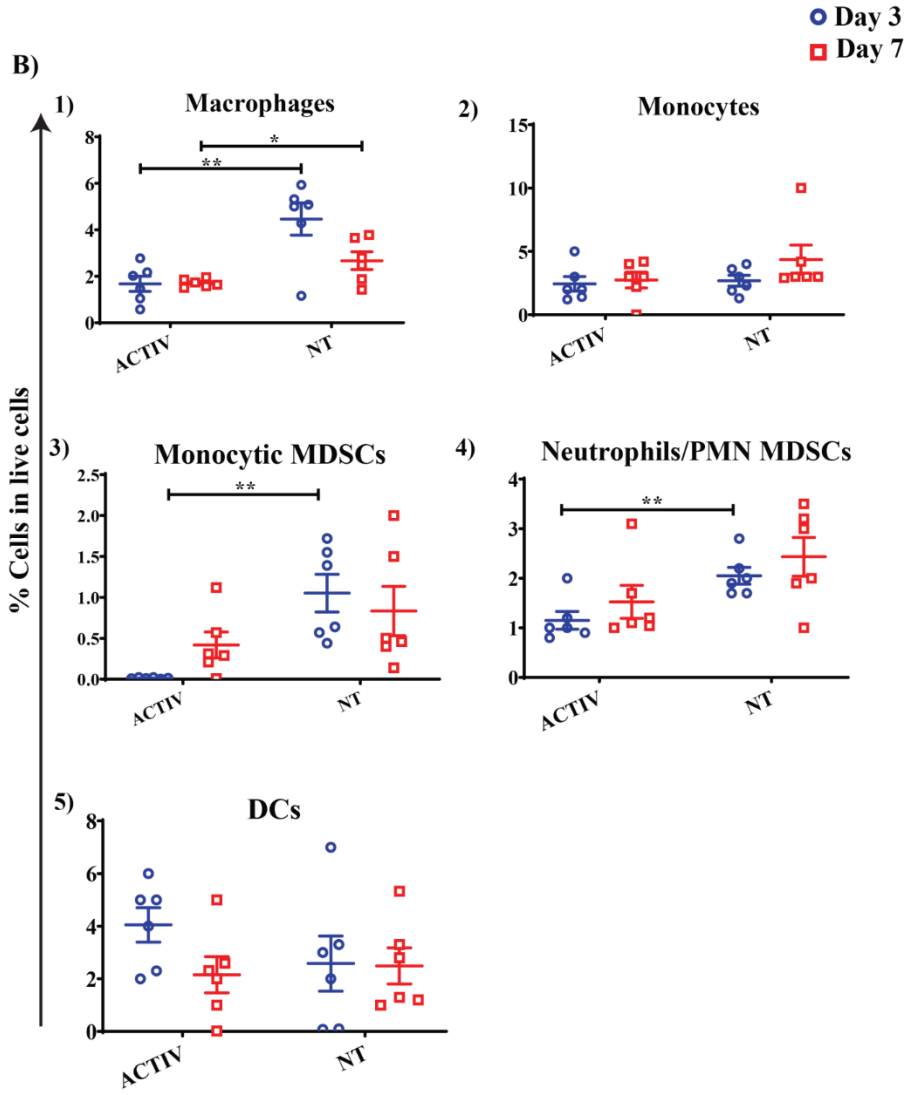


Figure 3.14. Infiltration of various leukocyte populations in Panc02-Her2 tumours following ACTIV therapy

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy or no treatment (NT). Tumours were harvested and analysed using flow cytometry, with gating as shown in **Figure 3.13**, to determine the percentage of (**A1**) $CD45.2^+$, (**A2**) B cells, (**A3**) NK cells, (**A4**) T cells, (**A5**) $CD4^+$, (**A6**) $CD8^+$, (**A7**) $Thy1.1^+$,$CD8^+$ in tumour, (**A8**) $Thy1.1^+$,$CD8^+$ in spleen, (**B1**) macrophages, (**B2**) monocytes, (**B3**) monocytic MDSCs, (**B5**) neutrophils/PMN MDSCs (**B6**) DC subsets of live endogenous cells from tumours on days three and seven post treatment. Data are presented as mean ± SEM of three mice per group pooled from two independent experiments. Statistical analyses were performed using the Student *t* –test (* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.2.10. Tumour relapse post ACTIV treatment was not due to Her2 antigen loss on the tumour cells

To investigate if tumour relapse was due to antigen loss on the tumour cells, which is a common phenomenon observed in other adoptive cell transfer trials [307-309], I injected the Panc02-Her2 cancer cell lines subcutaneously in C57BL/6-Her2 mice and allowed the tumours to grow for nine days. ACTIV therapy was performed as previously described and presented in the schematic (**Figure 3.7**). Six days post ACTIV treatment, tumours were harvested and enzymatically digested into single cell suspensions and incubated in normal tissue culture media for three weeks to reduce infiltrating leukocytes and fibroblasts. After that, I analysed Her2 expression using flow cytometry. Her2 antigen remained stably expressed on the tumour cells from mice treated with ACTIV therapy (**Fig 3.15 A**).

Moreover, Panc02-Her2 or Panc02-Her2 (post ACTIV) cancer cells were injected subcutaneously into C57BL/6 human-Her2 transgenic. Nine days following tumour injection the mice received ACTIV therapy. Panc02-Her2 and Panc02-Her2 (post ACTIV) tumours had similar growth rates and responses to ACTIV therapy (**Figure 3.15 B and C**), indicating that Panc02 regrowth was not because of the loss of Her2 expression.

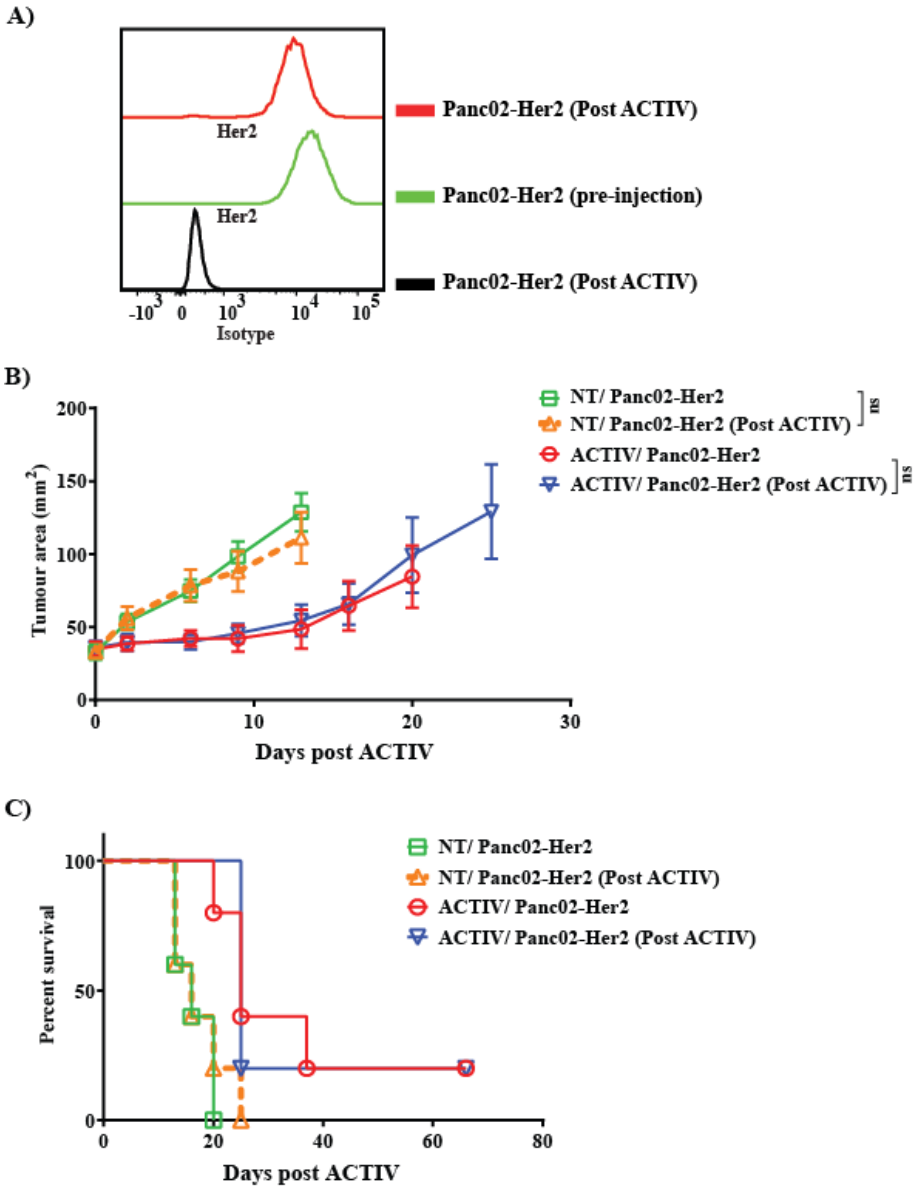


Figure 3.15. Her2 expression on Panc02-Her2 tumour cells remains stable *in vivo* following ACTIV therapy

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic. Nine days following tumour injection the mice received ACTIV. Tumours were harvested from mice following treatment with ACTIV therapy on day six post treatment. Tumours were enzymatically digested into single cell suspensions and incubated in normal tissue culture media for three weeks to reduce infiltrating leukocytes and fibroblasts. (**A**) Flow cytometry was performed to assess Her2 expression on viable tumour cells. Data are presented as histograms. FACS plots are gated on live cells. Pre-injected Panc02-Her2 tumour cells were used as a positive control. (**B**) Panc02-Her2 or Panc02-Her2 (post ACTIV) cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic. Nine days following tumour injection the mice received ACTIV therapy. As a control, a cohort of mice was left untreated. Tumour area was measured at the indicated times, and data is from one experiment. Shown is the mean ± SEM of five mice/group (**C**) Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (n = 5 mice/group, ns= not significant).

3.3. Discussion

Adoptive cell therapy using CAR T-cells has shown robust anti-tumour activity and significant responses in haematological malignancies [240-244, 310, 311]. However, CAR T cells has had limited success in solid tumours [245, 249-255, 286] including pancreatic cancer [129, 245, 246, 285]. The inefficiency of CAR T cells against solid cancers is believed to be due to several factors including an immunosuppressive TME, inadequate T cell trafficking into tumours, as well as limited T cell expansion and persistence [245, 249-255, 286, 312].

To address these limitations related with T cells; our laboratory created ACTIV therapy which includes dual-specific CARaMEL T cells that express the gp100 TCR and anti-Her2 CAR. This dual-specificity of CARaMEL T cells enabled them to expand in response to a VV-gp100 vaccine through the TCR and kill tumours through the CAR. ACTIV therapy showed significant anti-tumour activity and eradicated large solid $Her2^+$ orthotopic breast tumours, subcutaneous sarcoma, and large liver tumours expressing the human Her2 antigen in immunocompetent mice [287].

Pancreatic cancer was the next challenging target for ACTIV therapy because of its known highly immunosuppressive TME with its remarkable desmoplastic reaction and dense stroma, poor infiltration of effector T cells and the significant myeloid inflammation [274-276]. Therefore, I aimed to determine the efficacy of ACTIV therapy against mouse pancreatic cancers.

One of the important aspects in CAR-T cell therapy is to choose an appropriate targeted TAA as not all TAAs are completely tumour-specific, raising the possibility of potential toxicity against normal tissues. In my study I chose Her2 as my targeted antigen for modelling purposes, because I have CAR T cells against Her2 antigen, and the Her2 immunocompetent mice. I generated two $Her2^+$ murine pancreatic cancer models KPC-Her2 and Panc02-Her2, which both served as targets to investigate CARaMEL T cell activity *in vitro* and *in vivo*. Her2 expression is observed in 20–60% of human pancreatic cancer cases and therefore, it is a potential target for CAR T cell treatment, although the gene is rarely amplified in this malignancy [313-316]. However, there are also many alternative overexpressed pancreatic cancer TAA's including: MSLN [317], PSCA [318], CEA [319], MUC1 [320, 321], CD47 [322, 323] and CD24 [324, 325]. Also, FAP which is expressed on tumour stromal fibroblasts of pancreatic cancers can be targeted using CAR T cell therapy [326]. Ideally, to mitigate any on-target/off-tumour toxicity associated with CAR T cell therapy, the targeted TAA should be

uniquely or highly expressed on the tumour cell surface compared with normal tissues and also be involved in maintaining the oncogenic tumour phenotype to limit tumour escape [327].

On-target/off-tumour toxicity has been reported in patients who received CAR T cell therapy against CD19 in B cell malignancies [328-330], CAIX in renal cell carcinoma [331], NY-ESO-1 in metastatic synovial cell sarcoma and melanoma patients [332] and Her2 in colorectal cancer [333]. On-target/off-tumour toxicity related to anti-Her2 CAR-T cell infusion led to death of a patient with colon cancer metastases in the lungs and liver. The patient was preconditioned using cyclophosphamide and fludarabine and then treated with a dose of 10^{10} of third generation CAR T cells. However, this resulted in acute uncontrollable respiratory distress and subsequent death. An autopsy report found lung ischemia and injury and an increase in multiple cytokines including IFN-γ, IL-6, and TNF-α in serum post CAR T cells administration, which was consistent with an acute reaction against pre-existing low levels of Her2 antigen [333]. This case led to the suspension of CAR T cell immunotherapy targeting the Her2 antigen until another study utilizing lower numbers of second generation anti-Her2 CAR T cells in 19 patients with $Her2^{+}$ sarcoma demonstrated safety [334].

In ACTIV therapy I used mice expressing human Her2, which provided a physiologically relevant model enabling evaluation of possible toxicity [260]. C57BL/6-Her2 immunocompetent mice express human Her2 as a self-antigen in normal tissues including normal breast and the brain, enabling me to determine potential on-target/off-tumour toxicity in my model. I observed some transient toxicity in mice that received ACTIV therapy including unsteady movement, weight loss and lethargy. These manifestations were also observed in our previous study of ACTIV therapy against breast, sarcoma and liver tumours which was found to be due to infiltration of CARaMEL T cells into the cerebellum and their reaction against $Her2^{+}$ normal tissues of the brain [287]. Strategies can be employed to avoid on-target/off-tumour toxicity including the use of inhibitor chimeric antigen receptors (iCARs), which are CAR T cells that are inhibited by antigens expressed on normal tissue [335], and split CAR systems using complementary CARs possessing a chimeric costimulatory receptor (CCR) that recognizes a second antigen [336]. In addition the use of suicide genes, or safety systems that can be triggered by small molecule drugs, can be used to minimize the toxicity associated with recognition of normal tissues [337, 338].

Her2 transgenic mice are fully immunocompetent mice, which allowed us to determine the CARaMEL T cell-mediated anti-tumour response in the presence of a normal complement of

immune regulatory mechanisms. Also, this mouse model could potentially provide insight into potential contributions from the endogenous immune system, recruiting additional immune cells, production of pro-inflammatory cytokines from other immune cells and epitope spreading, which can enhance the anti-tumour activity [339-341].

In ACTIV therapy I hypothesized that CARaMEL T cells were activated and expanded through the TCR-MHC interaction with APCs in lymphoid tissue, which may lead to optimal CARaMEL T cell activity. In my system I used a TCR specific for the gp100 antigen as a model system, since transgenic mice expressing this TCR were available. However, the gp100 TCR is not the best option to be used in the clinic due to potential reactivity to this melanocyte antigen, which may destroy normal gp100-expressing cells in the skin, eyes, and ears leading to vitiligo, ocular toxicity and hearing loss [342, 343]. A safer option in the clinic may be the use of TCRs specific for foreign antigens such as influenza virus.

As an appropriate source of CARaMEL T cells, I utilized splenocytes of C57BL/6-CARaMEL mice, in which both CAR and TCR are expressed at consistent levels. CARaMEL mice were generated by crossing CAR mice which express an anti-Her2-CD28-CD3ζ CAR [344] and pMEL mice which express a TCR specific for the human gp100 antigen [261]. CARaMEL T cells were shown to be mostly $CD8^+$ and have an altered CD4:CD8 phenotypic ratio, likely due to the lack of interaction of the CD4 co-receptor with the MHC-I-restricted TCR. However, in my *in vivo* experiments using largely $CD8^+$ CARaMEL T cells I showed high anti-tumour activity in ACTIV therapy suggesting mainly a $CD4^+$ T-cell independent anti-tumour mechanism. Previous studies have sometimes demonstrated enhanced anti-tumour activity using a combination of $CD8^+$ and $CD4^+$ T cells [345], but there are also reports in cancer and other disease settings [346, 347] in which $CD8^+$ T cells can provide self-help if they are present at a sufficiently high frequency [347]. However, I cannot exclude the help of the endogenous $CD4^+$ T cells which were reconstituted after lymphodepletion.

The recombinant vaccinia virus (VV) encoding human gp100 [348] (VV-gp100) was the viral vaccine used in this model system. VV-gp100 is an attenuated virus that was generated by insertion of the human gp100 minigene by homologous recombination and subsequent purification of recombinant progeny. The gp100 minigene was incorporated into the virus ribonucleotide reductase gene which led to its deficiency and subsequent reduced virulence while maintaining the potential oncolytic ability of the virus [263]. I hypothesized that VVgp-100 may have contributed to the anti-tumour activity via several mechanisms, including

activation and expansion of CARaMEL T cells, induction of tumour cell death and initiation of inflammation. Therefore, I carried out experiments to gain insight into potential mechanisms involved in ACTIV therapy. I showed that VV-gp100 failed to infect and lyse Panc02-Her2 cells directly. Also, CARaMEL T cells significantly expanded in the spleens of mice that received VV-gp100 with CARaMEL T cells. These results together suggest that VV-gp100 has a crucial role in ACTIV therapy through the activation and expansion of CARaMEL T cells but not through direct oncolysis. In the clinic, there are different recombinant oncolytic viruses that can be used for intravenous use including vaccinia virus, adenovirus, reovirus and herpes virus [349]. For example, it has been demonstrated that vaccinia virus JX-594 has an ability and selectivity to infect, replicate and express transgene products in patients' tumours [350].

In vivo persistence of CAR-modified lymphocytes has great impact on therapy efficacy [351]. In my study, I provided exogenous IL-2 to support T cell growth and engraftment. IL-2 is produced mainly by $CD4^+$ T cells [352], can support T-cell growth and activation [261] and promote the expansion and function of tumour-specific T cells *in vitro* and *in vivo* and amplify $CD8^+$ T cell responses [229, 352]. Also, IL-2 can optimize both effector T cell generation and differentiation into memory cells and it plays an important role in the maintenance of peripheral self-tolerance [353]. One study showed that five doses of IL-2 was associated with tumour regression and no significant benefit to ≥6 doses [354]. In ACTIV therapy I used four doses of IL-2. In humans and especially in older patients, high-doses of IL-2 can cause significant side effects, such as renal toxicity [355]. Therefore, IL-2 can be substituted with less toxic alternate cytokines that may better support adoptively transfused T cells such as IL-7, IL-15, and IL-21 [354]. Several studies have reported that γ(c) cytokines including IL-2, IL-7, IL-15, and IL-21 are critical regulators of immunity, which can enhance T cell *in vivo* persistence and augment their anti-tumour effects [356-361].

Preconditioning with systemic chemotherapy and/or total body irradiation have been used prior to ACT to create lymphoid "space" and better engraftment of transferred T cells, leading to enhanced anti-tumour efficacy [362, 363]. Several studies showed that transient eradication of endogenous lymphocytes can augment the function of adoptively transferred $CD8^+$ T cells [222, 261, 340, 364, 365]. Several mechanisms may explain this augmented efficacy of T cells in the lymphopenic environment. These mechanisms include the removal of immunosuppressive cells such as $CD4^+$ $CD25^+$ T_{reg} cells [366], which compete for cytokines and serve as cellular cytokine sinks [294]. This can lead to sustained or increased persistence of transferred T cells [362, 363, 367], as well as T cell expansion and enhanced effector

function [294, 362, 368-371]. In addition, lymphodepletion can cause apoptosis or necrosis of tumour cells, and APCs can take up and present tumour antigens to tumour-reactive T cells from adoptive or endogenous sources [372]. Although lymphodepletion can initially decrease APCs numbers *in vivo*, it can increase their function and availability [373-375].

In ACTIV therapy I used total body irradiation; however in the clinical setting using cyclophosphamide as the precondition step is more convenient and less invasive than total body irradiation which can have oncogenic effects [376, 377] and cause polyostotic and/or generalized bone changes in epiphyseal/metaphyseal regions in the spine of patients [378]. Conditioning following cyclophosphamide is usually transient and associated with a rapid reformation of a new T_{reg} cell population [379] and using cyclophosphamide alone in solid tumours was inappropriate and led to a poor outcome [251]. In patients with metastatic melanoma, Rosenberg *et al*. have improved the therapeutic response of ACT with autologous tumour-infiltrating lymphocyte (TILs) to more than 70%, using a combined sub-lethal dose of radiation (12 Gy) and cyclophosphamide and fludarabine [355]. Therefore, further experiments using cyclophosphamide as a preconditioning regimen in ACTIV therapy would be necessary to determine its efficacy and suitability as a substitute for total body irradiation.

Tumour destruction and inflammation can lead to a phenomenon known as epitope-spreading, in which a broader immune response is induced against antigens other than the original target antigen [380, 381]. In our previous experiments including breast, sarcoma and liver tumours, ACTIV-treated surviving mice were partially resistant to rechallenge with the same tumour lacking Her2 expression, indicating that combined CARaMEL T cell and vaccine helped in immune memory generation which may have occurred during the initial response and involved epitope spreading [380]. Immunoblot and ProtoArray have been used by different investigators to detect anti-tumour immunity following various immunotherapies [245, 382-384]. Further studies in future can use these approaches to evaluate the development of humoral and cellular epitope-spreading induced by CARaMEL T cells and VV-gp100, which may have significant implications for the role of ACTIV therapy in stimulating broad anti-tumour adaptive immunity and tumour rejection.

Antigen loss can be one of the major obstacles in ACT therapy which was demonstrated in different CAR T cell clinical trials [307-309, 385, 386]. However, in this study I showed that Panc02 regrowth post ACTIV treatment was not because of Her2 antigen loss, as it remained stably expressed on the tumour cells given it is under the control of the MSCV-LTR promoter.

However, weak promoters could be used to induce this antigen loss to investigate this phenomenon in future studies. These results suggest that Panc02 regrowth was due to other reasons, such as loss of activation of CARaMEL T and/or the immunosuppressive microenvironment including metabolic reprogramming conditions, hypoxia and immunosuppressive signalling through cell checkpoint receptors [387-392].

Tumour heterogeneity may have played a role in antigen loss and subsequent tumour relapse. For example, Grupp *et al.* reported a clinical relapse of a $CD19^-$ tumour in an acute lymphoblastic leukaemia patient who was administrated with CD19-directed CAR T cells [385]. Also, CD19 CAR-T cells cannot recognize CD19 even if it is still present. This can happen due to the loss of the cell surface fragment having the cognate epitope as a result of an alternative RNA splicing or mutations [393]. In my study, Her2 was detected using flow cytometry with the 24D5monoclonal antibody. While 24D5 CAR binds to the juxtamembrane region of Her2, the FRP5 CAR can detect a discontinuous epitope within residues 11-169 of the Her2 protein facing away from the cell surface [394]. Therefore, we cannot exclude the possibility of that the FRP5 epitope was lost by mutation in the tumour escape variants, and these variants could still retain Her2 expression as judged by the 24D5 antibody. It would be of interest to sequence the Her2 gene in relapsing tumours in future experiments.

Similar effects of this phenomenon were also observed in solid tumours. A previous study reported that infusion of T cells specific for the melanoma-associated peptide, gp100 was followed by the outgrowth of tumours lacking the target antigen [395]. To model the phenomenon of tumour immune escape due to antigen heterogeneity different investigators targeted multiple TAAs expressed by haematological [396] or solid tumours as observed in a pancreatic cancer study which targeted MUC-1 and PSCA [339].

In conclusion, in this chapter I assessed the combination of dual-specific CAR T cells and an indirect vaccine (ACTIV therapy) in murine pancreatic cancer and showed how Panc02-Her2 and KPC-Her2 tumours responded to ACTIV therapy, leading to significant inhibition of tumour growth and increased survival. However, this tumour inhibition was temporary as KPC-Her2 and Panc02-Her2 tumours relapsed several days after ACTIV therapy. In considering the results of **Chapter 3**, I hypothesized that the resistance of pancreatic tumours to ACTIV therapy may have been due to suboptimal function of CARaMEL T cells, due to tumour-derived local immunosuppressive factors, and/or due to tumour cell-intrinsic anti-apoptotic mechanisms.

There are several ways to enhance apoptosis in tumour cells that include epigenetic manipulation, involving the use of histone deacetylase inhibitors (HDACi), which are a class of drugs that can affect gene transcription and protein function [164, 397]. HDACi may offer a better therapeutic window for immunotherapy through sensitizing tumour cells, as HDACi have been shown to upregulate tumour antigen expression and shift gene expression to a pro-apoptotic profile [398, 399]. In addition, HDACi have been shown to modulate the immunogenicity and improve anti-tumour immune responses [182, 183]. HDACi have also been reported to enhance $CD8^+$ T cell activation and function through enhancing their cytolytic abilities [185-188, 400]. Moreover, HDACi have been reported to enhance the susceptibility of tumour cells to oncolytic virus infection including vaccinia virus and act synergistically to kill tumour cells [401, 402]. In my case, this may enhance VVgp-100 infection of Panc02-Her2 tumour cells and induce cell death. Therefore, I hypothesized that HDACi may enhance ACTIV therapy against pancreatic cancer through these mechanisms.

Immunosuppressive cells and elements in TME of pancreatic cancer can be modulated by targeting CD40 [141, 403, 404]. CD40 is a member of the TNFR superfamily that is expressed on B cells and APCs such as DCs and macrophages and has an important role in T-cell-independent and T-cell-dependent immune mechanisms of tumour regression. Also, CD40-CD40L interaction can induce cellular maturation, activation and production of pro-inflammatory cytokines [133-135, 141, 149, 405-408]. Various studies have showed that CD40 agonistic antibodies alone or combined with chemotherapy altered the tumour microenvironment and suppressed tumour growth in both spontaneous and transplantable pancreatic tumour models [132, 139, 409]. In those studies, anti-tumour activity was mediated either by invoking productive T cell-mediated anti-tumour responses [139] or macrophage-mediated anti-tumour responses [132]. Therefore, I hypothesized that an agonist CD40 monoclonal antibody may enhance ACTIV therapy against Panc02-Her2 tumour through these mechanisms.

In the following **Chapter 4**, I aimed to enhance the anti-tumour activity of ACTIV therapy through using the HDACi, Panobinostat, and a CD40 agonist antibody.

CHAPTER 4: Enhancing the Anti-tumour Activity of ACTIV Therapy Using HDACi and CD40 Agonist

4.1. Introduction

Tumorigeneses and cancer progression can be stimulated through specific events that are related to histone modifications such as deacetylation, methylation and phosphorylation of the histones [410, 411]. The acetylation status of histones and non-histone proteins is determined by histone acetyl-transferases (HATs) which add acetyl groups to lysine residues and promotes a more relaxed and open chromatin structure, allowing transcriptional activation. Conversely, histone deacetylases (HDACs) are a distinct class of enzymes that remove the acetyl groups [163, 164] and act as transcription repressors through chromatin condensation [412, 413].

HDACs can be divided into two groups: the zinc-dependent HDACs including Class I (HDACs 1, 2, 3 and 8), Class II a/b (HDACs 4, 5, 6, 7, 9 and 10) and Class IV (HDAC11); and the zinc-independent, NAD-dependent Class III sirtuin enzymes [414]. HDAC1, HDAC2, HDAC3, and HDAC7 have been found to be overexpressed in pancreatic cancer cell lines [165-167]. Hyperactivity of these HDAC proteins can impair cell death regulation and induce proliferation in pancreatic cancer cells [168, 169].

Histone deacetylase inhibitors (HDACi) are small molecules or compounds that can influence the epigenetic modification of chromatin [164, 397] and alter gene transcription. HDACi represent an emerging class of anticancer drugs that have exhibited prominent anti-tumour activity by modulating transcriptional events resulting in cell growth arrest and apoptosis [410, 415]. HDACi have been shown to enhance anti-tumour immunity by increasing expression of MHC molecules and other molecules involved in antigen processing and presentation, and increasing expression of molecular components involved in tumour necrosis factor (TNF) superfamily signalling [416-422].

Among pan-deacetylase inhibitors, Panobinostat (LBH589, Novartis Pharmaceuticals) is considered a highly effective inhibitor of most HDAC enzymes associated with cancer progression [177]. Panobinostat is a potent inhibitor against Class I, II, and IV HDAC enzymes at low nanomolar concentrations across hematologic malignancies, such as lymphoma, multiple myeloma and acute myeloid leukaemia [174-178]. Panobinostat was approved by the US Food and Drug Administration (FDA) as a third-line treatment of multiple myeloma [179].

At present, Panobinostat is in clinical development with favourable clinical responses, limited toxicity [423-425] and a longer elimination time than other HDACi that have been investigated [426]. Panobinostat has demonstrated clinical efficacy in patients with advanced cutaneous T-cell lymphoma [174]. Panobinostat can stop a range of cancer related molecular pathways and target epigenetic events involved in cancer progression [177]. Moreover, Panobinostat can selectively induce apoptosis in cancer cells; but normal cells, including epithelial cells, fibroblasts, and peripheral blood mononuclear cells, are relatively resistant to this drug [413, 427, 428].

In addition to these multiple cytotoxic actions in cancer cells, HDACi have been shown to enhance the susceptibility of tumour cells to oncolytic virus infection and act synergistically to kill tumour cells [401, 402] through increasing the acetylation of histones and other proteins that inhibit the ability of tumour cells to mount a productive anti-viral response and augmentation of virus-induced apoptosis [412, 429]. I showed previously in Chapter 3 that VV-gp100 has an important role in ACTIV therapy through activation of CARaMEL T cells but not through viral oncolysis, despite the potential oncolytic ability of VV-gp100 through the deletion of the ribonucleotide reductase gene [263] (**Figure 3.10** and **Figure 3.11**). Therefore, I hypothesized that HDACi may enhance ACTIV therapy through increasing infection of tumour cells by VV-gp100 and oncolysis of the pancreatic cancer cells.

In addition to augmenting viral replication, HDACi have also been shown to modulate immunogenicity and improve anti-tumour immune responses [182, 183]. HDACi were reported to enhance $CD8^+$ T cell activation and function through various mechanisms, including improving their cytotoxicity effects, enhancing pro-inflammatory cytokines, increasing memory function of $CD8^+$ T cells and restoring immune functions of exhausted $CD8^+$ T cells [185-188, 400]. A previous study demonstrated that the addition of Panobinostat to adoptive T cell transfer therapy significantly decreased tumour burden, enhanced the proliferation, polyfunctional status and survival of adoptively transferred tumour specific T cells and decreased intra-tumoural T_{reg} cells [187].

Panobinostat has also been shown to induce the death of pancreatic tumour cells by apoptosis and achieve a significant reduction in pancreatic tumour growth as efficiently as gemcitabine in a subcutaneous xenograft mouse model [180].

Overall, given that HDACs are overexpressed in pancreatic cancer [165] and that HDACi may reduce the survival of pancreatic cancer cells *in vitro* and *in vivo*, increase the spread of the

oncolytic VV-gp100 in pancreatic cancer cells, as well as enhance CARaMEL T cell proliferation and function, I set out to investigate the anti-tumour activity of the combination of the HDACi Panobinostat with ACTIV therapy against murine pancreatic cancer.

In addition, in this chapter, I used a CD40 agonist antibody in an attempt to enhance the anti-tumour efficacy of ACTIV therapy. As I described previously in **Chapter 1 section 1.4.1**, an agonist CD40 monoclonal antibody can stimulate dendritic cell maturation to permit cross-presentation of antigens to cytotoxic T cells. In addition, CD40 agonists can lead to direct tumour killing by macrophages [141, 149, 150]. Various studies showed that CD40 agonistic antibodies alone or combined with chemotherapy reprogrammed the tumour microenvironment resulting in suppressing tumour growth in both spontaneous and transplantable KPC pancreatic tumour models [132, 139, 409]. Furthermore, CD40 agonistic antibodies have demonstrated early signs of clinical activity in pancreatic cancer patients when combined with the chemotherapy drug gemcitabine [159]. This anti-tumour activity was reported to be mediated by invoking productive T cell-mediated anti-tumour responses [139] and inducing macrophage-mediated anti-tumour responses [132].

On the basis of these prior observations, I hypothesized that an agonist CD40 monoclonal antibody (FGK.45) can enhance ACTIV therapy against Panc02-Her2 tumour through modulation of the tumour microenvironment. I sought to determine the mechanistic contribution of T cell-mediated and/or macrophage-mediated mechanisms towards the activity of the CD40 agonist/ACTIV therapy combination.

4.2. Results

4.2.1. Panobinostat enhanced ACTIV therapy against pancreatic cancer in vivo

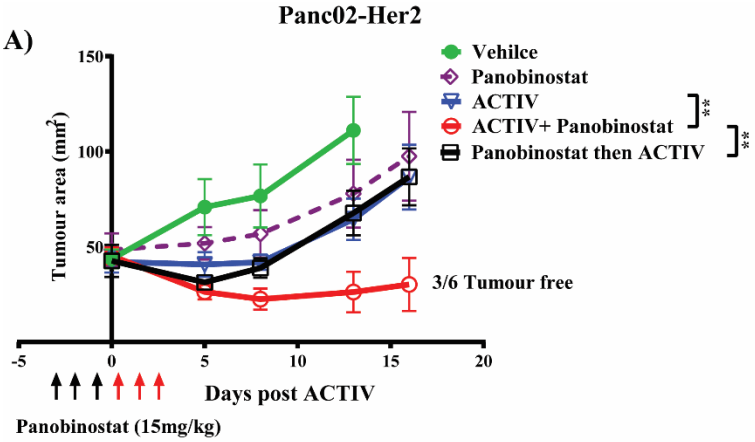
In order to study the efficacy of combining Panobinostat with ACTIV therapy against pancreatic tumours, I used two transplantable murine tumour systems which were previously described in **Chapter 3 section 3.2.1.**

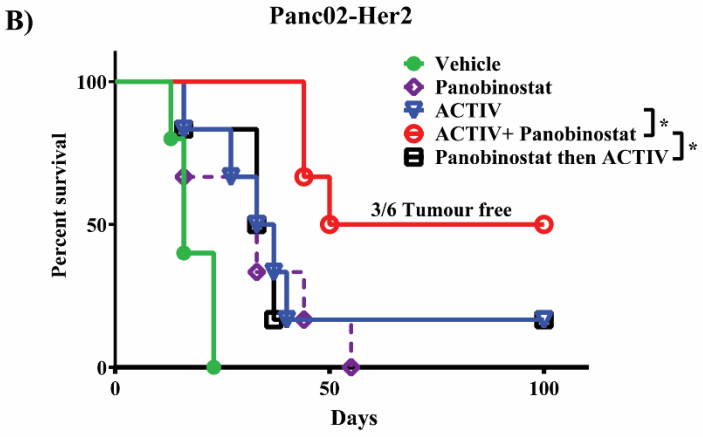
I injected either the Panc02-Her2 or KPC-Her2 cancer cell lines subcutaneously in C57BL/6-Her2 mice and allowed the tumours to grow for nine days to reach 35-55 mm^2 in size. ACTIV therapy was performed as previously described and presented in the schematic (**Figure 3.7**). In considering the dose of Panobinostat to use, I referred to previous studies, which described that Panobinostat was well tolerated and rapidly cleared from the plasma of mice bearing HCT116 subcutaneous xenograft tumours that received a 19.8 mg/kg dose of Panobinostat, with a four hour half-life and no detectable levels 48 hours post-treatment. However, Panobinostat levels remained elevated in tumours suggesting elevated retention in some tissues following systemic administration [430]. These kinetics and bio-distribution were considered reasonable for my tumour models, and based on this pharmacokinetics, and to avoid any undue toxicity in mice with higher doses, I used three doses of 15 mg/kg over three consecutive days.

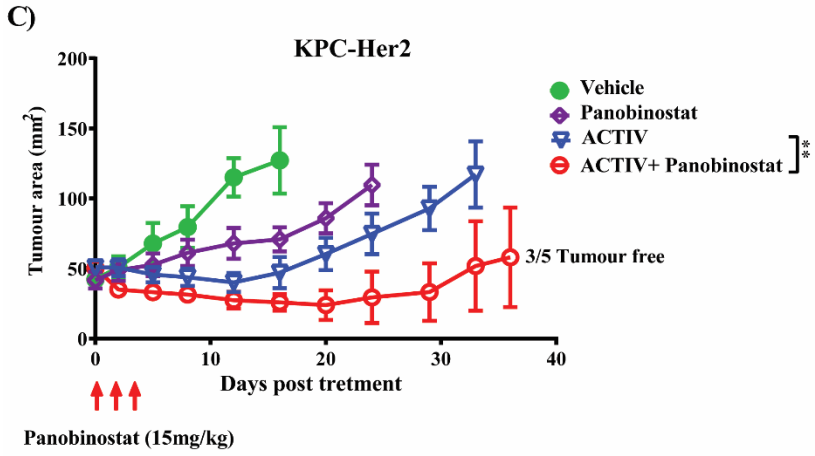
In addition to the dose, there are other factors that may affect the efficacy of the combined treatment of HDACi inhibitors and immunotherapy, which include the timing of HDAC inhibitor administration. Several studies have showed that the inhibitory effect of HDACi seems to be less effective during the activation of immune cells and better before or after the activation of immune cells [431-434]. To check the timing hypothesis, two groups of mice received intraperitoneal injections of Panobinostat over three consecutive days before ACTIV or with ACTIV therapy. As controls, cohorts of mice were injected with three doses of Panobinostat alone or vehicle (dextrose) over three consecutive days. Tumour area and mouse survival were then recorded with the endpoint being tumour area reaching ≥ 150 mm^2.

Complete tumour clearance and significantly prolonged survival was achieved in a proportion of mice in both tumour models, when treated with the combination of Panobinostat and ACTIV therapy, which was more effective than ACTIV therapy alone (**Figure 4.1 A-D**). In contrast to other studies [431-434] I found that the administration of Panobinostat before ACTIV therapy was less effective than administration during ACTIV therapy (**Figure 4.1 A-D**), which indicates the potential importance of timing of HDAC inhibitor treatment when combining

HDAC inhibitors with ACTIV therapy or potentially other immunotherapies. The importance of ACTIV therapy in the combination treatment was evident from the observation that single-agent Panobinostat alone showed little effect in suppression of Panc02-Her2 or KPC-Her2 tumour growth. Overall, this result is consistent with my hypothesis that Panobinostat can enhance the therapeutic effects of ACTIV therapy.


A)
Panc02-Her2
Vehilce
Panobinostat
ACTIV
ACTIV+ Panobinostat
Panobinostat then ACTIV
**
**
Tumour area (mm2)
150
100
50
3/6 Tumour free
-5
0
5
10
15
20
Days post ACTIV
Panobinostat (15mg/kg)


B)
Panc02-Her2
Vehicle
Panobinostat
ACTIV
ACTIV+ Panobinostat
Panobinostat then ACTIV
*
*
Percent survival
100
50
0
3/6 Tumour free
0
50
100
Days


C)
KPC-Her2
Vehicle
Panobinostat
ACTIV
ACTIV+ Panobinostat
**
Tumour area (mm2)
200
150
100
50
0
3/5 Tumour free
0
10
20
30
40
Days post tretment
Panobinostat (15mg/kg)

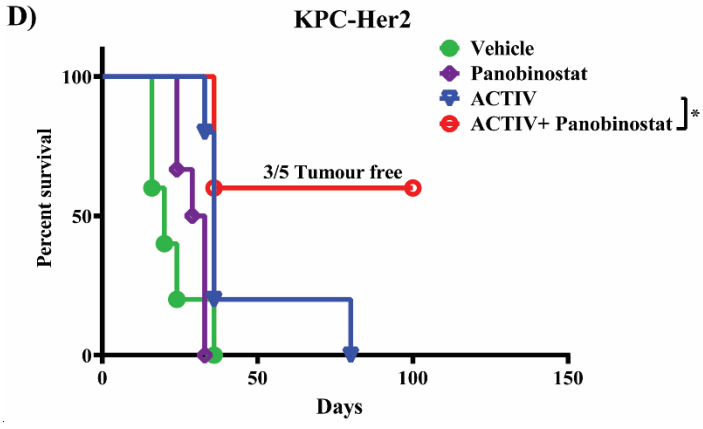

D)
KPC-Her2
Vehicle
Panobinostat
ACTIV
ACTIV+ Panobinostat
*
Percent survival
100
50
0
3/5 Tumour free
0
50
100
150
Days

Figure 4.1. Panobinostat enhances the anti-tumour activity of ACTIV therapy

C57BL/6 human-Her2 transgenic mice were subcutaneously injected with (**A and B**) Panc02-Her2 or (**C and D**) KPC-Her2 tumour cells (1×10^6 cells/mouse). The tumours were allowed to grow for nine days. On day 9, the tumour-bearing mice were treated with ACTIV therapy. Intraperitoneal injections of Panobinostat (15 mg/kg) were also given to some mice before ACTIV or with ACTIV therapy over three consecutive days. As controls, cohorts of mice were injected with three doses of Panobinostat (15 mg/kg) alone or vehicle (dextrose) for three consecutive days. Tumour areas were measured at the indicated times, and data are presented as the mean ± SEM of 5-8 mice/group, representative of four independent experiments. Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (* $p \leq 0.05$, ** $p \leq 0.01$). (**A and C**) Tumour size panel. (**B and D**) Mice survival panel.

4.2.2. Panobinostat did not enhance the infection of VVgp-100 in Panc02-Her2 cells

Having determined the effect of Panobinostat in ACTIV therapy, we next set out to gain insight into how Panobinostat enhanced ACTIV therapy. As we showed in **Figure 3.10** in the previous chapter, VV-gp100 alone did not infect and lyse Panc02-Her cells *in vitro,* compared to control cells; so, we investigated if Panobinostat can render murine pancreatic cancer cells more susceptible to VV-gp100 infection and lysis. To investigate this, we treated Panc02-Her2 with different concentrations of Panobinostat (**Figure 4.2 A**) or with 2.5 µM Panobinostat (**Figure 4.2 B**) at 37ºC for 3 hours to give enough time for Panobinostat to have an effect on Panc02-Her2 cells without stopping their proliferation or killing them. Then, we added different concentrations of VV-gp100 and incubated the cells at 37ºC for 72 hours to check if any plaques formed because of the VV-gp100 infection. In further experiments, cell viability post-virus exposure was analysed using a Resazurin reduction assay that measures the relative fluorescence of cultures after 2 hours. In this experiment, Panobinostat failed to enhance the ability of VV-gp100 to lyse or inhibit the growth of either Panc02-Her2 cells or the control cells 293A (**Figure 4.2 A and B**).

In this experiment, different concentrations of Panobinostat, ranging from 0.1 to 1.0 µM did not enhance the ability of VV-gp100 to lyse Panc02-Her2 cells, when assayed for viral plaque formation (**Figure 4.2 A**). Indeed, no viral plaques were observed in the presence of Panobinostat at these concentrations. At the highest concentration, 10 µM, Panobinostat completely abolished growth of Panc02-Her2 cells, which identified the limits of potential conditions to use. Interestingly, Panobinostat also failed to increase the ability of VV-gp100 to lyse 293A, which acted as a control cell line that is widely accepted as susceptible to vaccinia virus (**Figure 4.2 A**). These results indicated that Panobinostat was not able to enhance VV-gp100-mediated lysis of cells *in vitro*.

To further investigate the effect of Panobinostat on cell susceptibility to virus, I used another method to determine cell viability using a Resazurin reduction assay. In agreement with the plaque assays, I found that Panobinostat did not enhance the ability of VV-gp100 to inhibit the growth of either Panc02-Her2 cells or the control cells 293A when (**Figure 4.2 B**).

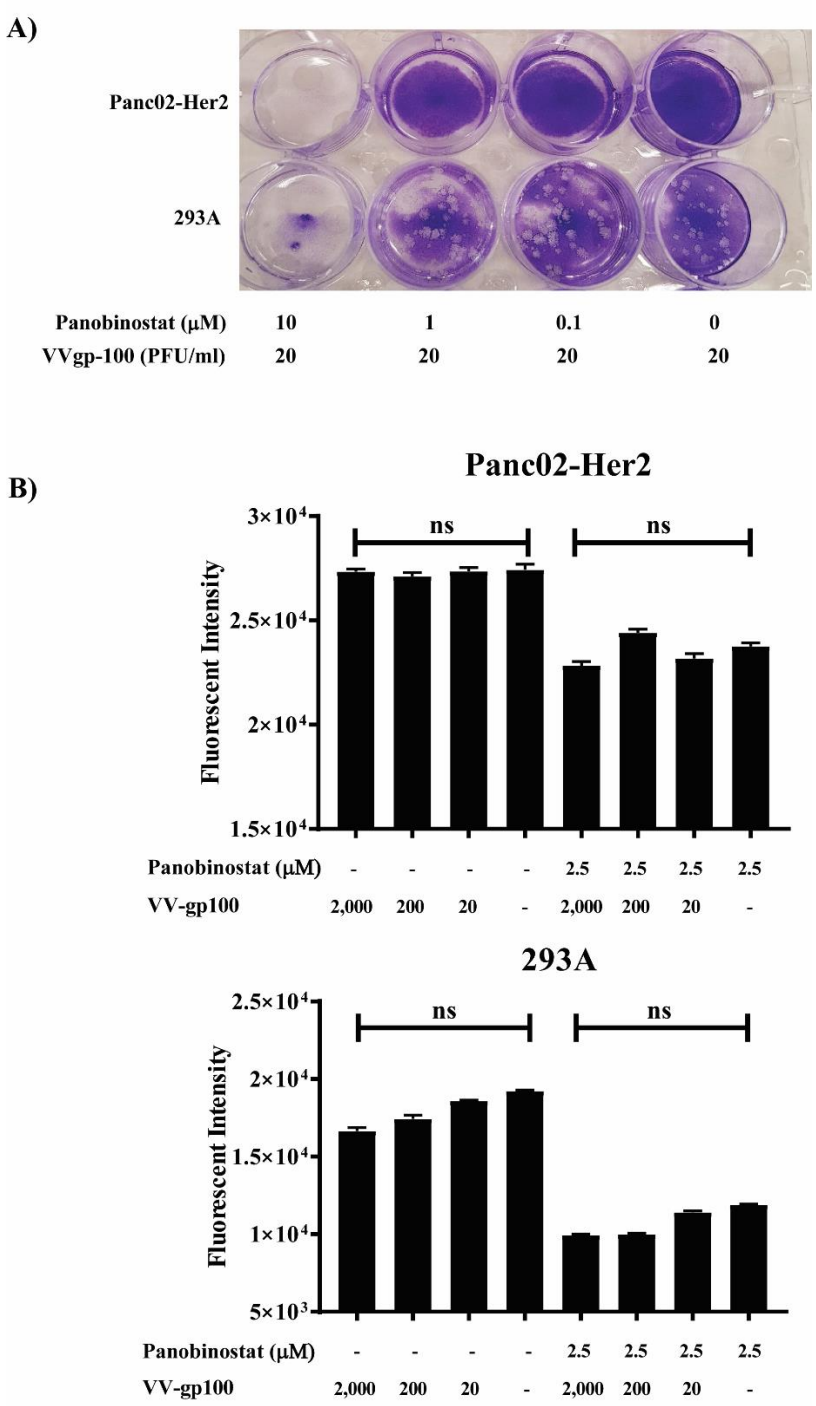


Figure 4.2. Panobinostat failed to enhance the infection of VVgp-100 in Panc02-Her2 cells

(**A**) Monolayers of Panc02-Her2 and 293A cells were pre-treated with Panobinostat as listed concentrations, after three hours different concentrations of VV-gp100 were added. Seventy-two hours post-infection; cells were fixed with ice-cold methanol and then stained with 0.1% crystal violet for plaque visualization. (**B**) Monolayers of Panc02-Her2 and 293A cells were pre-treated with 2.5 μM Panobinostat and after three hours different concentrations of VV-gp100 were added. Resazurin solution was added to cells 72 hours post-infection and relative fluorescent units were measured after two hours. Data are representative of two independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (ns= not significant).

4.2.3. Panobinostat did not increase Her2 and MHCI expression on tumour cell lines

As I discussed previously, HDACi including Panobinostat can modulate the expression of different TAAs, and components of the tumour antigen processing and MHC presentation pathway in human and murine tumour cell lines *in vitro and in vivo.* This modulation can result in helping tumour specific T cells to identify and destroy tumour cells [416, 419, 435-437]. Therefore, I assessed the effect of Panobinostat on the expression of Her2 *in vitro and in vivo.* First, I performed flow cytometry to assess the expression of Her2 *in vitro* on Panc02-Her2 tumour cells after incubation with different concentrations of Panobinostat overnight and stained them on the following day with anti-Her2 antibody. Panobinostat failed to change the expression of Her2 *in vitro* even when a high concentration (10 µM) of Panobinostat was used (**Figure 4.3**).

Second, I assessed the expression of Her2 and MHCI *in vivo* in the presence or absence of Panobinostat. Tumour-bearing mice were treated with ACTIV therapy with or without three doses of Panobinostat (15 mg/kg) over three consecutive days. Another two control groups received either Panobinostat alone (15 mg/kg) or vehicle alone over three consecutive days. Seventy-two hours after ACTIV and three Panobinostat injections which is considered enough time to ensure that Panobinostat can affect the expression of genes involved in antigen presentation or co-stimulatory molecules [438, 439], tumours were harvested, stained with specific antibodies, and analysed using flow cytometry to determine the expression of Her2 and MHCI ($H2D^b$).

In this experiment, Panobinostat did not increase the expression of MHCI ($H2D^b$) (**Figure 4.4 A and B**) in tumours of mice receiving Panobinostat with ACTIV therapy relative to tumours of mice receiving ACTIV therapy alone or mice receiving vehicle. Moreover, Her2 expression was also not increased in Panc02-Her2 tumours in mice receiving Panobinostat with ACTIV therapy (**Figure 4.4 C and D).**

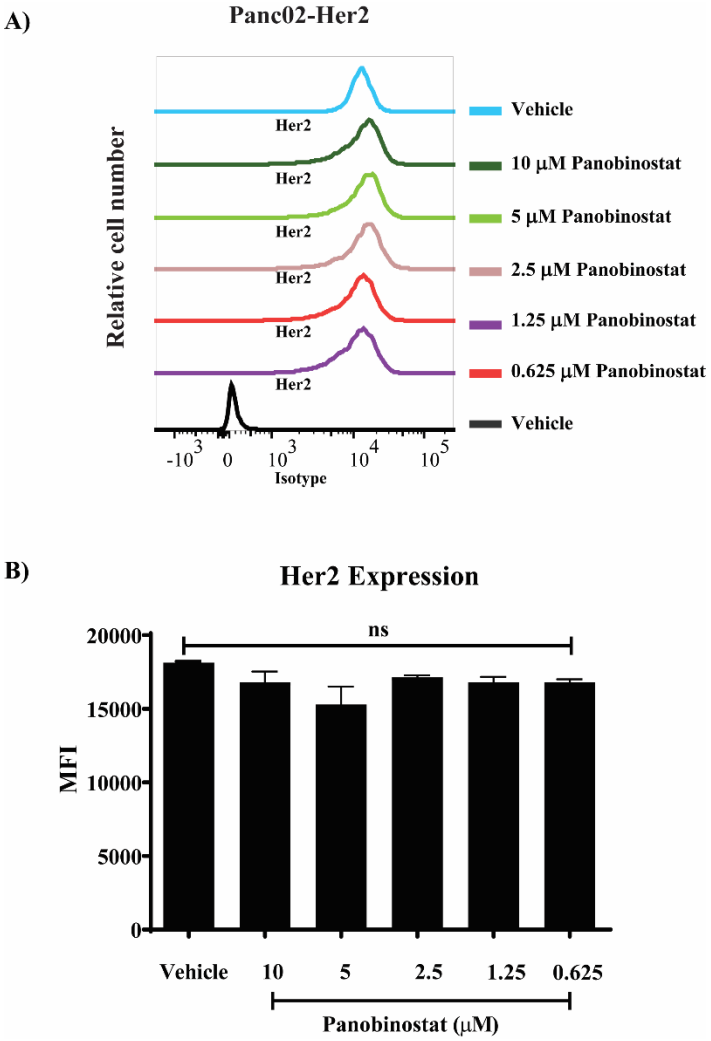


Figure 4.3. Panobinostat did not increase Her2 expression *in vitro*

Panc02-Her2 cells were treated with the indicated concentrations of Panobinostat and incubated at 37ºC overnight. On the following day, cells were stained with anti-Her2 antibody or isotype control. Cells treated with vehicle (dextrose) were used as a negative control. Flow cytometry was performed to assess the expression of Her2 on Panc02-Her2 cells after incubation with different concentrations of Panobinostat. (**A**) Histograms displaying Her2 expression on tumour cells. (**B**) Quantitative data depicting mean fluorescence intensity, presented as mean ± SEM of triplicate samples of one experiment. FACS plots are gated on live cells. Statistical analyses were performed using the Student *t*-test (ns= not significant).

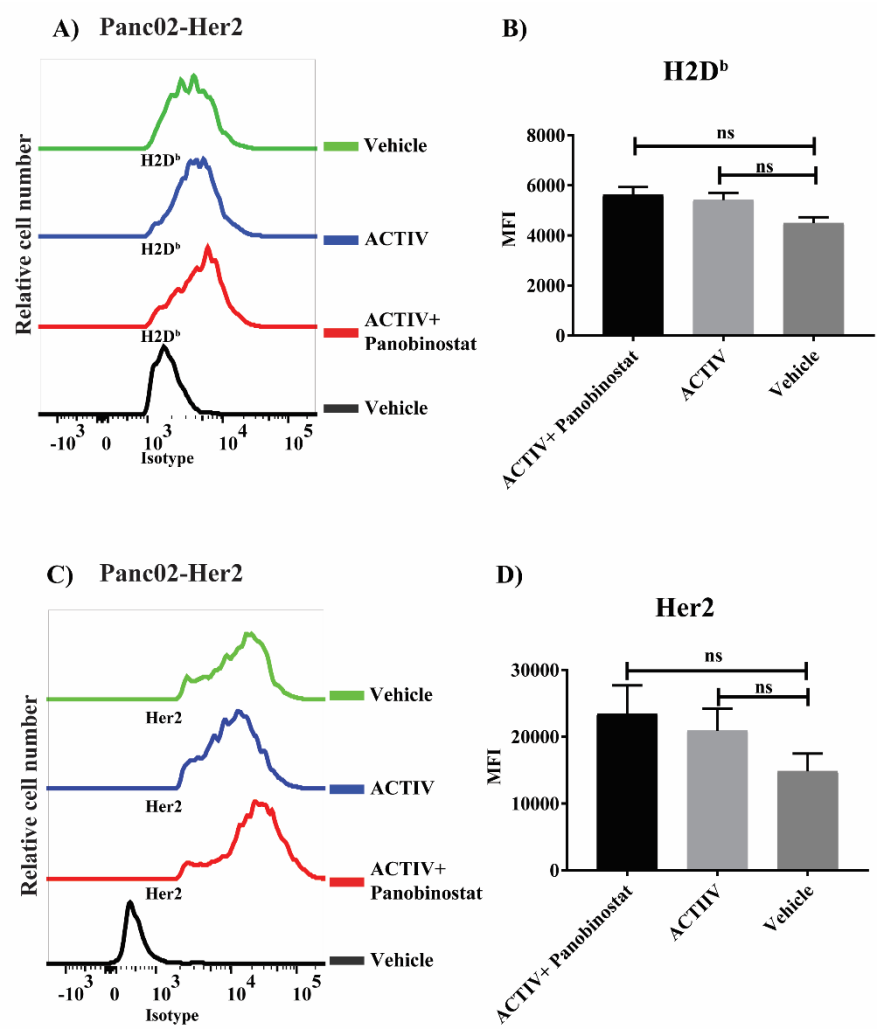


Figure 4.4. Panobinostat did not increase MHCI or Her2 expression *in vivo*

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy with or without three doses of Panobinostat (15 mg/kg) over three consecutive days. As a control, a cohort of mice were injected with three doses of vehicle (dextrose). Tumours were harvested on day 4, enzymatically dissociated and analysed using flow cytometry to determine the expression of Her2 and $H2D^b$. (A) Histograms displaying $H2D^b$ expression on tumour cells. (**B**) Data are representative of two independent experiments and shown as mean ± SEM of triplicate samples. (**C**) Histograms displaying Her2 expression on tumour cells. (**D**) Data are representative of two independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (ns= not significant).

4.2.4. Panobinostat combined with CARaMEL T cells potently suppressed Panc02-Her2 tumour cell growth

HDACi can have direct effects on tumour cells and lead to inhibition of tumour cell growth through various mechanisms including cell cycle arrest and the activation of apoptosis. Hence, I next assessed the direct effect of Panobinostat on Panc02-Her2 tumours. First, I evaluated the impact of Panobinostat on cell viability of Panc02-Her2 cells using an Alamar blue assay. In **Figure 4.5**, Panc02-Her2 cells were treated with Panobinostat diluted to concentrations of 10, 5, 2.5, 1.25 and 0.625 µM for 24 hours and 0.625, 0.1, 0.01 and 0.001 µM for 48 hours in complete medium. Concentrations of 0.001-10 µM Panobinostat were chosen based on those used in previous studies in a range of cell lines [177, 440, 441].

Relative to vehicle-treated control cells, Panc02-Her2 exposure to 0.625 - 10 µM Panobinostat for 24 hours exhibited a 14-23% reduction in metabolic activity or growth of the cells (**Figure 4.5 A and B**). In addition, incubation of 0.1 and 0.625 µM Panobinostat with Panc02-Her2 for 48 hours reduced the viability of cells about 17-39% (**Figure 4.5 C and D)**. I found that the viability of Panc02-Her2cells did not change significantly with the dosage below 0.1 µM at the 48-hour time-point (**Figure 4.5 C and D)**. These results indicated that Panobinostat inhibited the proliferation of Panc02-Her2 cells in a dose- and time-dependent manner.

I next investigated the cooperative effects of CARaMEL T cells and Panobinostat together against Panc02-Her2 cells. The addition of CARaMEL T cells to Panc02-Her2 (1:1) -which had no effect on Panc02-Her2 cell viability in the absence of Panobinostat- tripled the growth suppression of Panc02-Her2 cells in the presence of 10, 5, 2.5, 1.25 and 0.625 µM Panobinostat for 24 hours (**Figure 4.6 A and B**) and 0.625, 0.1 µM for 48 hours (**Figure 4.6 C and D**).

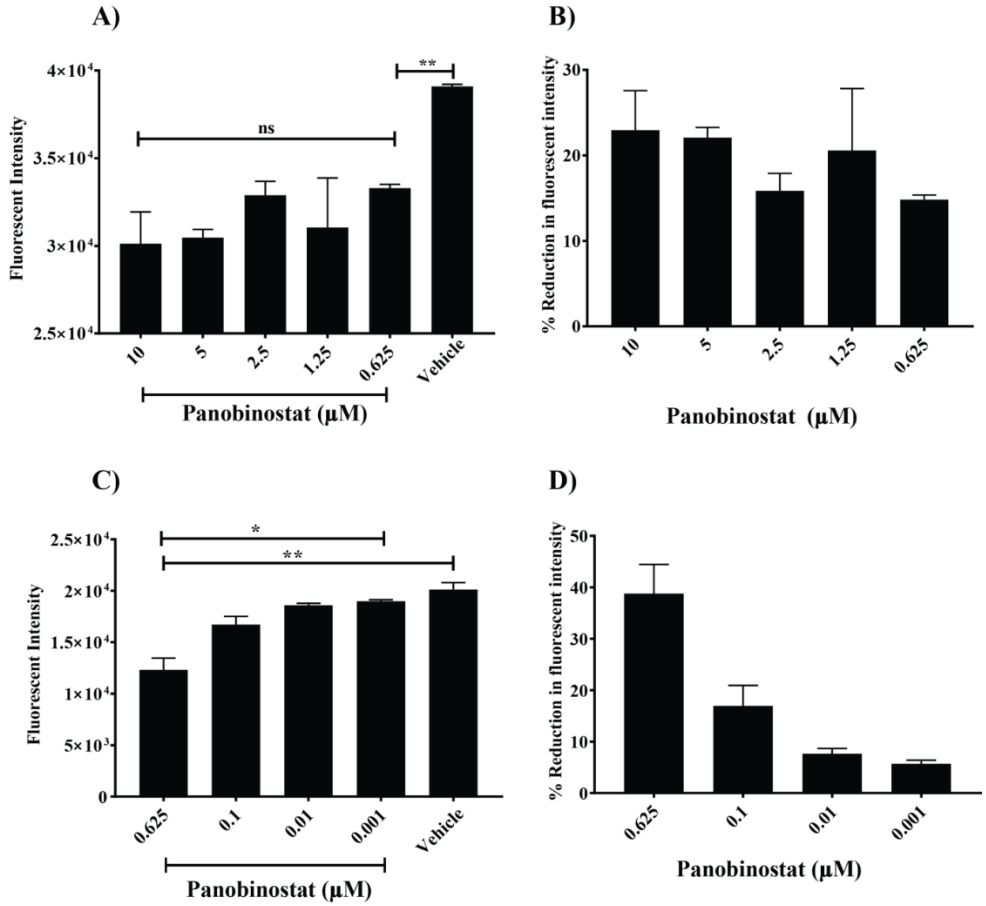


Figure 4.5. Panobinostat suppressed Panc02-Her2 cells' tumour cell growth *in vitro*

Panc02-Her2 cells were seeded at 1 × 10^5 cells in a 96-well plate with the indicated concentrations of Panobinostat for (**A and B**) 24 hours and (**C and D**) 48 hours. Resazurin solution (10% of cell culture volume) was added to the plate and relative fluorescent units were measured after two hours. Data in (**B**) is derived from (**A**) and expressed as percent reduction in fluorescence, and the data in (**D**) is derived from (**C**). Data are representative of two independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (ns= not significant, * $p < 0.05$, ** $p < 0.01$).

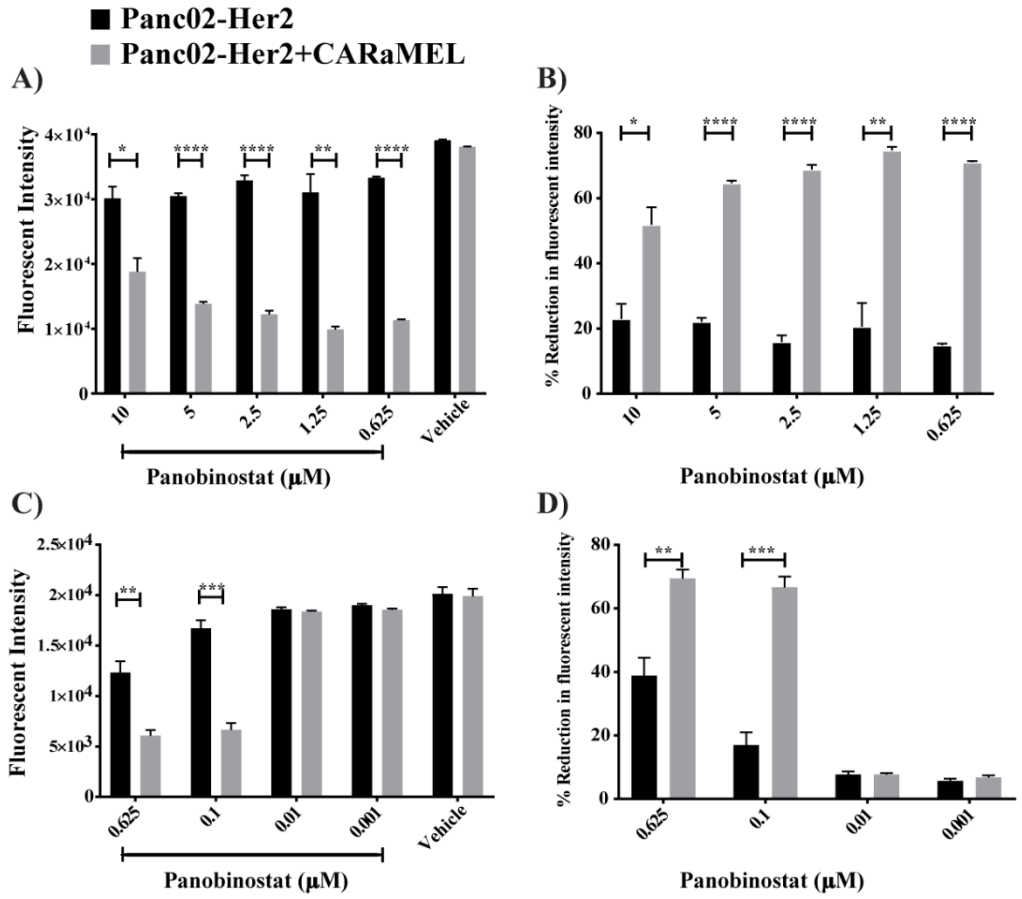


Figure 4.6. Panobinostat increased CARaMEL T cell-mediated growth suppression of Panc02-Her2 cell *in vitro*

Panc02-Her2 cells and activated CARaMEL T cells were seeded (1:1) at 1×10^5 cells in a 96-well plate with the indicted concentrations of Panobinostat for (**A** and **B**) 24 hours and (**C** and **D**) 48 hours. Resazurin solution (10% of cell culture volume) was added to the plate and relative fluorescent units were measured after two hours. (**A and C**) Fluorescent intensity. (**B and D**) Reduction in fluorescent intensity of data from panels **A** and **C** respectively. Data are representative of three independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.2.5. Panobinostat induces apoptosis in Panc02-Her2 cells in vitro

To define the growth inhibition of Panc02-Her2 cells by Panobinostat, I investigated the involvement of apoptosis and cell cycle arrest. To characterize Panobinostat-induced apoptosis, Panc02-Her2 cells were incubated with different concentrations of Panobinostat for 24 hours or 48 hours and stained with Annexin V-APC and 7AAD, to detect cells at an early and late stage of apoptosis respectively, and then analysed using flow cytometry. The results indicated that Panobinostat increased apoptosis in Panc02-Her2 cells (**Figure 4.7 A**), and the percentages of apoptotic cells (early and late apoptotic phases) at 10, 5, 2.5, 1.25, 0.625 and 0.1 for 24 hours were 68, 60, 55, 56, 52 and 40% respectively. In addition, I found that the viability of Panc02-Her2 cells did not change with the dosage below 0.1 µM at the 24- or 48-hours' time points (**Figure 4.7 B**). Moreover, I also found that the exposure to Panobinostat for 48 hours resulted in a marked increase in the percentages of apoptotic cells at the concentrations of 0.1 µM and 0.625 µM (67 and 96% respectively) (**Figure 4.7 C**).

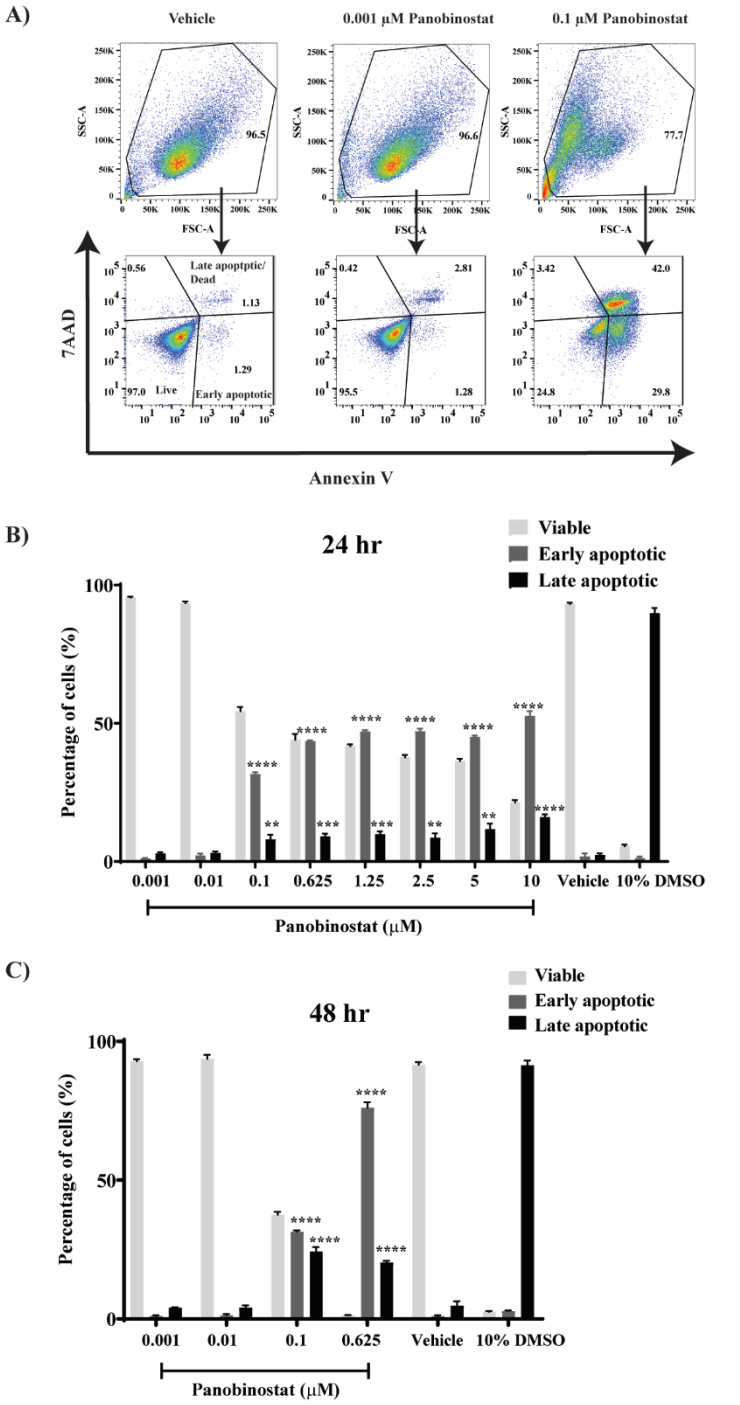


Figure 4.7. Panobinostat induced apoptosis in Panc02-Her2 cells

Panc02-Her2 cells were seeded at 2 × 10^6 cells in a 6-well plate with the indicated concentrations of Panobinostat, stained using Annexin V/7AAD and subjected to FACS analysis. (**A**) Representative FACS plots are gated on morphology (FSC/SSC plot). Percentage of early apoptotic cells (Annexin V^+) and late apoptotic cells (Annexin V^+ and $7AAD^+$) with indicated Panobinostat concentrations. The total percentage of apoptotic cells was analysed (**B**) 24 hours or (**C**) 48 hours. Cells treated with vehicle were used as a negative control. Data are representative of four independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.2.6. Combination of CARaMEL T cells and Panobinostat significantly increases apoptosis in Panc02-Her2 cells in vitro but not in vivo

I next examined the combinatorial effects of Panobinostat and CARaMEL T cells on apoptosis of Panc0-Her2 cells *in vitro*. As shown in **Figure 4.8**, the addition of CARaMEL T cells to Panc02-Her2 cells (at a 1:1 ratio) with 0.01 and 0.1 µM of Panobinostat for 48 hours significantly increased the apoptosis of Panc02-Her2 cells approximately two and three fold respectively when compared to T cells without Panobinostat (**Figure 4.8 A**). Interestingly, I also found that the addition of CARaMEL T cells to a breast cancer cell line, E0771-Her2 (1:1) in the presence of 0.1 µM of Panobinostat for 48 hours significantly increased the apoptosis of E0771-Her2 cells when compared to T cells without Panobinostat (**Figure 4.8 B**). Moreover, I found similar results when I incubated CARaMEL T cells with another pancreatic cancer cell line, KPC-Her2 (1:1) with 0.1 µM of Panobinostat for 48 hours. The cytotoxic ability of CARaMEL T cells was significantly increased, which was indicated by an increase in the apoptosis of KPC-Her2 mediated by CAR T cells in the presence of Panobinostat (**Figure 4.8 C**). These results indicated that the effect of Panobinostat on CARaMEL T cell cytotoxic ability was not specific to Panc02-Her2 cells only, but also applied to another cancer cell line.

To further investigate the mechanisms of cell death in my *in vivo* model, I performed immunohistochemical staining for cleaved caspase-3 (CC3), a marker of apoptosis (**Figure 4.9**). I treated Panc02-Her2 tumour bearing mice with ACTIV therapy in the presence or absence of three doses of Panobinostat (15 mg/kg) over three consecutive days. Another two control mice groups received either Panobinostat (15 mg/kg) or vehicle over three consecutive days. After five days, mice were sacrificed, and tumours were harvested and analysed for CC3 IHC staining. CC3 did not show higher induction of apoptosis in tumours of the combination group (ACTIV + Panobinostat) compared with ACTIV therapy alone group (p= 0.3583) (**Figure 4.9 B**) or the Panobinostat group compared to the vehicle group. This suggested that Panobinostat could not induce apoptosis in Panc02-Her2 tumours *in vivo* at this particular time point. However, a trend to have more CC3 staining was observed in tumours of the combination group (ACTIV + Panobinostat), which may be significant with a larger sample size at this time point. Therefore, further investigations at this and other time points would be necessary in the future to make any firm conclusions.

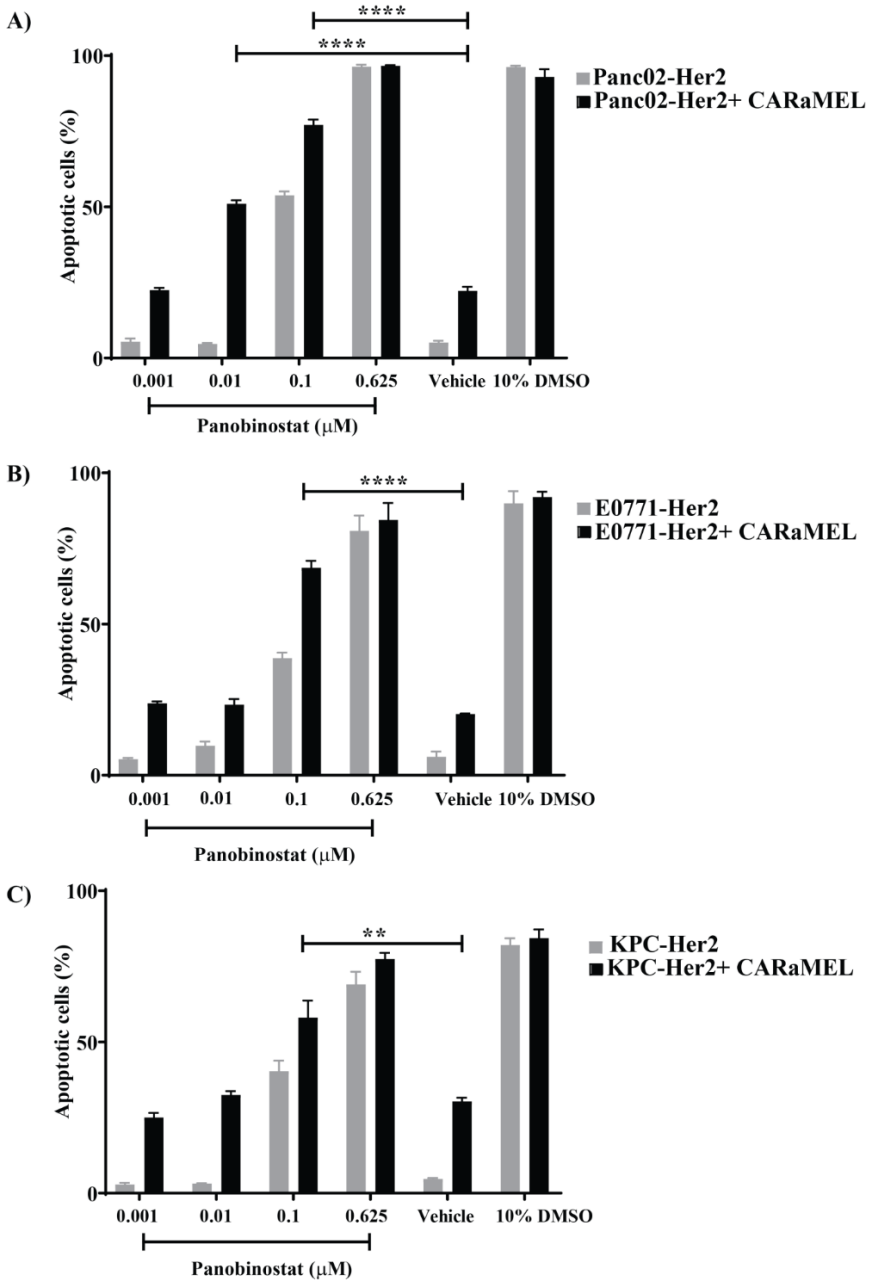


Figure 4.8. Combination of CARaMEL T cells and Panobinostat significantly increased the apoptosis of Panc02-Her2, E0771-Her2 and KPC-Her2 cells *in vitro*

(A) Panc02-Her2, **(B)** E0771-Her2 or (**C**) KPC-Her2 tumour cells and activated CARaMEL T cells were seeded (1:1) at 2×10^6 in a 6-well plate with the indicated concentrations of Panobinostat, followed by FACS analysis to determine Annexin V/7AAD staining. Cells treated with vehicle were used as a negative control. FACS plots are gated on $CD8^-$ cells (to exclude lymphocytes). The total percentage of apoptotic cells = percentage of early apoptotic cells (Annexin V^+) + late apoptotic cells (Annexin V^+ and $7AAD^+$). Data are representative of four independent experiments and are shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (** $p \leq 0.01$, **** $p \leq 0.0001$).

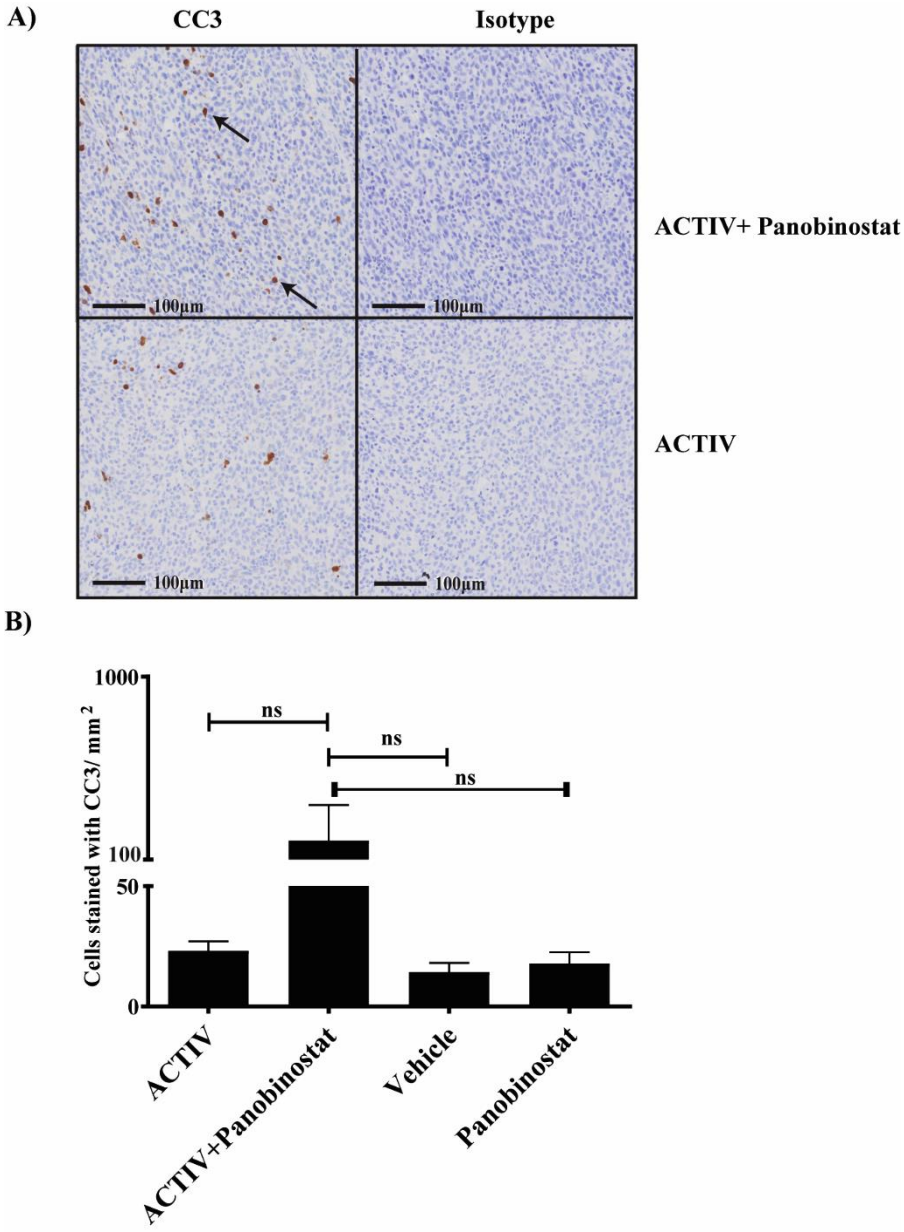


Figure 4.9. *In vivo* immunohistochemical staining analyses of CC3 in Panc02-Her2 tumours subjected to ACTIV therapy with or without Panobinostat

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, mice received ACTIV therapy with or without three intraperitoneal injections of Panobinostat (15 mg/kg) over three days. For comparison, other cohorts of mice were injected with Panobinostat alone (15 mg/kg) or vehicle (dextrose) over three days. After five days, tumours were harvested and analysed (**A**) Representative sections of tumours from mice treated as indicated in the panels and captured at 20 × magnification. Arrows indicate representative positively stained cells (Brown). (Scale bar = 100 µm; tumours from five mice per group, three sections per mouse). (**B**) Whole IHC section $CC3^+$ cell counts from three random sections of five different tumours per group enumerated using HALO. Data are presented as mean ± SEM of five mice per group pooled from two independent experiments. Statistical analyses were performed using the Student *t*-test (ns= not significant).

4.2.7. Panobinostat induces G1/S cell cycle arrest in Panc02-Her2 tumour cells

In order to investigate the involvement of cell cycle arrest on the growth suppression of Panc02-Her2 cells by Panobinostat, I treated the cells with the drug at 10 µM or vehicle in the presence of bromodeoxyuridine (BrdU), which is incorporated into DNA during the S phase overnight. This was followed by labelling with anti-BrdU and 7AAd and then analysis by FACS to determine the cell cycle profiles. My results showed that cell cycle distribution was markedly changed with Panobinostat treatment (**Figure 4.10**). Compared with the vehicle alone group, Panobinostat arrested Panc02-Her2 cells in G1 phase as shown by the negative BrdU staining. This suggests that, in addition to increasing apoptosis of cancer cells, Panobinostat-treated cells did not exit G1 and did not enter S phase. Furthermore, there was decrease in the number of S phase cells, indicating a G1/S phase arrest.

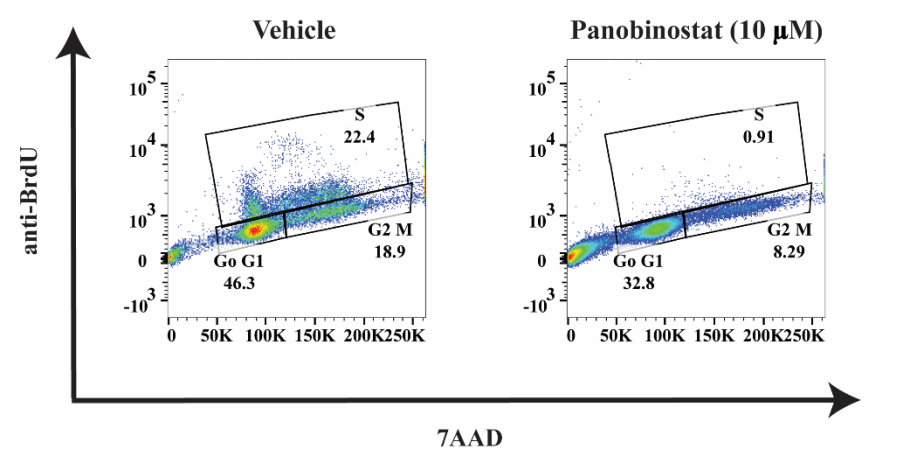


Figure 4.10. Panobinostat induced G1/S cell cycle arrest in Panc02-Her2 tumour cells

Panc02-Her2 cells were treated with either Panobinostat at 10 µM or vehicle with BrdU overnight, followed by labelling with anti-BrdU and 7AAd, and then analysed by FACS. The right panel depicts the average change in Panc02-Her2 cells treated with Panobinostat-compared to DMSO-treated cells in the left panel. Data are representative of two independent experiments.

4.2.8. Panobinostat has inhibitory effects on CARaMEL T cells

As discussed previously, the role of $CD8^+$ T cells is crucial for effective anti-tumour immunity. However, HDAC inhibitors have been observed to modulate $CD8^+$ T cell responses, which have been reported in various studies [421, 442]. HDACi can suppress or promote T cell responses, depending on the activation state of T cells [431-434]. In order to assess if Panobinostat had direct effects on CARaMEL T cell viability and function, I treated CARaMEL T cells with a range of concentrations of Panobinostat for 48 hours in complete medium. I then added Resazurin solution to the culture and assayed it after two hours. I found that exposure to 0.01 µM or higher concentrations of Panobinostat for 48 hours resulted in a marked inhibition of the proliferation of CARaMEL T cells in a dose-dependent manner (**Figure 4.11**).

In order to confirm this growth inhibitory effect of Panobinostat on CARaMEL T cells and to characterize Panobinostat-induced apoptosis, I incubated the cells with different concentrations of Panobinostat for 48 hours and stained with Annexin V-APC and 7AAD and then analysed by flow cytometry. The results indicated that Panobinostat significantly increased apoptosis in CARaMEL T cell, and the percentages of apoptotic cells (early and late apoptotic phases) at 0.01, 0.1 and 0.625 µM for 48 hours were 26, 74 and 81% respectively (**Figure 4.12**).

In order to assess the effect of Panobinostat on functional parameters of CARaMEL T cells, I examined the effect of Panobinostat on the secretion of the cytokine IFN-γ from CARaMEL T cells following stimulation with Panc02-Her2 tumour cells. My results revealed significant suppression of IFN-γ release by CARaMEL T cells by Panobinostat at a concentration of 0.01 µM or more (**Figure 4.13**). These results suggest that Panobinostat could also have some effect on the function and viability of CARaMEL T cells *in vivo*. However, as discussed previously, Panobinostat is rapidly cleared from the plasma of mice bearing subcutaneous tumours [430], suggesting that any effect on T cells in the periphery might be transient, although it is difficult to draw definitive conclusions in the absence of detailed pharmacokinetic data in my model system.

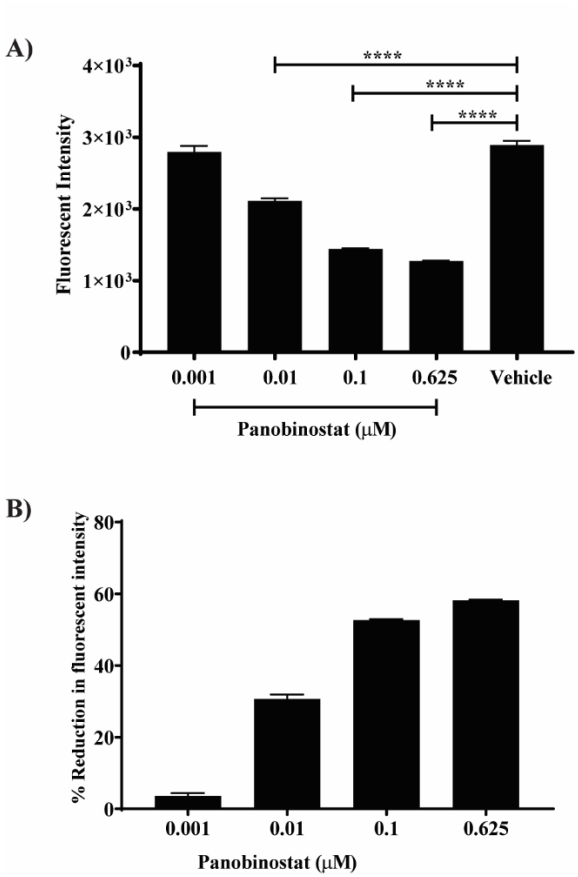


Figure 4.11. Panobinostat inhibited proliferation of CARaMEL T cells

CARaMEL T cells were activated for five days in culture with hgp100 $_{25\text{-}33}$ and then cells were seeded at 1×10^5 in a 96-well plate with the indicated concentrations of Panobinostat or vehicle for 48 hours. Resazurin solution (10% of cell culture volume) was added to the plate and relative fluorescence was measured after two hours. (**A)** Fluorescent intensity**. (B**) Reduction in fluorescent intensity. Data are representative of three independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (**** $p \leq 0.0001$).

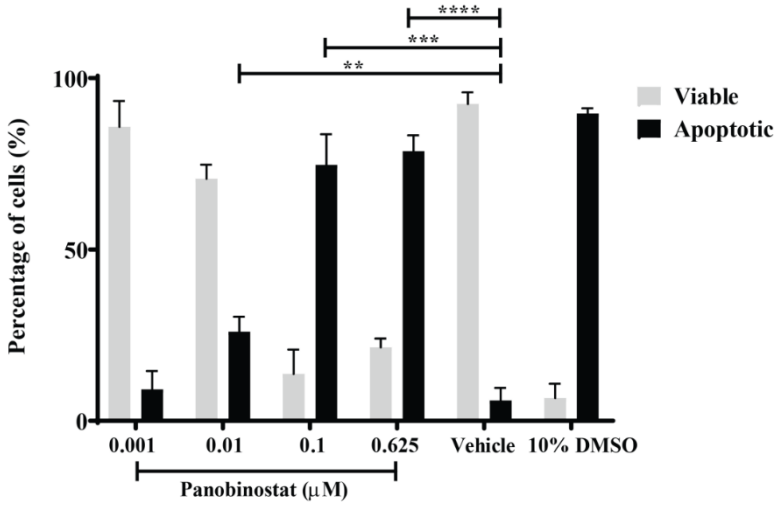


Figure 4.12. Panobinostat induced apoptosis in CARaMEL T cells *in vitro*

Activated CARaMEL T cell were seeded at 2 × 10^6 in a 6-well plate with the listed concentrations of Panobinostat or vehicle for 48 hours, followed by staining with Annexin V/7AAD and then analysed by FACS. The total percentage of apoptotic cells = percentage of early apoptotic cells (Annexin V^+) + late apoptotic cells (Annexin V^+ and $7AAD^+$). Data are representative of three independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

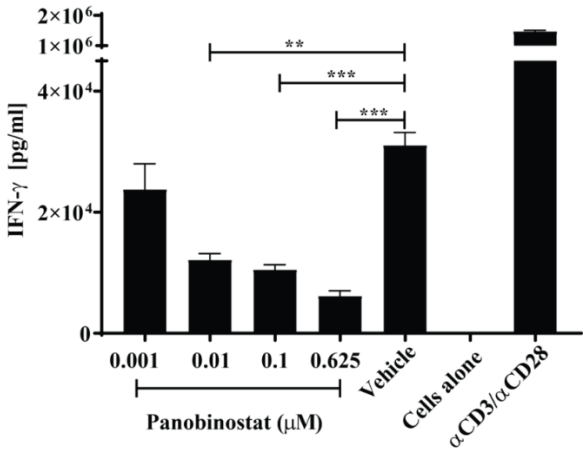


Figure 4.13. IFN-γ secretion by CARaMEL T cells in response to tumour cells is decreased in the presence of Panobinostat

Panc02-Her2 cells and activated CARaMEL T cells were seeded (1:1) at 1 × 10^5 in wells of a 96-well plate with the listed concentrations of Panobinostat for 48 hours. Controls included cells treated with vehicle (negative control), or αCD3/αCD28-stimulated cells (positive control). IFN-γ levels in the culture supernatants were analysed by ELISA. Data are presented as mean ± SEM of triplicate samples; data here are representative of three independent experiments. Statistical analyses were performed using Student *t*-test (** $p < 0.01$, *** $p \leq 0.001$).

4.2.9. Panobinostat affects the proliferation and distribution of CARaMEL T cells in vivo

To gain further understanding of how the co-administration of Panobinostat with ACTIV therapy enhanced the anti-tumour response, I studied the leukocyte infiltrate of tumours before and after treatment, including whether the co-administration of Panobinostat influenced the expansion of CARaMEL T cells and other endogenous leukocyte populations *in vivo*. These experiments involved subcutaneous inoculation of Panc02-Her2 tumour cells into Her2 transgenic mice and then evaluation of the percentage of infiltration by a variety of leukocytes as described below. I treated a group of mice with ACTIV therapy with or without three intraperitoneal injections of Panobinostat over three consecutive days. Tumours were collected on day three and seven post ACTIV therapy and enzymatically digested into single cell suspensions, and then stained with specific antibodies recognising various immune populations for FACS analysis. In addition, the spleens of mice receiving ACTIV, with or without Panobinostat, were collected to investigate CARaMEL T cell expansion. My flow cytometry gating strategy of leukocyte populations is shown in **Figure 3.13**. I present the data as percentage of cells in the live cell gate (**Figure 4.14 A and B).**

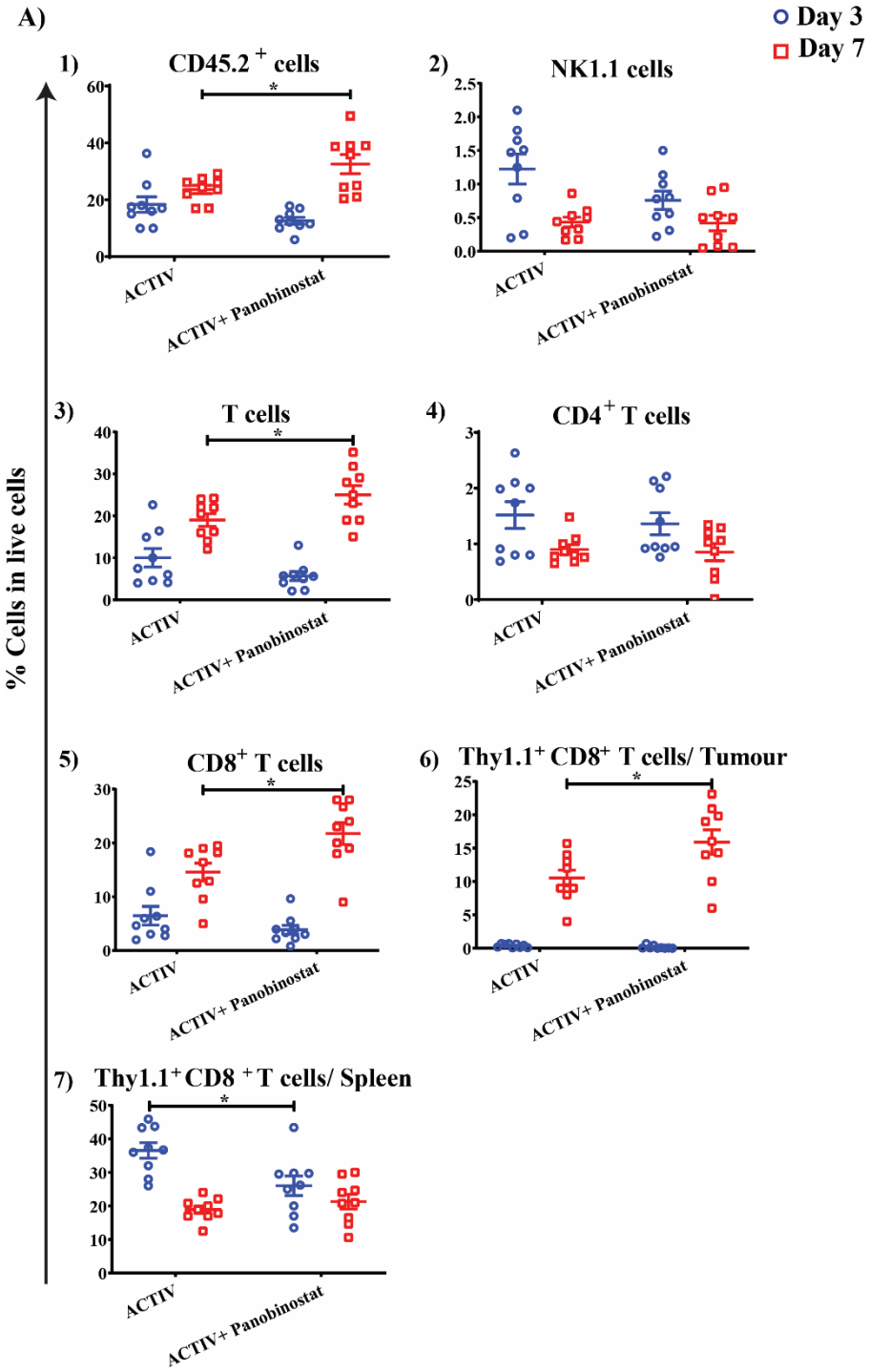
The percentage of leukocytes ($CD45.2^+$ cells) on day seven significantly increased in tumours of mice receiving the combination treatment (Panobinostat + ACTIV therapy) compared with mice receiving ACTIV therapy alone (**Figure 4.14 A1).** There was no significant difference in NK cells between the groups receiving ACTIV therapy with or without Panobinostat (**Figure 4.14 A2**) suggesting that the anti-tumour activity enhancement of Panobinostat was not through NK cells following the combinatorial treatment.

In addition, there was an increase in tumour-infiltrating T cells by day seven in Panc02-Her2 tumours of mice receiving combination therapy (ACTIV+ Panobinostat) compared with mice receiving ACTIV therapy alone (**Figure 4.14 A3**). This increase in T cells was not reflected in an increase in $CD4^+$ T cells (**Figure 4.14 A4**), but there was a significant increase in endogenous $CD8^+$ T cells and CARaMEL T cells ($Thy1.1^+$ $CD8^+$) (**Figure 4.14 A5, A6)**. Moreover, in the spleens, a marked decrease in CARaMEL T cells was observed on day three in mice receiving Panobinostat with ACTIV therapy compared to mice receiving ACTIV therapy alone (**Figure 4.14 A7**).

In contrast, there was no difference in percentage of myeloid lineage cells infiltrating Panc02-Her2 tumours of mice receiving combination therapy (ACTIV+ Panobinostat) compared with mice receiving ACTIV therapy only (**Figure 4.14 B**).

Having investigated the percentage of tumour-infiltrating CARaMEL T cells by flow cytometry, I next sought to confirm these findings, and determine $CD8^+$ T cell distribution throughout the tumour, using immunohistochemistry. Tumour sections were prepared from Panc02-Her2 tumours of mice that received ACTIV therapy with or without Panobinostat and other control groups were subjected to immunohistochemical staining for $CD8^+$ T cells within live area (excluding the necrotic area) and then enumerated through Halo software (**Figure 4.15**). As shown in **Figure 4.15,** the tumour sections of mice receiving combination treatment contained an even distribution (**Figure 4.15A**) and significantly higher numbers of $CD8^+$ T cells (**Figure 4.15B**) on day seven compared with the group of mice that received ACTIV therapy only.

In summary, these data suggest that Panobinostat significantly increased the percentage of infiltrated leukocytes that was reflected by a significant increase at day seven in both host $CD8^+$ T cells and CARaMEL T cells in Panc02-Her2 tumours of mice receiving combinational therapy (ACTIV+ Panobinostat) compared with the control groups, but in contrast there was no difference in percentage of myeloid lineage cells infiltrating Panc02-Her2 tumours at this time point.


A)
Day 3
Day 7
% Cells in live cells
1) CD45.2 + cells
2) NK1.1 cells
3) T cells
4) CD4+ T cells
5) CD8+ T cells
6) Thy1.1+ CD8+ T cells/ Tumour
7) Thy1.1+ CD8 + T cells/ Spleen
ACTIV
ACTIV+ Panobinostat
*

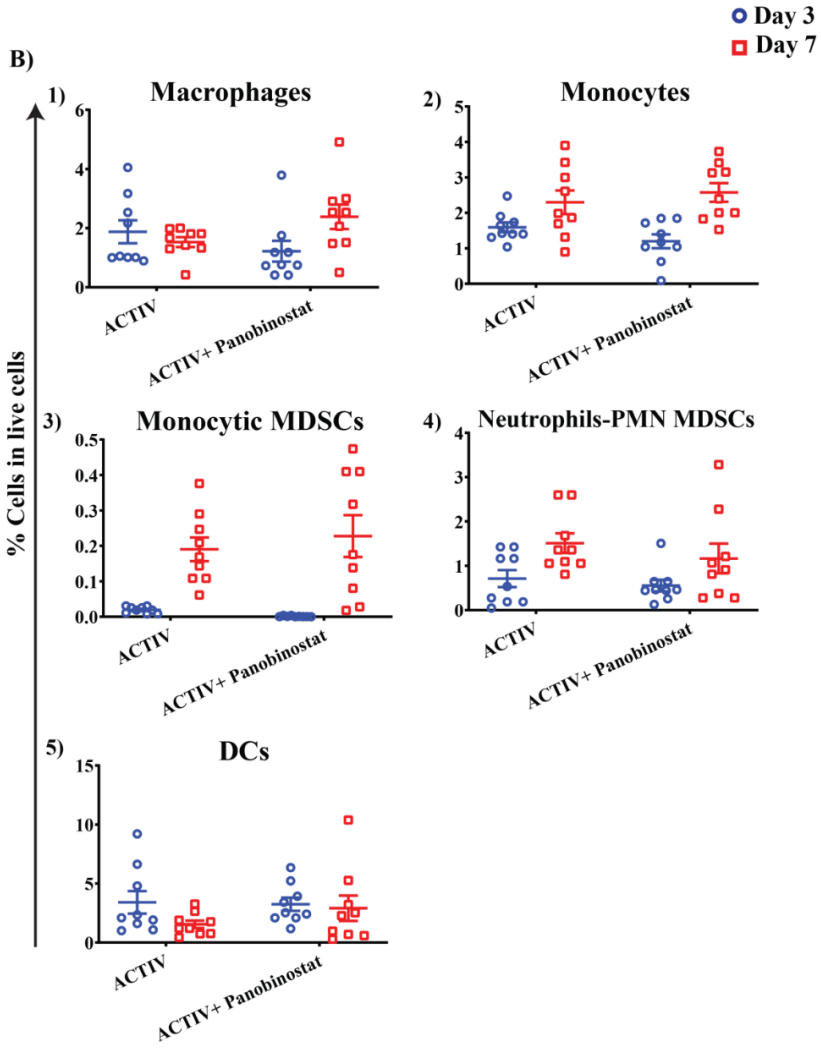


Figure 4.14. Leukocyte infiltration in Panc02-Her2 tumours following ACTIV therapy in combination with Panobinostat

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy with or without three intraperitoneal injections of Panobinostat (15 mg/kg) over three consecutive days. As a control, a cohort of mice were injected with three intraperitoneal doses of Panobinostat (15 mg/kg) alone or vehicle (dextrose) over three days. Tumours and spleens were harvested and analysed using flow cytometry, with gating as shown in **Figure 3.13**, to determine the percentage of (**A**) Lymphocyte populations: (**A1**) Leukocytes ($CD45.2^+$) (**A2**) NK cells ($CD45^+$, $NK1.1^+$), (**A3**) Total T cells, ($CD45^+$, TCR^+), (**A4**) endogenous $CD4^+$ ($CD45^+$, TCR^+, $Thy1.1^-$, $CD4^+$), (**A5**) endogenous $CD8^+$ ($CD45^+$, TCR^+, $Thy1.1^-$, $CD8^+$), (**A6**) $CD8^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD8^+$) in tumour (**A7**) $CD8^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD8^+$) in spleen, (**B**) Myeloid lineage populations: (**B1**) macrophages ($CD45^+$, $MHCII^+$, $CD11b^+$, $Ly6C^-$, $F4/80^{+/hi}$), (**B2**) monocytes ($CD45^+$, $CD11b^+$, $Ly6C^{intr}$, $Ly6G^-$, $MHCII^{low}$), (**B3**) monocytic MDSCs ($CD45^+$, $CD11b^+$, $Ly6C^{hi}$, $Ly6G^-$, $MHCII^-$), (**B4**) neutrophils/PMN MDSCs ($CD45^+$, $MHCII^{-/low}$, $CD11b^+$, $Ly6G^+$, $Ly6C^{low}$), (**B5**) DC subsets ($CD45^+$, $MHCII^+$, $CD11c^+$, $Ly6C^-$, $F4/80^{+/low}$) of live cells from tumours on days three and seven post treatment. Data are presented as mean ± SEM of nine mice per group, data pooled from three independent experiments. Statistical analyses were performed using Student *t*-test (* $p < 0.05$).

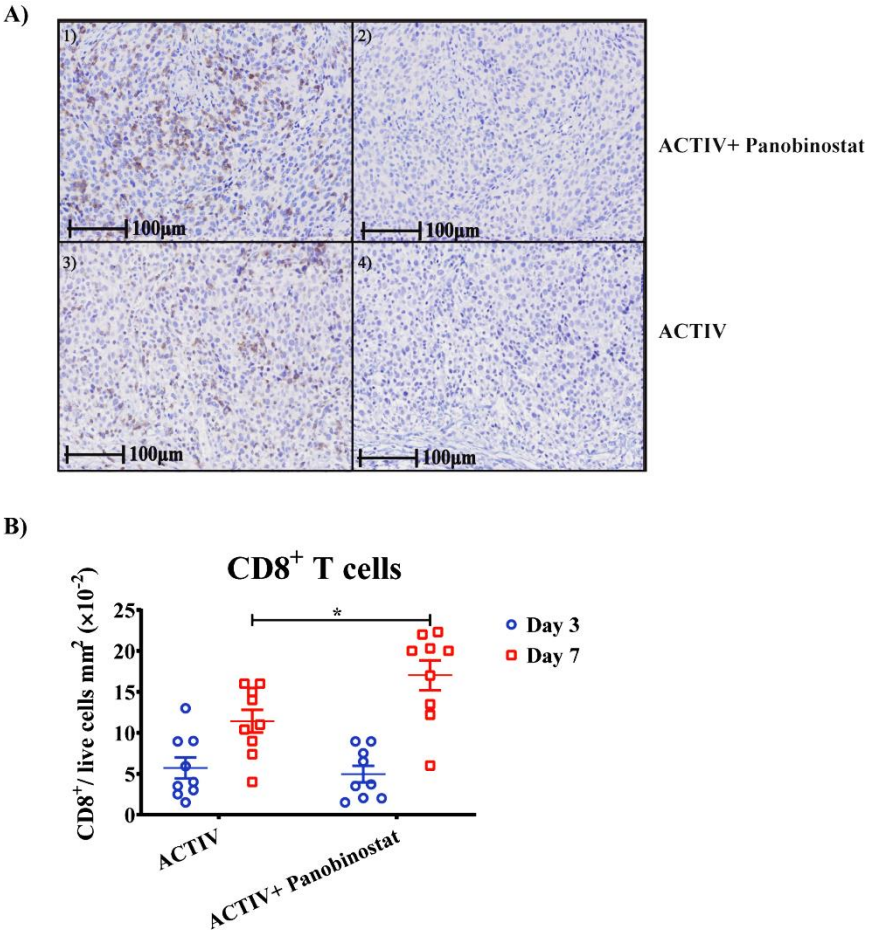


Figure 4.15. The frequency of CD8$^+$ T cells increases in Panc02-Her2 tumours subjected to ACTIV therapy in combination with Panobinostat

Panc02-Her2 cancer cells (1 × 10^6 cells/mouse) were injected subcutaneously into C57BL/6 human Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy with or without three intraperitoneal injections of Panobinostat (15 mg/kg) over three consecutive days. As a control, a cohort of mice were injected with three doses of Panobinostat (15 mg/kg) alone or vehicle (dextrose) for three consecutive days. Tumours were harvested and stained with an anti-CD8 antibody or matched isotype control. (**A**) Representative sections of tumours from mice treated with Panobinostat and ACTIV therapy (top) or ACTIV therapy alone (bottom) and stained with anti-CD8 (**A1 and A3**) or matched isotype antibody (**A2 and A4**) captured at 20× (brown; scale bar = 100 µm; Representative of tumours from nine mice per group, 1 section per mouse). (**B**) Quantitative data of whole IHC sections depicting CD8$^+$ cell counts/mm^2 of live area from nine mice per group at each time point enumerated using HALO. Data are presented as mean ± SEM pooled from three independent experiments. Statistical analyses were performed using the Student *t*-test (* $p < 0.05$).

4.2.10. IFN-γ plays a critical role in the enhancement of ACTIV therapy by Panobinostat

To gain a better understanding of the influence of Panobinostat on ACTIV therapy, I explored the relative importance of some cytokines (IFN-γ and TNF-α) and other potential contributors, including chemokine receptors (CXCR3) using neutralizing antibodies. The dose and schedule for neutralising antibodies was chosen based on previous effective protocols used in our research lab. The activity of the antibodies was confirmed by colleagues working on separate parallel experiments at the same time.

I have shown previously that IFN-γ is an important cytokine in potentiating anti-tumour immunity which has been shown to have effects on both innate and adaptive immunity [290-292]. IFN-γ signalling can inhibit tumour growth by various mechanisms such as the induction of tumour cell apoptosis, cycle arrest, ischemia or necroptosis [443-447]. In addition, IFN-γ can induce a number of signals enabling T cells and APCs to function effectively [291, 444-446]. Moreover, IFN-γ can inhibit the function of some suppressive immune cells, such as T_{reg} and MDSCs [444-446, 448, 449], and can polarize macrophages toward a tumour-inhibitory M1 phenotype [450].

In this experiment I treated groups of mice with ACTIV therapy and three intraperitoneal injections of Panobinostat (15 mg/kg) over three consecutive days. Different groups were intraperitoneally injected with 250 µg of anti-IFN-γ antibody or control antibody on day -1, 0, five and then weekly (**Figure 4.16**). I found an important role of IFN-γ in my combined therapy (Panobinostat and ACTIV therapy) as the group of mice treated with anti-IFN-γ antibody had significantly larger tumours and shorter overall survival compared with the control group (mice receiving Panobinostat, ACTIV therapy and control antibody) ($p= 0.0272$) **(Figure 4.16 A and B)**.

Cancer cell immunogenicity and anti-tumour immune responses may have been altered by Panobinostat. Panobinostat may have sensitized Panc02-Her2 tumours; as various studies have demonstrated a role for Panobinostat in resensitizing cancer cells to other therapies [451-453]. Panobinostat may have enhanced the release of IFN-γ [454, 455] from CARaMEL T cells, or other immune cells, and it may have also mediated pro-inflammatory actions on immune cells and target tissues.

In addition, I investigated whether TNF-α played a role in the combination treatment using ACTIV therapy and Panobinostat. TNF-α is a pleiotropic cytokine with various roles in onco-

immunology. TNF-α can be secreted by a range of immune cells including T and B lymphocytes, dendritic cells, monocytes, neutrophils and mast cells. TNF has both pro-inflammatory and anti-inflammatory functions, and can play roles as either an anti-cancer factor [456], or an immunosuppressive cytokine [457-461]. TNF was initially identified as a cytotoxic soluble factor [456] of natural killers and $CD8^+$ T lymphocytes; but recently, TNF has been shown to be an immune suppressor cytokine that can facilitate the accumulation of T_{reg} and MDSCs [460]. Therefore, in this experiment I neutralized TNF-α by injecting a group of mice with 500 µg of anti-TNF-α daily for one week. I found that TNF-α had no impact on the therapeutic effect of the combinatorial therapy (Panobinostat and ACTIV therapy) compared with the control group (mice receiving Panobinostat, ACTIV therapy and control antibody), and had no effect on overall survival (**Figure 4.16 A and B**).

Furthermore, to investigate the chemokine receptor requirements during CARaMEL T cell migration to tumour sites I used a neutralizing anti-CXCR3 antibody. CXCR3 is a chemokine receptor expressed on Th1-type $CD4^+$ T cells and effector $CD8^+$ T cells. Migration of activated T cells *in vitro* and *in vivo* can be induced through the binding of CXCR3 to interferon-inducible chemokines CXCL9 and CXCL10 [462]. As shown in **Figure 4.16,** the group of mice that received the combinatorial treatment of Panobinostat and ACTIV therapy together with anti-CXCR3 antibody had no reduction in the anti-tumour activity (**Figure 4.16 A**). In addition, the survival of mice was not reduced after CXCR3 blockade suggesting that CXCR3 had no role in CARaMEL T cell migration to the tumour site.

In summary, this data suggested that IFN-γ played an important role in my combined therapy (Panobinostat and ACTIV therapy). In contrast, TNF-α and CXCR3 appeared to have little role in the anti-tumour response mediated by the combined therapy.

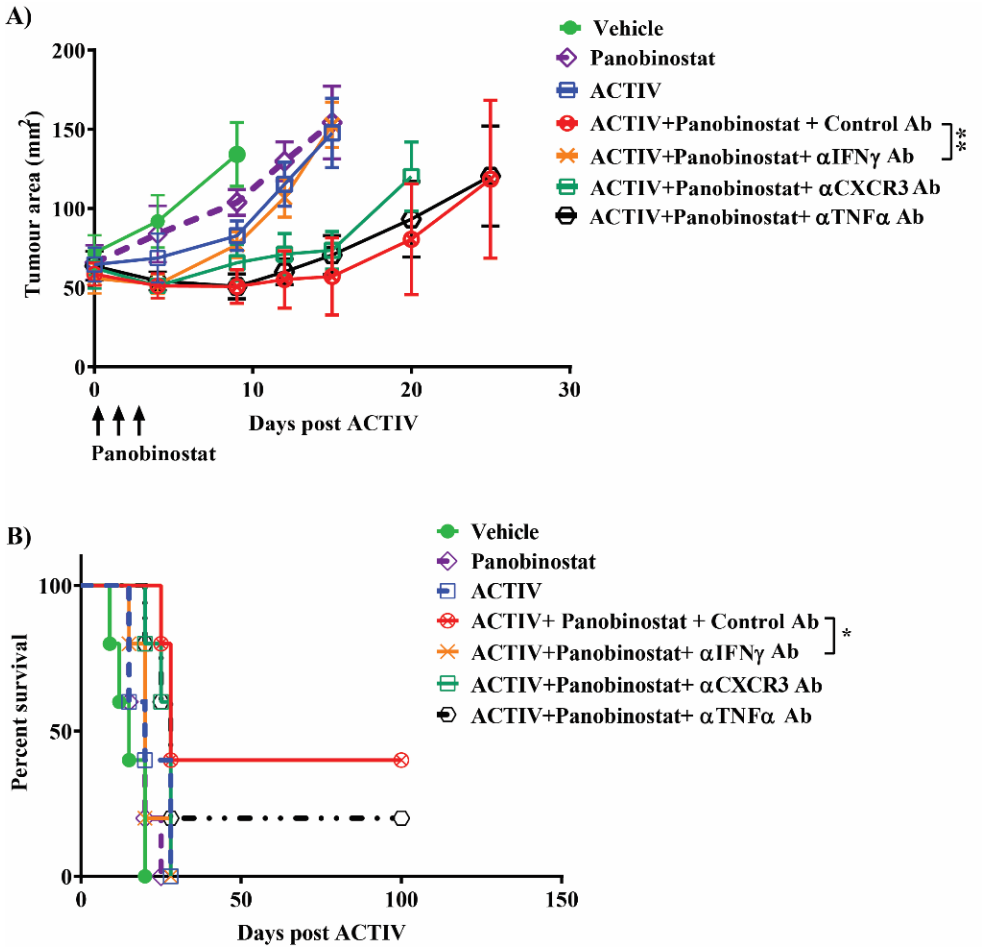


Figure 4.16. IFN-γ is important for the enhanced anti-tumour activity mediated by ACTIV + Panobinostat therapy

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, the mice received ACTIV therapy with or without three intraperitoneally injections of Panobinostat (15 mg/kg) over three consecutive days. As a control, a cohort of mice were intraperitoneally injected with three doses of vehicle for three consecutive days. Different groups were intraperitoneally injected with the indicated neutralizing antibodies. Tumour areas were measured at the indicated times, and data are presented as the mean ± SEM of 4-5 mice/group, representative of two independent experiments. Percent survival was determined with the endpoint being tumour area reaching ≥200 mm^2. Statistical analyses were performed using the Student *t*-test for tumour size and the Log-rank (Mantel-Cox) Test for Kaplan-Meier survival curves (* $p < 0.05$, ** $p \leq 0.01$). (**A**) Tumour size panel. (**B**) Mice survival panel.

4.2.11. Panobinostat rendered Panc02-Her2 tumours more sensitive to ACTIV therapy

Cytotoxic effector T cells can kill tumour cells through a variety of cytotoxic mechanisms, including exocytosis of cytotoxic secretory granules containing perforin and granzymes. When cytotoxic effector T cells recognize and conjugate to a targeted cell, an immunological synapse is formed and cytotoxic granules are released resulting in initiation of apoptosis in the targeted cells [463]. In light of the importance of IFN-γ for anti-tumour immunity in the combined therapy (Panobinostat and ACTIV therapy), I next investigated how CARaMEL T cells carry out their killing function whether through perforin or secretion of IFN-γ. In addition, I investigated whether IFN-γ was secreted mainly by adoptively transferred CARaMEL T cells, rather than endogenous lymphocytes.

To examine this, I subcutaneously inoculated Panc02-Her2 tumour cells into Her2 transgenic mice. Nine days following tumour injection, I treated groups of mice with ACTIV therapy with either: wild type CARaMEL T cells, CARaMEL T cells from IFN-γ gene-deficient mice (IFN-$\gamma^{-/-}$ CARaMEL) or CARaMEL T cells from perforin gene-deficient mice ($PFP^{-/-}$ CARaMEL) (**Figure 4.17**). All these groups were injected with three intraperitoneal injections of Panobinostat (15 mg/kg) over three consecutive days. Tumour areas were measured to monitor the growth of tumours until they reached a pre-determined endpoint (tumour area ≥ 150 mm^2). This endpoint was used to determine survival of the mice.

The group of mice receiving Panobinostat with either IFN-$\gamma^{-/-}$ or $PFP^{-/-}$ CARaMEL T cells did not reduce the anti-tumour activity compared with the control group (mice receiving Panobinostat and wild type CARaMEL T cells) (**Figure 4.17**). My results showed that neither IFN-γ secreted by CARaMEL T cells nor perforin compromised the anti-tumour effects of the combination therapy against Panc02-Her2 tumours. Nevertheless, my previous observations indicated that IFN-γ was necessary for optimal tumour growth inhibition. This suggests that IFN-γ secreted by other endogenous immune cells played an important role. Such endogenous cells may include CD4 Th1 and CD8 cytotoxic T cells, NK cells, natural killer T (NKT) [464] or macrophages [465]. Panobinostat may have epigenetically affected or stimulated these immune cells to release IFN-γ [466].

These findings indicate a contribution from tumour-intrinsic anti-proliferative and apoptotic mechanisms on the growth inhibitory effects induced by the combined treatment of Panobinostat and ACTIV therapy in Panc02-Her2 tumours. However, in addition, these results

suggested that Panobinostat may render Panc02-Her2 tumour cells more sensitive to the treatment and may work cooperatively with ACTIV therapy to enhance anti-tumour responses.

In summary, I examined the combined anticancer activity and mechanisms of ACTIV therapy and HDACi (Panobinostat) in mice bearing established $Her2^+$ subcutaneous pancreatic tumours. Here, I demonstrate the novel tumour inhibitory capacity of this combination therapy and its ability to induce durable anti-tumour responses against Panc02-$Her2^+$ tumours. This set of experiments demonstrated that Panobinostat did not enhance the infection of VVgp-100 in Panc02-Her2 cells or increase Her2 and MHCI expression on these tumours *in vitro* and *in vivo*. However, Panobinostat suppressed the growth of Panc02-Her2 cells by inducing apoptosis and G1/S cell cycle arrest *in vitro*. In addition, I showed that the combination of CARaMEL T cells and Panobinostat significantly increased growth suppression of Panc02-Her2 cells. I demonstrated that IFN-γ was important for the combinatorial treatment of Panobinostat and ACTIV therapy; and my results suggest the source of this cytokine was host derived. These findings led us to conclude that Panobinostat rendered Panc02-Her2 tumours more sensitive to ACTIV therapy and Panobinostat may work cooperatively with ACTIV therapy to enhance anti-tumour responses. These results suggest that the combination of HDACi and adoptive cell transfer may be an effective strategy for anti-cancer therapy.

As detailed previously in other studies, CD40 agonistic antibodies alone or combined with chemotherapy suppressed tumour growth in both spontaneous genetic and transplantable pancreatic tumour models [132, 139, 409]. This anti-tumour activity was reported to be associated with both T cell– mediated [139] or macrophage-mediated [132] anti-tumour responses. Therefore, in the next section I evaluated whether combining αCD40 antibody with ACTIV therapy could elicit increased anti-tumour responses against Panc02-Her2 tumour.

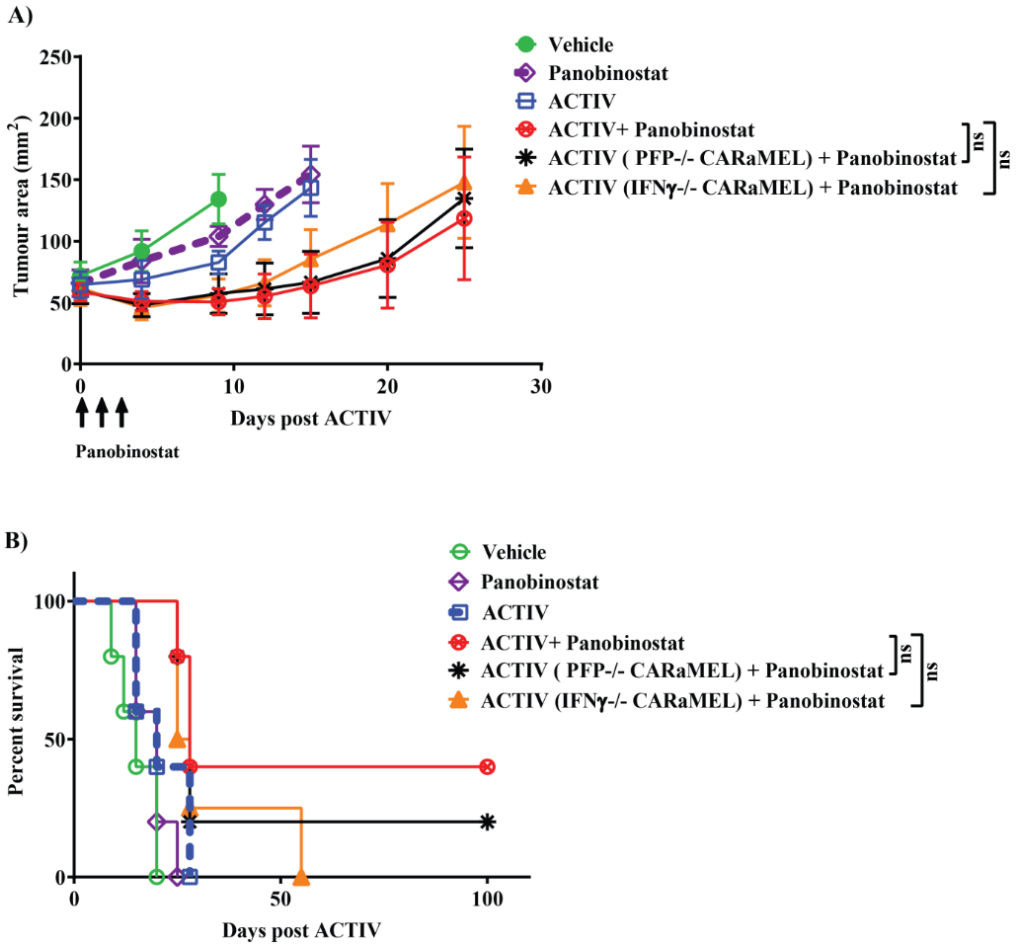


Figure 4.17. Neither IFN-γ nor perforin produced by CARaMEL T cells are required for optimal anti-tumour activity in the combined treatment using Panobinostat and ACTIV therapy

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, mice received ACTIV therapy in the presence or absence of three intraperitoneally injections of Panobinostat (15 mg/kg) over three consecutive days. Different groups were administered with either wild type CARaMEL T cells, CARaMEL T cells from IFN-γ gene-deficient mice (IFN-$\gamma^{-/-}$ CARaMEL) or CARaMEL T cells from perforin gene-deficient mice (PFP$^{-/-}$ CARaMEL). As a control, a cohort of mice were intraperitoneally injected with three doses vehicle (dextrose) for three consecutive days. Tumour areas were measured at the indicated times, and data are presented as the mean ± SEM of 4-5 mice/group, representative of two independent experiments. Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2 (ns= not significant). Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) Test for Kaplan-Meier survival curves (ns= not significant). (**A**) Tumour size panel. (**B**) Mice survival panel.

4.2.12. ACTIV therapy was enhanced by the co-administration of anti-CD40 agonist antibody in Panc02-Her2 bearing mice

I first sought to determine the efficacy of the addition of an αCD40 antibody to ACTIV therapy against murine pancreatic tumours. In this experiment, I injected the Panc02-Her2 cancer cell line subcutaneously into C57BL/6-Her2 mice and allowed the tumours to grow for nine days to reach 35-55 mm^2 in size. On day 0, ACTIV therapy was performed as explained in the schematic of ACTIV therapy (**Figure 3.6**). A group of mice received three intraperitoneal injections of αCD40 antibody (10 μg) on days 0, three and five of ACTIV therapy. As a control group, a cohort of mice were injected with three doses of αCD40 antibody (10 μg) alone or 2A3 isotype antibody (10 μg) on days 0, three and five of treatment. Tumour area and mouse survival were then recorded with the endpoint being tumour area reaching ≥ 150 mm^2 (**Figure 4.18**).

A significant reduction in tumour growth and significantly prolonged survival was achieved in some mice treated with the combination of αCD40 antibody with ACTIV therapy relative to ACTIV treated mice **(Figure 4.18 A and B)**. In contrast, single-agent αCD40 antibody therapy showed little effect in suppression of Panc02-Her2 tumours. Overall, this result is consistent with my hypothesis that αCD40 antibody can enhance the therapeutic effects of ACTIV therapy.

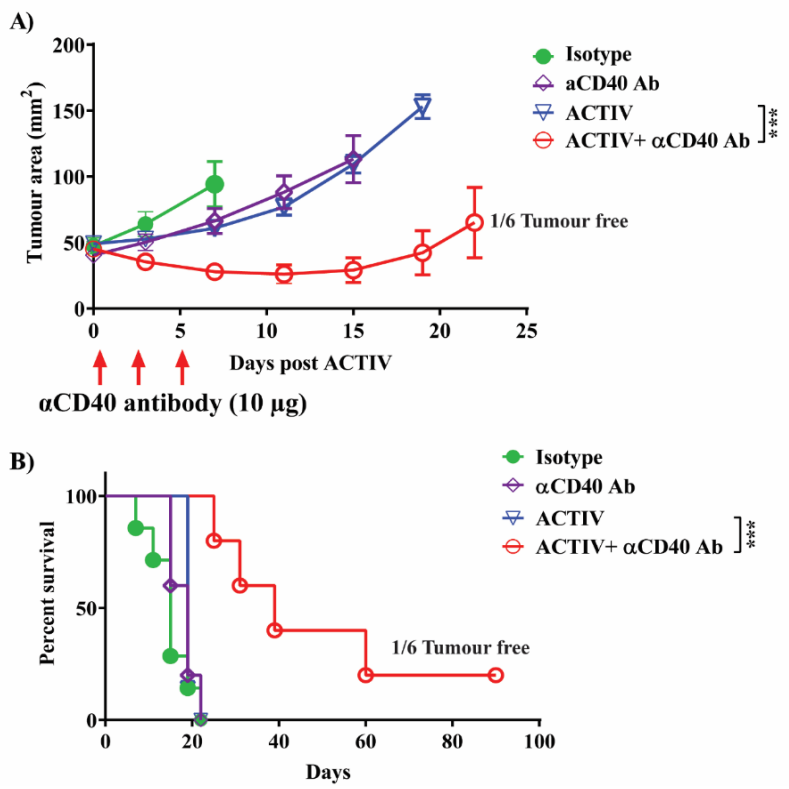


Figure 4.18. αCD40 antibody enhanced the anti-tumour activity of ACTIV therapy

C57BL/6 human-Her2 transgenic mice were subcutaneously injected with Panc02-Her2 tumour cells (1×10^6 cells/mouse). The tumours were allowed to grow for nine days. On day 9, the tumour-bearing mice were treated with ACTIV therapy. Three intraperitoneal injections of αCD40 antibody (10 µg) were also given with ACTIV therapy on days 0, three and five of treatment. As a control, a cohort of mice were injected with three doses of αCD40 antibody (10 µg) alone or 2A3 isotype antibody (10 µg) on days 0, three and five of treatment. (**A**) Tumour areas were measured at the indicated times, and data are the mean ± SEM of 5-6 mice/group, representative of five independent experiments. (**B**) Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (*** $p \leq 0.001$).

4.2.13. Effect of αCD40 antibody on the proliferation and distribution of CARaMEL T cells and other leukocytes in vivo

To gain insight into the mechanism behind the impact of αCD40 antibody on ACTIV therapy I investigated the phenotype and percentage of infiltrated CARaMEL T cells and macrophages.

I treated a group of mice with ACTIV therapy with or without three intraperitoneal injections of αCD40 antibody (10 µg) on days 0, three and five of ACTIV therapy. As controls, mice were injected with three doses of αCD40 antibody (10 µg) alone or 2A3 isotype antibody (10 µg) on days 0, three and five of treatment. Tumours were collected on days three and seven post ACTIV and enzymatically digested into single cell suspensions. The cells were stained with cell-type specific antibodies and analysed using flow cytometry to determine the percentage of leukocytes that infiltrated Panc02-Her2 tumours. My flow cytometry gating strategy of the leukocyte population is shown in **Figure 3.13**. I presented the data as percentage of live cells (**Figure 4.19 A, B).**

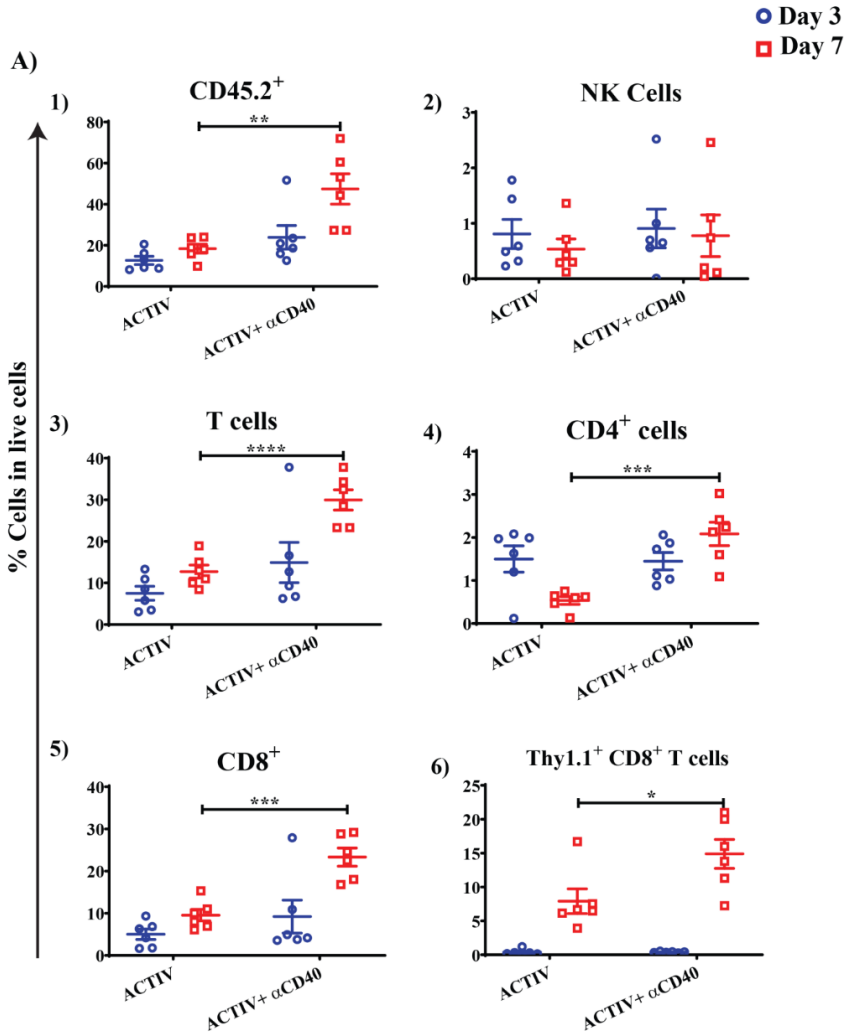
The percentage of leukocytes ($CD45.2^+$) significantly increased in tumours of mice receiving the combinatorial treatment of αCD40 antibody and ACTIV therapy compared with mice receiving ACTIV therapy alone (**Figure 4.19 A1).** There was no significant difference in percentage of NK cells between the groups receiving ACTIV therapy with or without αCD40 antibody (**Figure 4.20 A2**) suggesting that the enhanced anti-tumour activity of αCD40 was not through NK cells. In contrast, I found a sharp increase in the percentage of tumour-infiltrating T cells among live cells on day seven in Panc02-Her2 tumours of mice receiving the combinatorial therapy compared with mice receiving ACTIV therapy alone (**Figure 4.19 A3**). This increase in percentage of T cells was reflected by an increase of host $CD4^+$ T cells (**Figure 4.19 A4**), $CD8^+$ T cells (**Figure 4.19 A5**) and CARaMEL T cells ($Thy1.1^+$ $CD8^+$) (**Figure 4.19 A6)**. This suggests that CD40 agonist antibody enhanced ACTIV therapy through a T-cell-dependent mechanism by increasing the percentage of CARaMEL T cells in tumours.

I next examined the impact of treatment involving an agonist CD40 antibody on myeloid cells. I found a significant reduction in percentage of macrophages on day three and seven in Panc02-Her2 tumours of mice receiving the combinatorial treatment of αCD40 antibody and ACTIV therapy compared with mice receiving ACTIV therapy alone (**Figure 4.19 B1**). In further analyses, I investigated the possibility of the combinational therapy switching the phenotype of tumour-infiltrating macrophages from M2 (pro-tumour) to M1 (anti-tumour). To determine this, I analysed the expression of CD206 on the surface of the macrophages, which is associated

with an M2 phenotype [467]. The flow cytometry results showed no significant reduction in percentage of M2 macrophages in tumours of mice receiving both αCD40 antibody and ACTIV therapy (**Figure 4.19 B2**). These results suggested that while αCD40 reduced the total macrophage percentage in tumours, there was no specific reduction in M2 macrophages, based on CD206 expression. Nevertheless, it is possible that the percentage of pro-tumoural macrophages of other phenotypes were reduced following αCD40 treatment, which would be of interest to determine in future experiments.

In contrast to a reduction in percentage of macrophages I observed a significant increase in percentage of monocytes in Panc02-Her2 tumours of mice receiving αCD40 antibody with ACTIV therapy compared with mice receiving ACTIV therapy alone (**Figure 4.19 B3)**. Previous studies showed that monocytes can give rise to DCs after stimulation with anti-CD40 [468-470] or inflammatory macrophages, and may contribute to MDSCs associated with tumours [471]. Another study showed that anti-CD40 agonist antibody stimulated macrophages originating from monocytes and facilitated the depletion of extracellular matrix proteins that resulted in tumour regression in both mice and patients with pancreatic cancer [469]. Further experiments are needed to determine the role of monocytes in ACTIV therapy.

In summary, the flow cytometry analysis indicated an increase in percentage of tumour-infiltrating T cells (host $CD4^+$, $CD8^+$ T cells and $Thy1.1^+$ $CD8^+$ CARaMEL T cells) and monocytes in Panc02-Her2 tumours of mice receiving combinatorial treatment of αCD40 antibody and ACTIV therapy. In contrast, the inclusion of anti-CD40 to ACTIV therapy led to a reduction in percentage of macrophages in Panc02-Her2 tumours. Thus, the data suggested a predominant T cell-dependent mechanism contributed to enhanced ACTIV therapy mediated by CD40 agonist antibody.


A)
Day 3
Day 7
% Cells in live cells
1)
CD45.2+
**
2)
NK Cells
3)
T cells
****
4)
CD4+ cells
***
5)
CD8+
***
6)
Thy1.1+ CD8+ T cells
*
ACTIV
ACTIV+ αCD40

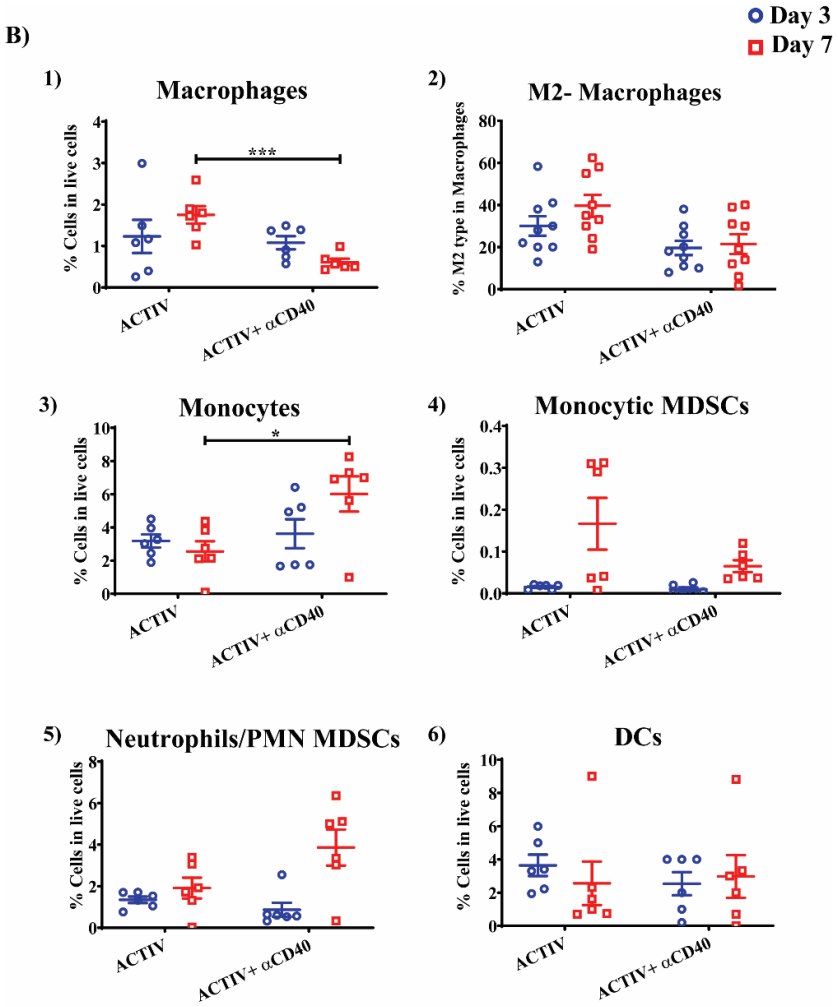


Figure 4.19. Characterization of leukocyte infiltration in Panc02-Her2 tumours receiving ACTIV therapy combined with an agonist αCD40 antibody

Panc02-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic. Nine days following tumour injection, mice received ACTIV therapy with or without three intraperitoneal injections of αCD40 antibody (10 µg) on days 0, three and five of ACTIV therapy. As a control group, mice were injected with three doses of αCD40 antibody (10 µg) alone or 2A3 isotype antibody (10 µg) on days 0, three and five of treatment. Tumours were harvested and analysed using flow cytometry, with gating as shown in **Figure 3.13**, to determine the percentage of (**A**) Lymphocyte populations: (**A1**) Leukocytes ($CD45.2^+$) (**A2**) NK cells ($CD45^+$, $NK1.1^+$), (**A3**) Total T cells, ($CD45^+$, TCR^+), (**A4**) endogenous $CD4^+$ ($CD45^+$, TCR^+, $Thy1.1^-$, $CD4^+$), (**A5**) endogenous $CD8^+$ ($CD45^+$, TCR^+, $Thy1.1^-$, $CD8^+$), (**A6**) $CD8^+$ CARaMEL T cells ($CD45^+$, TCR^+, $Thy1.1^+$, $CD8^+$) in tumour (**B**) Myeloid lineage populations: (**B1**) macrophages ($CD45^+$, $MHCII^+$, $CD11b^+$, $Ly6C^-$, $F4/80^{+/hi}$), (**B2**) M2-macrophages ($CD45^+$, $MHCII^+$, $CD11b^+$, $Ly6C^-$, $F4/80^{+/hi}$, $CD206^+$), (**B3**) monocytes ($CD45^+$, $CD11b^+$, $Ly6C^{intr}$, $Ly6G^-$, $MHCII^{low}$), (**B4**) monocytic MDSCs ($CD45^+$, $CD11b^+$, $Ly6C^{hi}$, $Ly6G^-$, $MHCII^-$), (**B5**) neutrophils/PMN MDSCs ($CD45^+$, $MHCII^{-/low}$, $CD11b^+$, $Ly6G^+$, $Ly6C^{low}$), (**B6**) DC subsets ($CD45^+$, $MHCII^+$, $CD11c^+$, $Ly6C^-$, $F4/80^{+/low}$) of live cells from tumours on days three and seven post treatment. Data are presented as mean ± SEM of 6-9 mice per group pooled from 2-3 experiments performed independently from the tumour growth experiments. Statistical analyses were performed using the Student *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.2.14. CD40 agonist antibody enhanced ACTIV therapy through a T-cell-dependent mechanism

The previous figure suggests that αCD40 antibody enhancement of ACTIV therapy was associated with an increase in the percentage of tumour-infiltrating T cells. To further investigate the contribution of T cells in the αCD40 antibody-mediated enhancement of ACTIV therapy, I determined the relative level of CARaMEL T cell proliferation and cytokine production. I treated a group of mice bearing Panc02-Her2 tumours with ACTIV therapy with or without three intraperitoneal injections of αCD40 antibody (**Figure 4.20**). Spleens and tumours were collected at various time points and flow cytometric analysis was performed to determine the expansion of CARaMEL T cells. A significantly higher percentage of CARaMEL T cells was observed in the spleens of mice receiving αCD40 antibody with ACTIV therapy on day five and nine post treatment compared with mice receiving ACTIV therapy alone (**Figure 4.20 A**). In addition, the percentage of CARaMEL T cells was significantly higher in Panc02-Her2 tumours in the mice receiving αCD40 antibody with ACTIV therapy on day five and nine post treatment (**Figure 4.20 B**). Furthermore, I observed a significantly higher percentage of IFN-γ producing $CD8^+$ T cells in Panc02-Her2 tumours in mice receiving αCD40 antibody with ACTIV therapy on day five post treatment compared with the group of mice receiving ACTIV therapy alone (**Figure 4.20 C and D**). Data in the previous figures (**Figure 4.20**) suggested that the enhancement of anti-tumour activity of αCD40 antibody enhancement of ACTIV therapy was through the higher percentage of CARaMEL $CD8^+$ T cells among live cells and better activation of $CD8^+$ T cells.

Having analysed the percentage of tumour-infiltrating CARaMEL T cells I next tried to confirm these findings and determine $CD8^+$ T cell distribution throughout the tumour using IHC. I prepared tumour sections from Panc02-Her2 tumours of ACTIV treated mice with or without αCD40 antibody. On day three and 7, I collected the tumours and performed immunohistochemical staining of CD8 only, to compare with flow cytometry findings, then enumerated cells using Halo software (**Figure 4.21**). In this analysis, I excluded necrotic areas, as there were large regions of necrosis found in sections of tumours receiving the combined treatment.

Tumour sections of mice receiving ACTIV therapy combined with αCD40 antibody contained an even distribution (**Figure 4.21 A**) and significantly higher numbers of $CD8^+$ T cells compared with the group of mice receiving ACTIV therapy only on day seven (**Figure 4.21**

B). In addition, larger regions of necrosis were observed in sections of tumours receiving the combined treatment (**Figure 4.21 C**). These results are consistent with my flow cytometry findings, which suggest that αCD40 enhanced the proliferation, activation and functional status of CARaMEL T cells in ACTIV therapy.

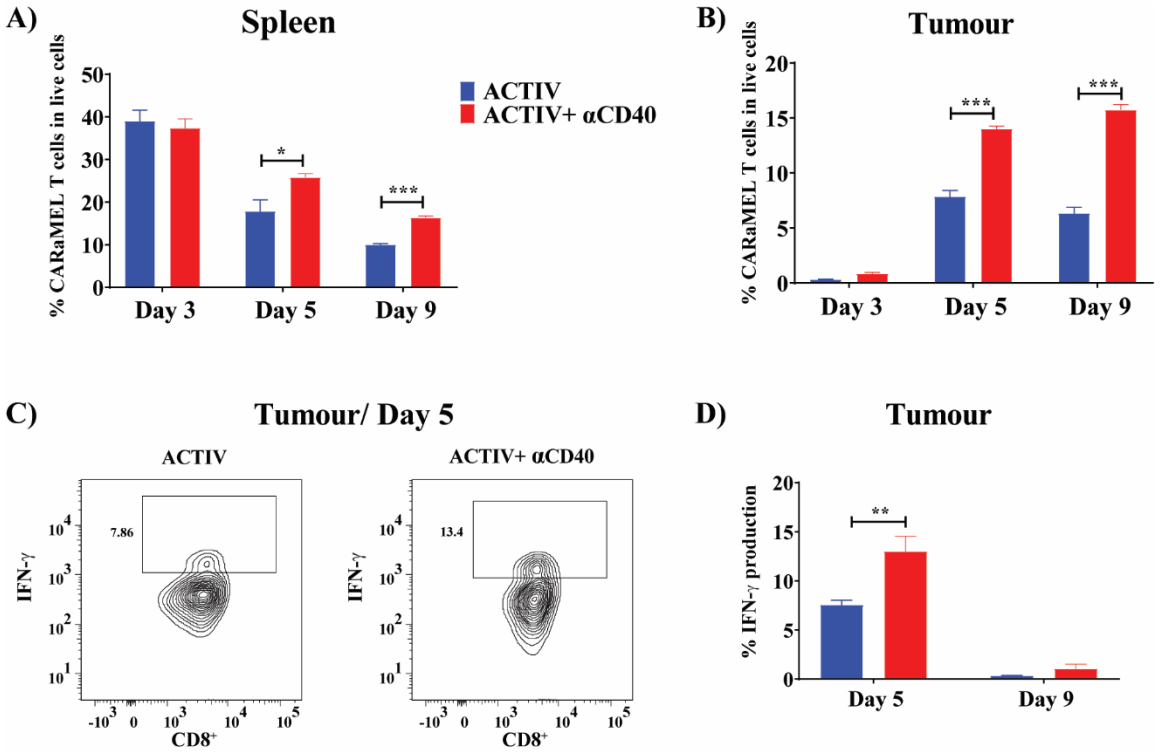


Figure 4.20. Addition of an CD40-agonist antibody to ACTIV therapy increases the accumulation of IFN-γ-producing CARaMEL T cells in tumours

Flow cytometric analysis of the spleens (**A**) and Panc02-Her2 tumours (**B**) subjected to ACTIV with or without αCD40 antibody (10 μg) on days 0, three and 6. Tumours and spleens were collected at the indicated time points and flow cytometric analysis was performed to determine the percentage of $CD8^+$/$Thy1.1^+$ T cells in live cells (**C**) Representative plots of T cells from Panc02-Her2 tumours showing the percentage of IFN- γ production from $CD8^+$ T cells (**D**) Data show the percentage of IFN-γ production in $CD8^+$ T cells following incubation with golgistop and golgiplug for four hours prior to being harvested for intracellular staining and analysis by flow cytometry. Data are representative of two experiments performed independently from the tumour growth experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (n=3) Student (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

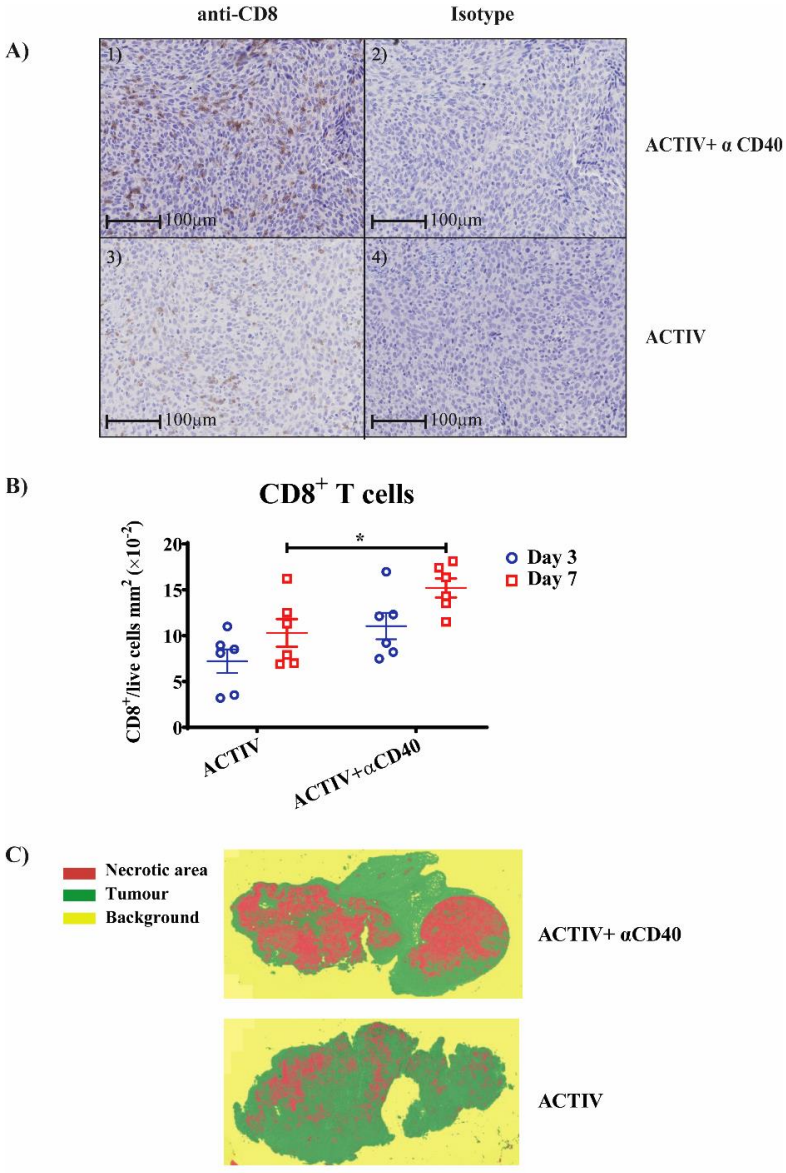


Figure 4.21. Addition of αCD40 agonist antibody to ACTIV therapy increases the frequency of tumour-infiltrating CD8$^+$ T cells

Panc02-Her2 cancer cells (1 × 10^6 cells/mouse) were injected subcutaneously into C57BL/6 human-Her2 transgenic mice. Nine days following tumour injection, mice received ACTIV therapy with or without three intraperitoneal injections of αCD40 antibody (10 µg) on days 0, three and five of ACTIV therapy. (**A**) Representative sections of tumours from mice taken on day three after ACTIV therapy and αCD40 antibody (top) or ACTIV therapy alone (bottom). Tumour section were stained with anti-CD8 (**A1 and A3**) or matched isotype control antibody (**A2 and A4**) captured at 40 × (CD8 staining indicated in brown; scale bar = 100 µm; tumours from six mice per group, one section per mouse). (**B**) Whole IHC section CD8$^+$ cell counts/mm^2 of live cell areas from six mice per group at each time point enumerated using HALO. (**C**) Representative section of tumours of mice on day seven receiving ACTIV therapy with or without αCD40 antibody showing necrotic areas (red), live tumour (green) of the treated tumours. Data are presented as mean ± SEM of six mice per group pooled from two experiments performed independently from the tumour growth experiments. Statistical analyses were performed using the Student *t*-test (* $p \leq 0.05$).

4.2.15. F4/80- or IL-12-neutralizing antibodies did not inhibit the enhancement of ACTIV therapy by anti-CD40 agonist antibody

Data in the previous figures (**Figure 4.19-4.21**) suggested that αCD40 antibody enhanced ACTIV therapy through a T cell dependent immune mechanism. In order to investigate this further, I targeted macrophages using an anti-F4/80 depleting antibody in mice that received ACTIV therapy in combination with αCD40. The F4/80 receptor, thought to be an adhesion molecule, is a murine pan-macrophage marker, and it is expressed on tumour-associated macrophages [472]. Anti-F4/80 antibodies were first reported in 1981 to bind to murine macrophage surface antigens, and have been shown to deplete F4/80^{+} macrophages [472, 473] and prevent entry of macrophages into tissue [474].

Our data suggested that macrophages had little impact on the therapeutic effect of the combinatorial therapy (αCD40 agonist and ACTIV therapy); as anti-F4/80 antibody administration did not reduce the anti-tumour activity in mice receiving the combination therapy. Furthermore, anti-F4/80 antibody had no significant effect on overall survival (**Figure 4.22 A and B**).

In addition, I determined whether IL-12 played a role in the combination αCD40 agonist antibody and ACTIV therapy. IL-12 is a heterodimeric cytokine produced primarily by activated APCs such as dendritic cells and macrophages in response to infection by various pathogens [475]. IL-12 promotes the proliferation and cytolytic activity of T and NK cells, and induces their IFN-γ production [476, 477]. However, as shown in **Figure 4.22 A and B,** neutralizing IL-12 had no significant impact on the anti-tumour response mediated by αCD40 agonist antibody and ACTIV therapy.

This data suggests that neither macrophages nor IL-12 were necessary for ACTIV and CD40 agonist-mediated tumour regression of Panc02-Her2 tumours. This suggests that CD40 agonist enhanced ACTIV therapy through mechanisms not involving macrophages and IL-12, as discussed in the next section. However, these results need to be confirmed in future experiments via analysis of the extent of macrophage depletion attained using anti-F4/80 antibody, and assessment of the neutralization ability of αIL-12 antibody using a bioassay.

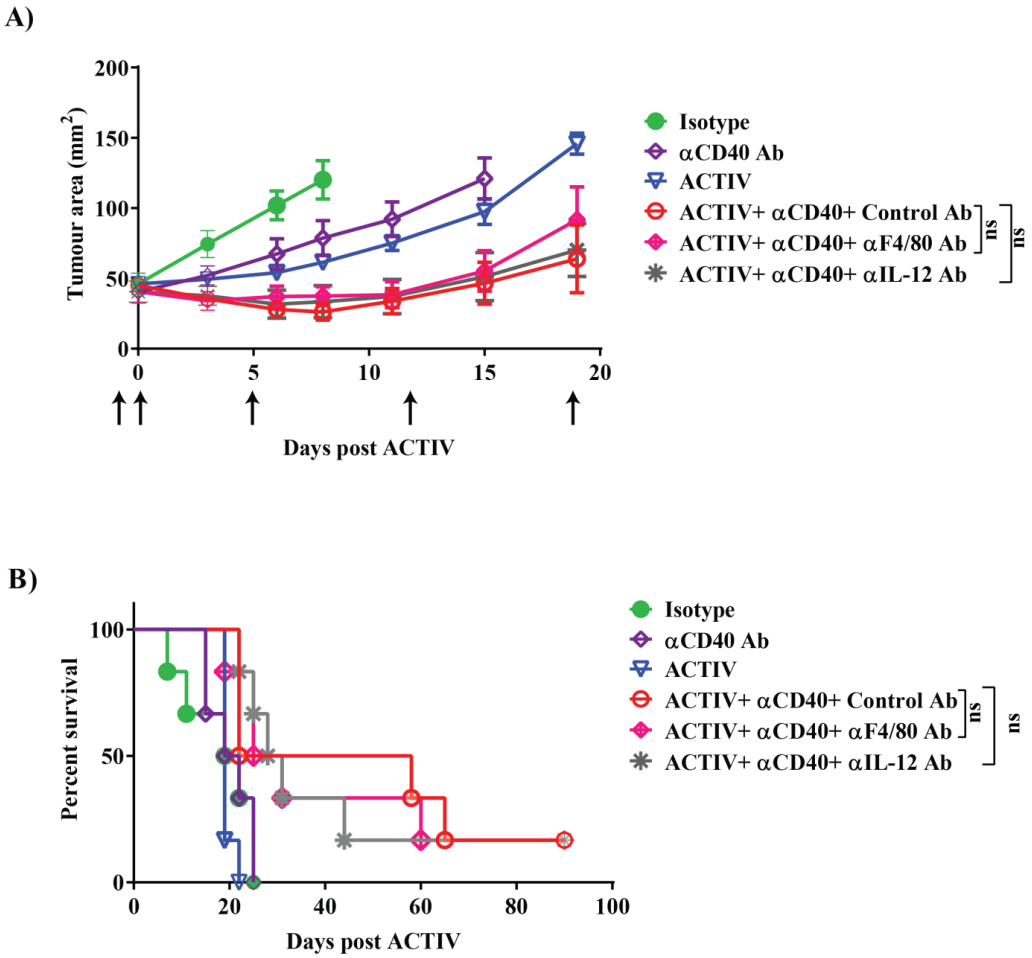


Figure 4.22. αF4/80 or αIL-12 antibodies did not abrogate the enhancement of anti-tumour activity of ACTIV therapy by αCD40 antibody

Panc02-Her2 tumour-bearing mice received ACTIV therapy with or without αCD40 antibody. As controls, cohorts of mice were injected with αCD40 antibody alone or 2A3 isotype. Different groups were injected intraperitoneally with the listed neutralizing antibodies as described in **Materials and Methods section 2.14.2**. Arrows indicate depletion antibody injection time-points. Tumour areas were measured at the indicated times, and data are mean ± SEM of six mice/group, data are from one experiment. Percent survival was determined with the endpoint being tumour area reaching ≥200 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) Test for Kaplan-Meier survival curves (ns= not significant). (**A**) Tumour size panel. (**B**) Mice survival panel.

4.2.16. CXCR3, IFN-γ and IFNAR-1 are important for the combined treatment of αCD40 antibody and ACTIV therapy

I next assessed the role of the chemokine receptor CXCR3 during CARaMEL T cells migration to tumour sites, as well as the role of IFNs in the combined therapy of αCD40 antibody and ACTIV therapy.

Byrne and Vonderheide previously reported that IFN-γ is required for T cell-dependent tumour destruction in the combined treatment of αCD40 antibody combined with chemotherapy in genetically engineered mouse models of pancreatic ductal adenocarcinoma [139]. Therefore, I determined the relative importance of IFN-γ and αIFNAR-1 (the receptor of type I IFNs in mice) in the combined treatment using αCD40 antibody and ACTIV therapy. In this experiment I treated groups of mice bearing Panc02-Her2 tumours with ACTIV therapy with or without αCD40 antibody. Different groups were intraperitoneally injected with anti-IFN-γ, or anti-IFNAR-1 (neutralizing antibodies) or control antibody on day -1, 0, 5 and then weekly (**Figure 4.23**). My data demonstrated that both IFN-γ and IFNAR1 are required for the combination of αCD40 antibody and ACTIV therapy. Mice treated with αIFN-γ or αIFNAR-1 antibodies had significantly larger tumours and shorter survival rates compared with the control group (mice receiving αCD40 antibody, ACTIV therapy and control antibody) **(Figure 4.23 A and B)**.

I also assessed the role of CXCR3 during CARaMEL T cells migration to tumour sites. As shown in **Figure 4.23 A and B,** the group of mice that received αCD40 antibody and ACTIV therapy with anti-CXCR3 antibody resulted in a reduction in anti-tumour efficacy compared with the control group as indicated by the larger tumours. In addition, the survival of the mice was shorter after CXCR3 blockade administration, suggesting that CXCR3 plays a critical role in CARaMEL T cell migration to the tumour site.

In summary, my data suggest that CXCR3 and type I/type II interferons played important roles in mediating Panc02-Her2 tumour regression in mice treated with the combination of αCD40 antibody and ACTIV therapy, as demonstrated by inhibition of responses when αCXCR3, αIFN-γ, αIFNAR-1antibodies were used. This data also supports that the anti-tumour activity of combination αCD40 and ACTIV therapy was predominantly through T-cell-dependent immune mechanisms.

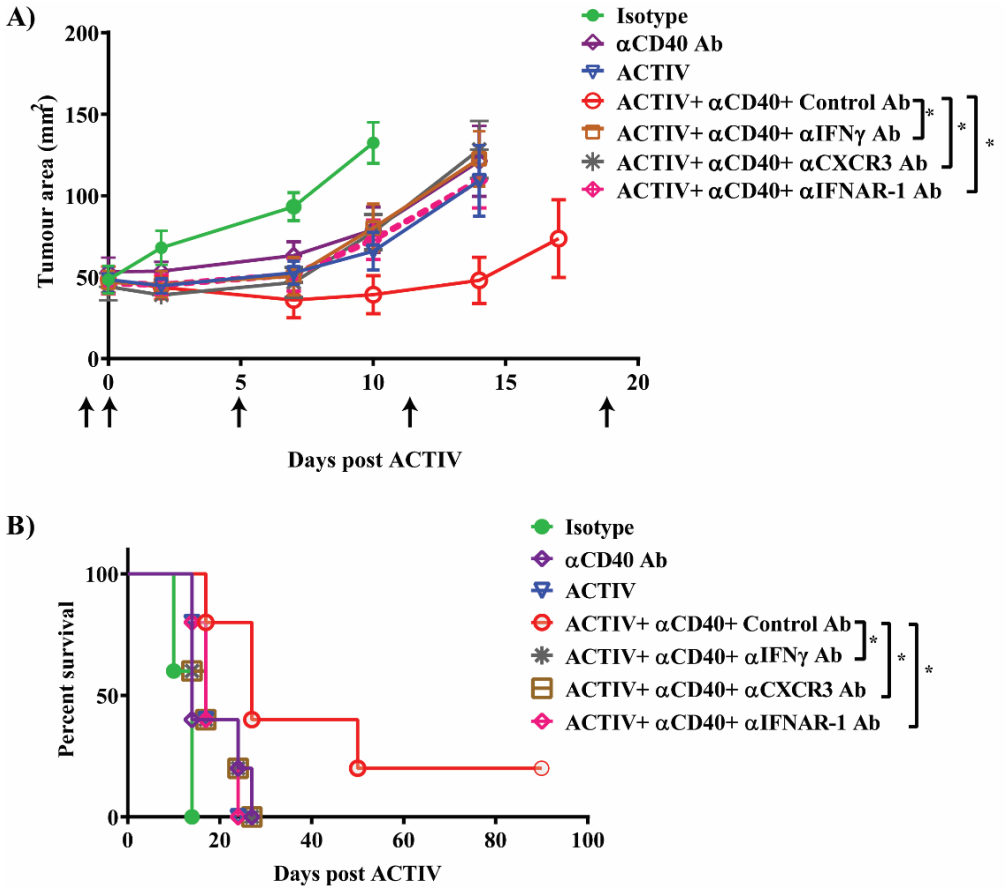


Figure 4. 23. Blockade of IFN-γ, CXCR3 and IFNAR-1 halted the enhancement of anti-tumour activity of αCD40 antibody in ACTIV therapy

Panc02-Her2 tumour-bearing mice received ACTIV therapy with or without αCD40 antibody. As controls, cohorts of mice were injected with αCD40 antibody alone or 2A3 isotype antibody. Different groups of mice were intraperitoneally injected with the listed depletion antibodies as described in **Materials and Methods section 2.14.2**. Arrows indicate depletion antibody injection time-points. Tumour areas were measured at the indicated times, and data are mean ± SEM of five mice/group, with data from one experiment. Percent survival was determined with the endpoint being tumour area reaching ≥200 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) Test for Kaplan-Meier survival curves (* $p \leq 0.05$). (**A**) Tumour size panel. (**B**) Mice survival panel.

4.2.17. CARaMEL T cell-derived IFN-γ is required for the anti-tumour activity of the combined treatment of αCD40 antibody and ACTIV therapy

Although the above studies demonstrated that IFN-γ was important for the therapeutic effects, it was not clear whether IFN-γ was derived from adoptively transferred CARaMEL T cells or from endogenous T cells as they were reconstituted following initial lymphodepleting preconditioning. For further investigation, I assessed the role of specific immune effector mechanisms of CARaMEL T cells. The role of IFN-γ and perforin was investigated using CARaMEL T cells derived from IFN-γ gene-deficient (IFN-$\gamma^{-/-}$ CARaMEL) or perforin gene-deficient ($PFP^{-/-}$ CARaMEL) mice. In this experiment I treated different groups of Panc02-Her2 bearing mice with αCD40 antibody and ACTIV therapy with either: wild type CARaMEL T cells, CARaMEL T cells from IFN-γ-deficient mice or CARaMEL T cells from perforin-deficient mice (**Figure 4.24**).

Our findings are consistent with previous studies which have revealed that anti-tumour activity of donor T cells is IFN-γ-dependent [478, 479]. The combination therapy of αCD40 antibody and ACTIV therapy was dependent on the release of IFN-γ; however, perforin played less of a role in control of tumour progression **(Figure 4.24 A and B)**.

In summary, I found that a CD40 agonist enhanced ACTIV therapy through increasing the infiltration and function of CARaMEL T cells.

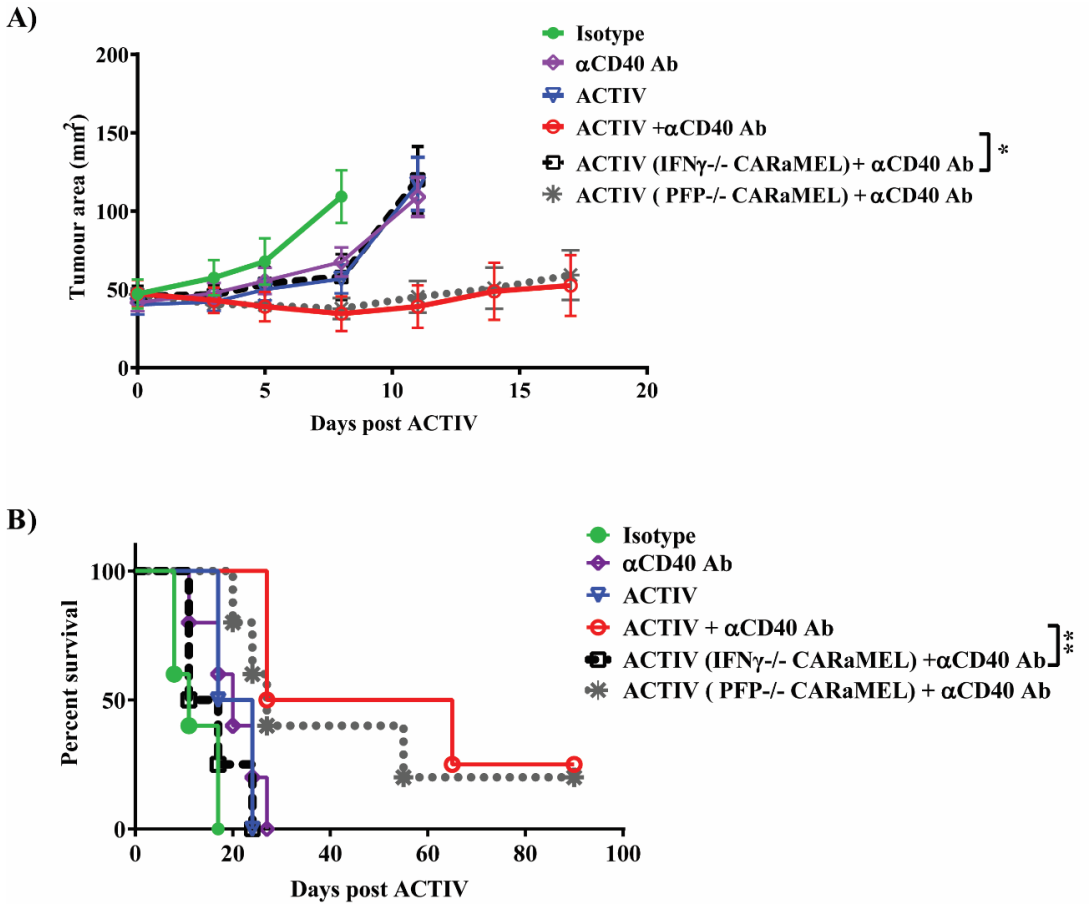


Figure 4.24. CARaMEL T cell-produced IFN-γ, but not perforin, is required for anti-tumour activity in the combined treatment using αCD40 antibody and ACTIV therapy

Panc02-Her2 tumour-bearing mice received ACTIV therapy with or without αCD40 antibody. As controls, cohorts of mice were injected with αCD40 antibody alone or 2A3 isotype. Different groups were administered with either wild type CARaMEL T cells, CARaMEL T cells from IFN-γ gene-deficient mice (IFN-$\gamma^{-/-}$ CARaMEL) or CARaMEL T cells from perforin gene-deficient mice ($PFP^{-/-}$ CARaMEL). Tumour areas were measured at the indicated times, and data are presented as the mean ± SEM of 4-5 mice/group, representative of two independent experiments. Percent survival was determined with the endpoint being tumour area reaching ≥150 mm^2 (* $p \leq 0.05$, ** $p \leq 0.01$). Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) Test for Kaplan-Meier survival curves. (**A**) Tumour size panel. (**B**) Mice survival panel.

4.3. Discussion

In this study, I describe the enhancement of an adoptive immunotherapy regimen using the HDACi, Panobinostat. The mechanism of action of HDACi involved direct cytotoxicity of cancer cells and their sensitization to CTL. *In vitro* treatment with Panobinostat significantly suppressed the growth of Panc02-Her2 cells and was associated with cell cycle arrest at the G1/S-checkpoint stage and an increase in apoptosis. Consistent with other tumour studies [174, 425, 480-484], I found that Panobinostat inhibited the viability of Panc02-Her2 cancer cells at nanomolar concentrations in a dose-dependent manner. *In vivo,* Panobinostat enhanced the efficacy of ACTIV therapy to significantly reduce tumour growth and prolong the survival of mice treated with the combined therapy.

A range of previous studies have combined HDACi to improve the efficacy of therapies such as chemotherapy, and have demonstrated an effect of HDACi, including Panobinostat, in sensitizing cancer cells to cell death [451-453]. Defective apoptotic pathways are characteristic of cancer cells that provide them with a survival advantage [485], which can provide a suitable target for therapeutic intervention [486]. HDACi have been used as anti-proliferative agents for cancer cells through increasing histone acetylation and affecting the expression of apoptotic proteins [487] to generate an intracellular pro-apoptotic program of events [175, 488-491].

The direct cytotoxic effects of Panobinostat in my study involved a G1 cell cycle arrest and induction of apoptosis in pancreatic cancer cells. HDACi have been reported to play an important role in inducing tumour cell apoptosis leading to reduction of tumour growth *in vivo* and improved the survival of tumour-bearing mice [442, 492]. Although pancreatic cancer cells are known to be resistant to apoptosis [493], several HDACi can efficiently induce apoptosis [494-497]. Panobinostat has been shown to inhibit the growth of various cancer cells and exhibit strong pro-apoptotic and anti-proliferative effects in various tumour models [4, 21, 22], including pancreatic cancer cell lines [169, 498-510].

In my study I found that Panobinostat induced cycle arrest at the G1/S-checkpoint in Panc02-Her2 cells. Consistent with my studies, Panobinostat has been shown to induce cell-cycle arrest at both G1/S and G2/M in a range of cell lines including pancreatic cancer [425, 480, 482, 500, 511].

In addition, I found that Panobinostat did not increase the immunogenicity of Panc02-Her2 tumour cells where Her2 expression was neither increased *in vitro* nor *in vivo*. HDACi have

been shown to upregulate TAA expression on other tumour cells, which can result in increased recognition and killing of tumour cells by CTL [182, 183]. However, this is not always the case, as other studies have demonstrated that HDACi can decrease TAA-expression on tumour cells. For example, Valproic acid downregulated the expression of the tumour antigen MUC-1 and reduced its recognition by MUC1-specific $CD8^+$ T cells *in vitro* [437]. Nevertheless, in my model the enhancement of ACTIV therapy mediated by Panobinostat was not simply due to an increase in Her2 expression.

Moreover, in my study I found that Panobinostat increased CARaMEL T cell-mediated suppression of Panc02-Her2 cells *in vitro* and increased the percentage of tumour-infiltrating $CD8^+$ CARaMEL T cells in Panc02-Her2 tumours. The type of HDAC inhibitor used, the activation status of $CD8^+$ T cells and timing of HDACi administration are all crucial factors in immunocombination therapy and can either enhance or inhibit $CD8^+$ T cells. Several studies have suggested that pan-HDACi can enhance $CD8^+$ T cell activation and function through a variety of mechanisms that include; 1) an increase in the cytotoxic effects, 2) enhancement of the pro-inflammatory cytokine environment and state of T cell responsiveness and 3) an increase in memory function of $CD8^+$ T cells and restoration of immune function of exhausted $CD8^+$ T cells [185-188]. For example, one study demonstrated that immunomodulation of adoptively transferred gp100 specific T cells by Panobinostat increased anti-tumour efficacy and enhanced the proliferation, retention, functional status and survival of these effector $CD8^+$ T cells [187].

In addition, I showed in my study that Panobinostat decreased IFN-γ secretion by CARaMEL T cells *in vitro* but neutralizing studies using anti-IFN-γ antibody *in vivo* revealed that IFN-γ played a crucial role in the combined Panobinostat and ACTIV therapy. However, IFN-γ was not necessary from CARaMEL T cells, since adoptively transferred IFN-$\gamma^{-/-}$ CARaMEL T cells did not negatively impact the anti-tumour activity of the combined therapy against Panc02-Her2 tumours. A potential interpretation is that IFN-γ was mainly sourced from other endogenous immune cells, for example CD4 Th1, CD8 cytotoxic T cells, NK cells, NKT or macrophages [464, 465]. Panobinostat may have epigenetically affected these immune cells resulting in an increase in IFN-γ production [466]. Further investigations would be necessary to gain insight into the source and requirements of IFN-γ.

In another aspect of my study, I found that the chemokine receptor CXCR3 had little if any role in the combined therapy of ACTIV and Panobinostat. In contrast to this finding, our

previous study of ACTIV therapy alone against other cancer types including the E0771-Her2 breast cancer tumours demonstrated that the anti-tumour activity of ACTIV therapy was dependent on CXCR3 but not IFN-γ [287]. From my study, it seems that different molecular contributors operated when combining Panobinostat with ACTIV therapy in the pancreatic cancer setting. It would be of interest, in further studies, to investigate more molecular contributors in anti-tumour activity against pancreatic tumours and to verify that the neutralising antibody used was hitting the right target.

As I discussed previously in **Chapter 3,** mice receiving ACTIV therapy for breast or colon cancers or sarcoma showed some transient toxicity. This toxicity was evident from unsteady movement, weight loss and lethargy. Toxicity was associated with CARaMEL T cell infiltration into the cerebellum [287]. This transient toxicity in mice began on the 5^{th} day following treatment and lasted for three days. Encouragingly, my study found that no further increase in toxicity was observed when combining Panobinostat with ACTIV therapy, raising the possibility of combining HDAC inhibitors with ACTIV therapy approach in a clinical setting. Panobinostat has been evaluated in many clinical trials, as either a single modality or in combination with other therapies in solid and hematologic malignancies [423, 424, 512-514]. Panobinostat demonstrated safer toxicity profiles than traditional chemotherapeutic agents [515]. Panobinostat has demonstrated favourable clinical responses, with manageable toxicity [423, 424, 516, 517]. Diarrhoea, thrombocytopenia, fatigue, nausea and vomiting have all been reported as side effects in patients or in mice receiving HDACi but these conditions are manageable [518-521]. Panobinostat was used in two clinical trials with pancreatic cancer patients: 1) NCT01056601: a single-arm, phase II study to determine the efficacy and safety of Panobinostat and bortezomib in patients with pancreatic cancer progressing on gemcitabine-based therapy. Seven patients were included in the study. The study was suspended because of the lack of funding and a lack of complete treatment responses and grade III or IV adverse events of thrombocytopenia (57%) and diarrhoea (29%). Median progression-free survival was 2.1 months [522]. 2) NCT03878524: a personalized medicine study for patients with advanced cancer of the breast, prostate, pancreas or those with refractory acute myelogenous leukaemia (SMMART), still recruiting.

Thus, it would be necessary in any future clinical trial, involving a combination of ACTIV therapy and Panobinostat to begin with dose escalations studies and carefully monitor patient progress.

In the second part of my study, I demonstrated that ACTIV therapy could also be enhanced by the co-administration of an anti-CD40 agonist antibody. CD40 is a member of the TNFR

superfamily, and it has an important role in several cell-mediated immune responses [133-135, 141, 149, 405-408]. In pancreatic cancer, CD40 agonists have been reported to mediate tumour regression through both T-cell-dependent [139] and T-cell-independent (macrophage-mediated) [132] immune mechanisms. Several studies have demonstrated that CD40 agonistic antibodies alone or combined with chemotherapy altered the tumour stroma and suppressed tumour growth in either spontaneous genetic or transplantable KPC pancreatic tumour models [132, 139, 409]. In these studies, CD40-activated macrophages inhibited tumour growth [132, 151-153] via reprogramming macrophages to an M1 (anti-tumour) status [140, 141, 154].

In my study, I found a significant reduction in percentage of macrophages in Panc02-Her2 tumours of mice receiving the combinatorial treatment of αCD40 antibody and ACTIV therapy, although I did not observe a switch in the phenotype of the infiltrating macrophages from M2 (pro-tumour) to M1 (anti-tumour), at least as classically defined. These results suggest that macrophages did not play a major role, which was supported when I used anti-F4/80 antibody to deplete macrophages, where the anti-tumour activity of αCD40 and ACTIV therapy was not affected. However further experiments would be desirable to confirm these results to confirm depletion of macrophages by anti-F4/80 antibody.

I found that combining αCD40 antibody with ACTIV therapy was associated with a massive influx in percentage of T cells into Panc02-Her2 tumours with an improvement in their capacity to produce IFN-γ, which was not observed in control-treated tumours. The CD40-CD40L interaction on T cells can stimulate immune responses [523]. CD40 agonists have been utilized to boost T cell responses to “cold” tumours, which are characterized by low immunogenicity and lack of infiltration of T cells and other immune cells. Byrne and Vonderheide showed that treating mice with αCD40 antibody resulted in a clonal effector T cell expansion and converted the TME of both spontaneous genetic and transplantable KPC pancreatic tumours to sites full of infiltrating T cells. Furthermore, the addition of gemcitabine and nab-paclitaxel to αCD40 antibody impacted antigen-loaded DCs and enhanced $CD8^+$ T cell function with increased production of TNF-α and IFN-γ. Also, αCD40 with dual chemotherapy also shifted the T cell phenotype toward a Th1 bias with almost complete collapse of the intra-tumoural T_{Reg} compartment [139].

In my model, anti-Her2 CAR expressed on CARaMEL T cells are MHC independent, however I cannot rule out the role of the endogenous T cells that reconstitute after preconditioning total body irradiation. Therefore, these endogenous T cells maybe enhanced the anti-tumour effect

against Panc02-Her2 tumours after the interaction of CD40-CD40L. My data suggest that further experiments to investigate the role of host T cells in the therapeutic effect mediated by ACTIV and anti-CD40 combined therapy are warranted.

Mice that received αCD40 antibody with ACTIV therapy showed similar manifestations of transient toxicity that were reported in my previous study of ACTIV therapy against breast, sarcoma and liver tumours but lasted longer, up to five days. Transient toxicity manifestations included unsteady movement, weight loss and lethargy due to CARaMEL T cell infiltration and reaction against $Her2^{+}$ normal tissues of the brain [287]. Multiple phase I studies of CD40 agonists revealed dose dependent and transient side effects. The most common side effect was mild to moderate cytokine release syndrome characterized by rigors, fever, and chills [132, 157, 524]. Other side effects of using αCD40 antibody in patients were reduction in monocytes, B cells, and platelets and liver injury due to the high pro-inflammatory cytokines levels including IL-6, TNF-α, and IFN-γ [525]. In mice, liver and platelet toxicity were demonstrated following increasing levels of αCD40 antibodies [265, 266]. In my study I did not investigate either of these potential toxicities; however, it would be interesting to assess cytokine levels in the serum of treated mice and evaluate platelet activation and liver function after the combined treatment. Overall, the transient nature of toxicity does not exclude the possibility of combining α CD40 antibody with ACTIV therapy in a clinical setting.

In conclusion, in this chapter I assessed the possibilities of enhancing ACTIV therapy, in a subcutaneous model of mouse pancreatic cancer, by co-administering an HDACi or αCD40 agonist. My reasoning behind these approaches was to potentially harness the T cell-dependent and –independent mechanisms of action of these two reagents to boost ACTIV therapy. My investigations revealed that the HDACi, Panobinostat significantly enhanced ACTIV therapy through a mechanism involving direct cytotoxic effects against cancer cells and their sensitization to CTL. *In vitro* treatment with Panobinostat significantly sensitized Panc02-Her2 cells to CARaMEL T cells even when low number of CARaMEL T cells were used. This effect of Panobinostat on T cells maybe an advantage in the clinic to boost the anti-tumour growth suppression of even low numbers of adoptively transferred T cells. Panobinostat with ACTIV therapy increased CARaMEL T cell infiltration into Panc02-Her2 subcutaneous tumours, reduced tumour growth and prolonged survival of mice treated with this combined therapy. In addition, *in vitro* assays showed that Panobinostat suppressed Panc02-Her2 tumour cell growth via cell cycle arrest and increased apoptosis.

In addition, an αCD40 agonist antibody enhanced ACTIV therapy through a mechanism involving a massive influx of T cells into Panc02-Her2 tumours with an improvement in IFN-γ production by these T cells. These results suggest that synergism between αCD40 antibody and ACTIV therapy was through a T-cell-dependent mechanism and not macrophage-mediated.

While treatment-associated toxicity was transient and manageable in the models used here, further studies would be necessary in other pancreatic tumour models including orthotopic and metastatic models to more closely represent the situation encountered in patients. With more safety data and further mechanistic insight that could come from future studies, it is possible that studies into adoptive cell transfer regimens incorporating epigenetic modifiers or CD40 agonists may offer insight into the design of more effective immunotherapies for pancreatic cancer.

CHAPTER 5: ACTIV Therapy of Pancreatic Cancer in a Humanized Xenograft Model in Mice

5.1. Introduction

A variety of clinical trials using CAR T cells therapy for pancreatic cancer have been initiated, largely in the USA and China. These trials are targeting various TAAs including: CEA, MSLN, MUC1, PSCA, Her2, CD133, Claudin 18.2, EpCAM and CD70 [129]. Although most of these trials are still in early stages, some studies have shown promising results. For example, in a phase I trial [253] , six chemotherapy-refractory metastatic pancreatic cancer patients were transfused with autologous mesothelin-specific CAR T cells (CARTmeso cells) three times per week for three consecutive weeks. The treatment with CARTmeso cells was well-tolerated and no dose-limiting toxicities were observed. The disease was stabilized in two patients with PFS of 3.8 and 5.4 months and one patient had total metabolic active volume decreased by 69.2%. Even though there was no detected effect on the primary tumour, however this study provided evidence for the potential anti-tumour activity of CAR-T cell therapy [129]. In another clinical trial used anti-Claudin-18.2 CAR also showed some objective responses and no obvious toxicity in patients [526]. In addition, anti-Her2-CAR T cells were used in another clinical trial to treat two pancreatic cancer patients and showed safety, with moderate responses [527]. However, not all the trials demonstrated safety. For example, a trial using CEA-CAR T cells resulted in respiratory toxicity and closure of the trial [246]. In general, clinical trials involving CAR T cell therapy targeting different pancreatic cancer TAAs have not demonstrated significant responses in patients to date [129, 245, 246]. Therefore, more efforts and other strategies are needed to expand the safety and efficacy of CAR T cell therapy against pancreatic cancer.

Various factors contributed to the limited success of these trials, which include an immunosuppressive TME that inhibits CAR T cell activity, poor CAR T cell trafficking into tumours and short T cell persistence [245, 249-255, 286]. Therefore, to eradicate solid tumours using CAR T cell approaches, my laboratory investigated the use of a vaccine to enhance the anti-tumour activity of CAR T cells by improving their persistence, expansion and infiltration into tumours.

In the previous chapters, I investigated ACTIV therapy against murine pancreatic cancer *in vitro* and *in vivo*. I was able to enhance the anti-mouse pancreatic tumour activity of ACTIV therapy using the epigenetic modifier (Panobinostat), or a CD40 agonist antibody. In the

current chapter I explored the translational potential for using ACTIV therapy by using human components. I hypothesized that human dual-specific T cells (human CARaMEL T cells) combined with an indirect vaccine would inhibit the growth of human pancreatic cancer cells *in vitro* and *in vivo*, using a tumour xenograft in immunodeficient mice. ACTIV therapy would involve adoptive cell transfer following isolation of PBMCs from cancer patients and re-engineering the T cells to express both a CAR specific for Her2 and a TCR specific for gp100. These dual-specific CARaMEL T cells would be reinfused back to the patients together with a gp100-expressing virus vaccine. However, Her2-CAR and gp100 TCR antigens are used here for modelling purposes, and other antigens would likely be used in the clinical setting. Therefore, this study represents a proof-of-concept of ACTIV therapy, in which dual-specific T cells undergo expansion mediated through their TCR using an indirect vaccine and attack tumour cells through their CAR.

In this chapter, I generated human CARaMEL T cells that express both a Her2-specific CAR and a gp100-TCR. Then I characterized human CARaMEL T cells according to CD4:CD8 composition, phenotypic maturation and immune receptor expression. I then assessed their *in vitro* functional activity, including their killing and cytokine secretion abilities. Finally, I determined their anti-tumour activity against subcutaneous human pancreatic cancer *in vivo*.

5.2. Results

5.2.1. Her2 is expressed on the human pancreatic cancer cell lines Panc1-Her2 and MiaPaCa2-Her2

In order to assess ACTIV therapy in human pancreatic cancer, I first used two pancreatic cancer cell lines: Panc1 and MiaPaCa2. Panc1 is a human pancreatic cancer cell line that was isolated from a pancreatic carcinoma of ductal cell origin from a 56-year-old female [528, 529], and the MiaPaCa2 cell line was derived from the pancreatic adenocarcinoma of a 65-year-old male [530]. In ACTIV therapy I targeted the human tumour-associated antigen, Her2, expressed on tumour cells. Using flow cytometry, I assessed the expression of Her2 on Panc1 and MiaPaCa2. As shown in **Figure 5.1**, MiaPaCa2 expressed a high level of Her2, and Panc1 have a low level of Her2 expression. In order to test my current Her2-CAR function on Her2-expressing human pancreatic cancer cells, Panc1 and MiaPaCa2 cell lines were transduced with Her2 using a retroviral vector described in **Chapter 2 section 2.5**. High expression of Her2 was demonstrated on both transduced Panc1-Her2 (**Figure 5.1 A**) and MiaPaCa2-Her2 (**Figure 5.1**

B) cell lines. These cells provided a model system to assess human CARaMEL T cell activity in human pancreatic cancer model systems *in vitro* and *in vivo*.

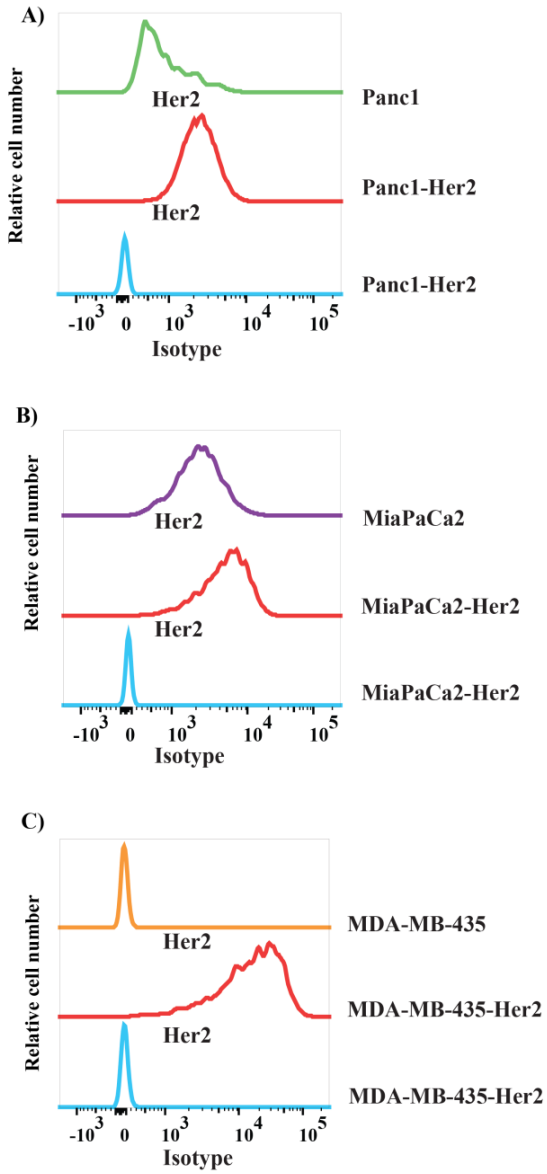


Figure 5.1. Her2 expression level on human pancreatic cancer cell lines Panc1 and MiaPaCa2

Tumour cells were stained with anti-Her2 antibody or isotype control. Flow cytometry was performed to assess the expression of Her2 on (**A)** Panc1 and (**B**) MiaPaCa2 and **(C)** MDA-MB-435-Her2 cells (positive control). Data are presented as histograms. FACS plots are gated on live cells.

5.2.2. Human CARaMEL T cells generation and phenotype

Having generated Her2 expressing human pancreatic tumour cell lines, I next generated human CARaMEL T cells expressing both an anti-Her2 CAR and a gp-100 TCR. Human PBMCs were isolated from healthy donor buffy coats or patient-derived PBMCs, using a Ficoll gradient, activated using an anti-CD3 monoclonal antibody (OKT3), and cultured in media supplemented with IL-2. These T cells were then transduced using with retroviral supernatant from two PG13 packaging cell lines producing retrovirus encoding gp-100 TCR genes (**Figure 5.2 A**) or anti-Her2 CAR genes (**Figure 5.2 B**). Detailed method is in **Chapter 2 section 2.9.**

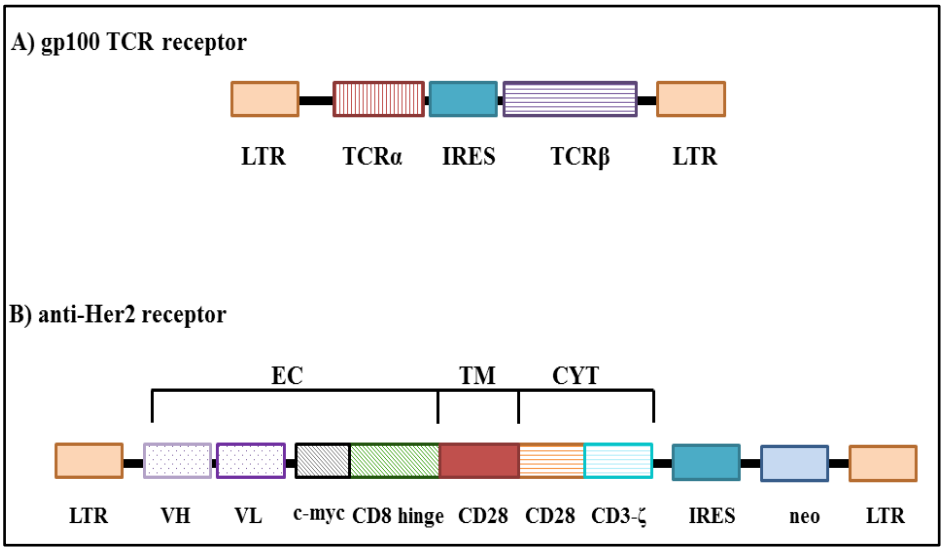


Figure 5.2. Chimeric gene constructs in retroviral vectors

(**A**) The gp100 TCR vector was composed of TCR α and TCR β separated by an internal ribosome entry site (IRES). (**B**) The anti-Her2 vector consisted of variable domains of heavy (VH) and light (VL) chains of anti-Her2 linked to a human c-Myc epitope, for use to detect CAR expression. These components were connected through a human CD8 hinge and CD28 transmembrane regions to the cytoplasmic domains of CD28 and CD3-ζ. Expression was driven by the viral long terminal repeat (LTR) promoter in both vectors. TCR: T cell receptor; IRES: Internal ribosomal entry site; LTR: Long-terminal repeat; VH: variable heavy domain of antibody; VL: variable light domain of antibody; neo: Neomycin; EC: extracellular domain; TM: transmembrane domain, CYT: cytoplasmic domain.

Cells were analysed for receptor expression using flow cytometry after 10 days in culture. Four types of T cells were produced: two monospecific T cells (expressing either anti-Her2 CAR or gp-100 TCR); a dual-transduced T-cell population (CARaMEL T cells containing both receptors: anti-Her2 CAR and gp-100 TCR) and T cells containing an empty-vector. T cells transduced with the empty-vector did not express either of anti-Her2 CAR or gp-100 TCR (**Figure 5.3 A**).

Expression of the anti-Her2 CAR and gp-100 TCR was detected on T cells following staining with anti-mouse TCRβ antibody and anti-c-Myc antibody. The representative plots from twelve different experiments of transduced T cells are shown. The expression of gp-100 TCR was ~ 80% on T cells transduced with gp-100 TCR viral supernatant (45-81%; mean: 71.4%; n = 12) (**Figure 5.3 B**). T cells transduced with viral supernatant of anti-Her2 CAR had ~ 67% expression of the anti-Her2 CAR (40-83.7%; mean: 63.9%; n = 12) (**Figure 5.3 C**). T cells transduced with both receptors had 43% of cells were dual-specific, expressing both anti-Her2 CAR and gp-100 TCR (43-70%; mean: 54.83%; n = 12) (**Figure 5.3 D**).

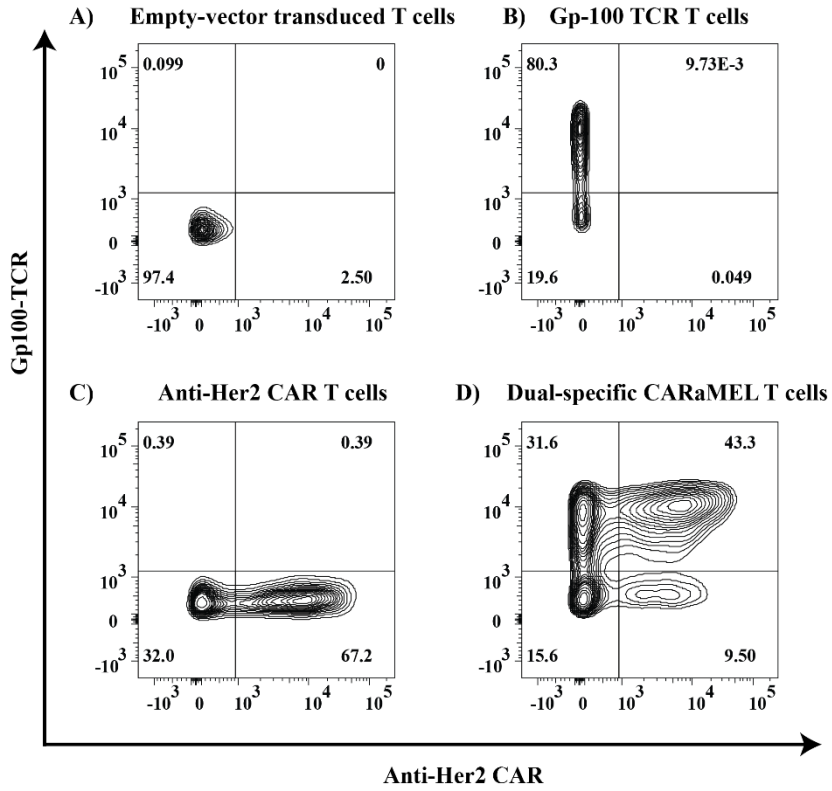


Figure 5.3. Human dual-specific T cells express gp100 TCR and anti-Her2 CAR

PBMCs were activated using anti-human CD3 antibody (clone: OKT3), then genetically modified with (**A**) negative control: empty-vector (**B**) gp100 TCR (**C**) anti-Her2 CAR and (**D**) dual-specific gp100 TCR and anti-Her2 CAR ('CARaMEL' T cells). Cells were stained with antibodies specific for mouse TCRβ and anti-c-Myc with a secondary PE-conjugated antibody. The cells were analysed by flow cytometer. FACS plots are gated on live cells. Data here are representative of results from one of 12 experiments.

In order to produce high numbers of pure mono- or dual-transduced T-cell populations expressing anti-Her2 CAR and/or gp-100 TCR, I sorted T cells on high expression of one or both receptors and expanded them using a single round of a rapid expansion protocol (REP) [531]. REP involved using OKT3, IL-2 in the presence of irradiated (50 Gy) allogeneic feeder cells (PBMCs) from 2-3 donors at a 200:1 ratio of PBMCs feeders to transduced T cells of healthy donors and one cancer patient. Sorting and rapid expansion resulted in pure population (>98%) mono- or dual-specific T cells expressing gp-100 TCR and/or anti-Her2 CAR (96-99 %; mean: 98%; n = 4) (**Figure 5.4 A**). Typically, this expansion of T cells from healthy donors or the cancer patient produced similar yields approximately 1×10^9 cells of each population. In addition, CAR and TCR expression on T cells persisted more than 60 days after sorting and expansion (**Figure 5.4 B**).

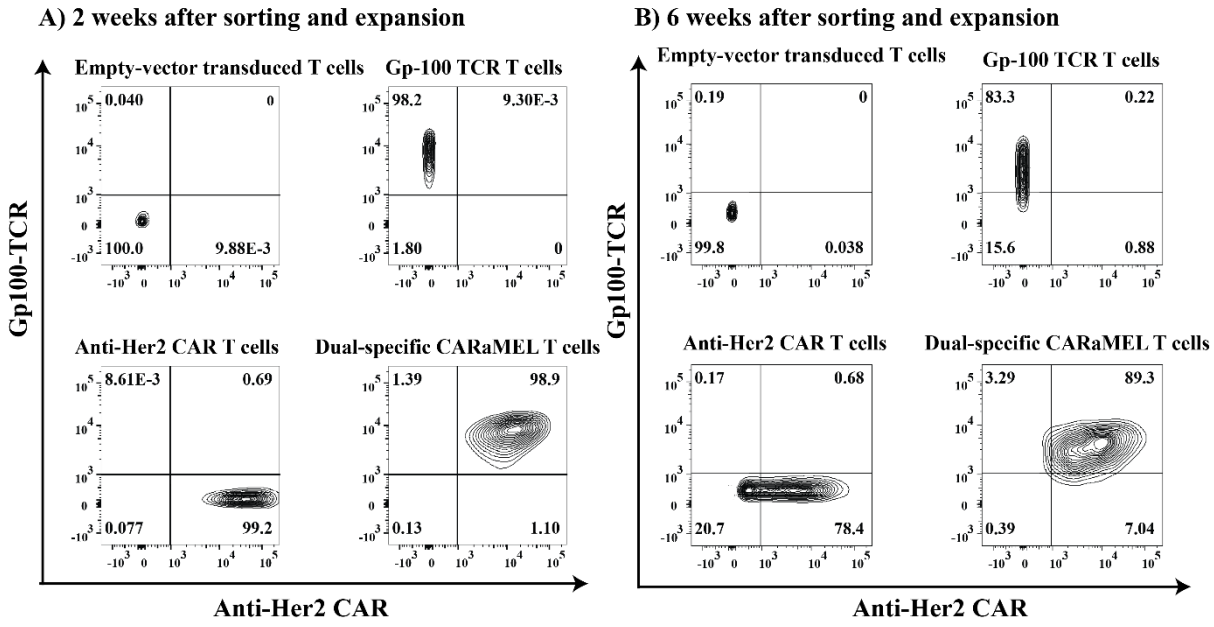


Figure 5.4. Sorting and rapid expansion resulted in pure populations of mono- or dual-specific T cells expressing gp-100 TCR and/or anti-Her2 CAR

PBMCs of healthy donors or patients were activated using OKT3, then T cells were transduced with anti-Her2 CAR and gp100 TCR and cultured for 10 days, followed by sorting for anti-Her2 CAR^+ and/or gp100-TCR^+. T cells were then expanded in culture using a rapid expansion protocol (REP). Flow cytometric analysis was performed to determine the expression of anti-Her2 CAR and/or gp100 TCR after (**A**) two weeks and (**B**) six weeks of expansion after sorting. Cells were stained with antibody specific for mouse β TCR and anti-c-Myc with a secondary PE-conjugated antibody. Empty-vector transduced T cells were used as negative control. FACS plots are gated on live cells. Data here represent results from one experiment of 12 experiments.

To determine if the different genes and culture conditions affected T cell phenotype, I determined the CD4:CD8 composition of the different populations using FACs analysis. The percentage of $CD4^+$ and $CD8^+$ T cells was determined among live cells before and during the process of activation, transduction and REP of T cells (between days 0 and 31). Representative plots of T cells from buffy coats from different individual donors or patient-derived PBMCs are shown in **Figure 5.5 A**. Of the total population of PBMCs on day 0, approximately 25–65% (mean= 44.16) of T cells were $CD4^+$, while 5-35% (mean= 27.5) of T cells were $CD8^+$. On day 10, approximately 18–30% (mean= 23.83) of T cells were $CD4^+$ and 62-77% (mean= 66.58) of T cells were $CD8^+$. This represented a significant enrichment in $CD8^+$ T cells compared to the starting population. After sorting and expansion 16–28% (mean= 20.41) of T cells were $CD4^+$ and 60-78% (mean= 67.66) of T cells were $CD8^+$ (**Figure 5.5 B and C**). These frequencies were not significantly different for cultures irrespective of the gene(s) used for transduction. The negative controls: OKT3 stimulated non-transduced T cells (NT) and the empty-vector transduced T cells showed similar CD4:CD8 ratios as CARaMEL T cells at the specified time points (**Figure 5.5 B and C**). This indicated that the initial use of soluble anti-CD3 OKT3 and expansion of T cells enriched for $CD8^+$ T cells [532] was not subsequently affected by CAR or TCR transduction (**Figure 5.5 B and C**).

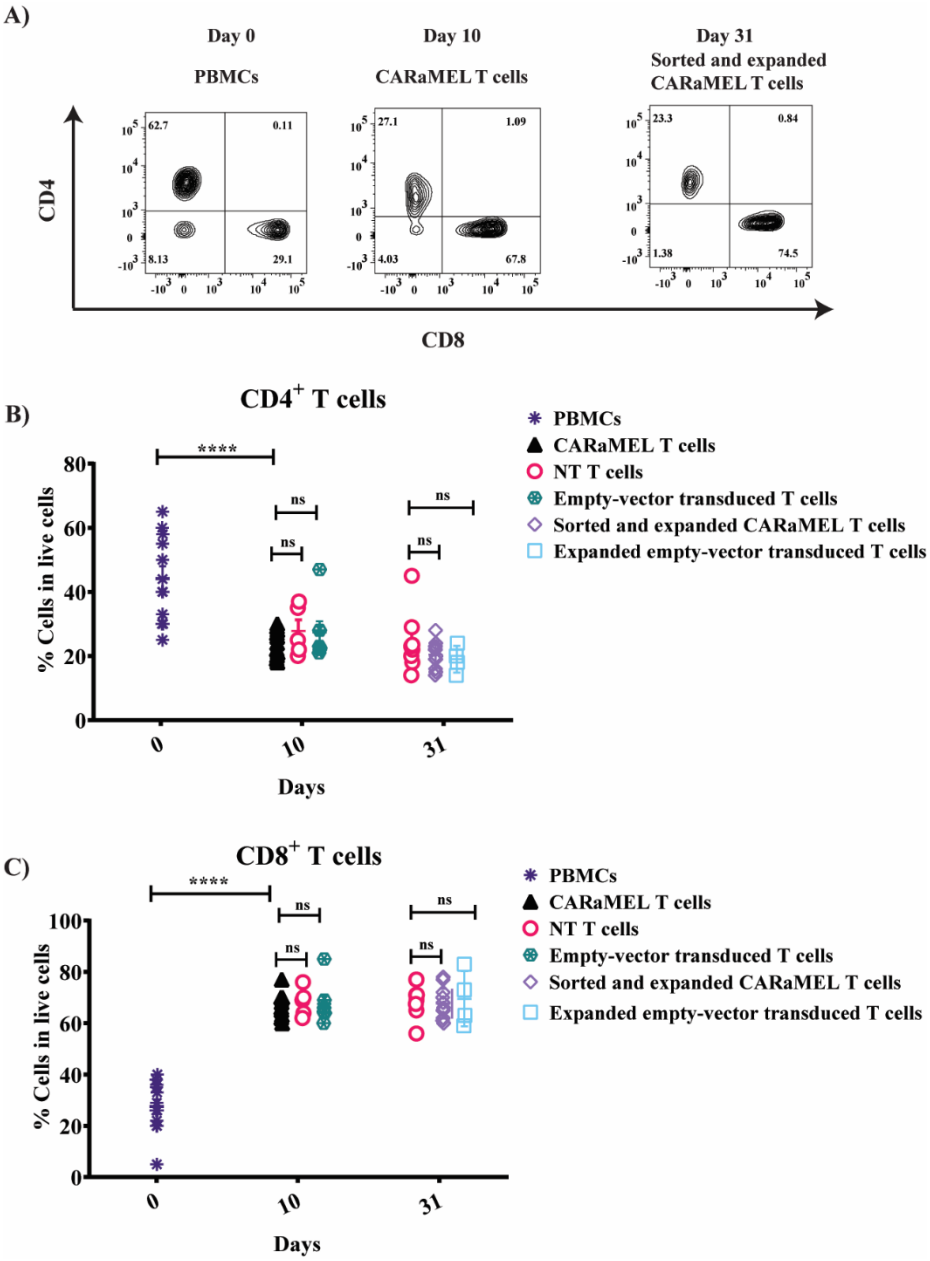


Figure 5.5. The percentage of CD4$^+$ and CD8$^+$ T cells among human CARaMEL T cells population during the process of activation, transduction and REP of T cells

PBMCs of healthy donors or patients were activated using OKT3, then T cells were transduced with anti-Her2 CAR and gp100 TCR and cultured for 10 days, followed by sorting for anti-Her2 CAR$^+$ and gp100-TCR$^+$ and expanded in culture for a further 21 days. Flow cytometric analysis was performed to determine the percentage of CD4$^+$ and CD8$^+$ T cells before activation (day 0) and during the process of activation, transduction and REP (days 10 and 31). (**A**) Representative plots of T cells of a donor showing the percentage of CD8$^+$ and CD4$^+$ T cells PBMCs on day 0, human CARaMEL T cells on day 10, sorted and expanded human CARaMEL T cells on day 31. (**B**) Scatter plot showing the percentage of CD4$^+$ cells in T cell cultures at the indicated time points. (**C**) Scatter plot showing the percentage of CD8$^+$ cells in T cell cultures at the indicated time points. Data are presented as mean ± SEM of 12 different donors or patients' samples. Statistical analyses were performed using the Student *t*-test (ns= not significant, **** $p \leq 0.0001$).

Various previous studies have indicated that ACT of T cell populations containing more naïve ($CD45RA^+$, $CCR7^+$), and central memory ($CD45RA^-$, $CCR7^+$) T cell subsets have better persistence and anti-tumour activity compared with more-differentiated effector memory ($CD45RA^-$, $CCR7^-$) and terminally differentiated effector cells ($CD45RA^+$, $CCR7^-$) [533-535]. Therefore, I next investigated the phenotypic differentiation characteristics of the transduced T cells using flow cytometry. The representative plots of T cells from one donor are shown in **Figure 5.6 A**. Approximately half of T cells in PBMCs were naïve before activation -day 0- (**Figure 5.6 B**). After 10 days of T cell activation, with soluble anti-CD3 antibody and IL-2, more effector memory T and terminally differentiated cells effector memory T cells developed. After expansion and re-stimulation of T cells with soluble anti-CD3; a greater frequency of effector memory cells developed with less expression of CCR7 and CD45RA (**Figure 5.6 B**). The negative control (empty-vector transduced T cells) had a similar differentiation status as CARaMEL T cells on day 10 and day 31, which indicated that this shift of differentiation status was due the use of soluble anti-CD3 and IL-2, instead of CAR or TCR gene transduction (**Figure 5.6 C**).

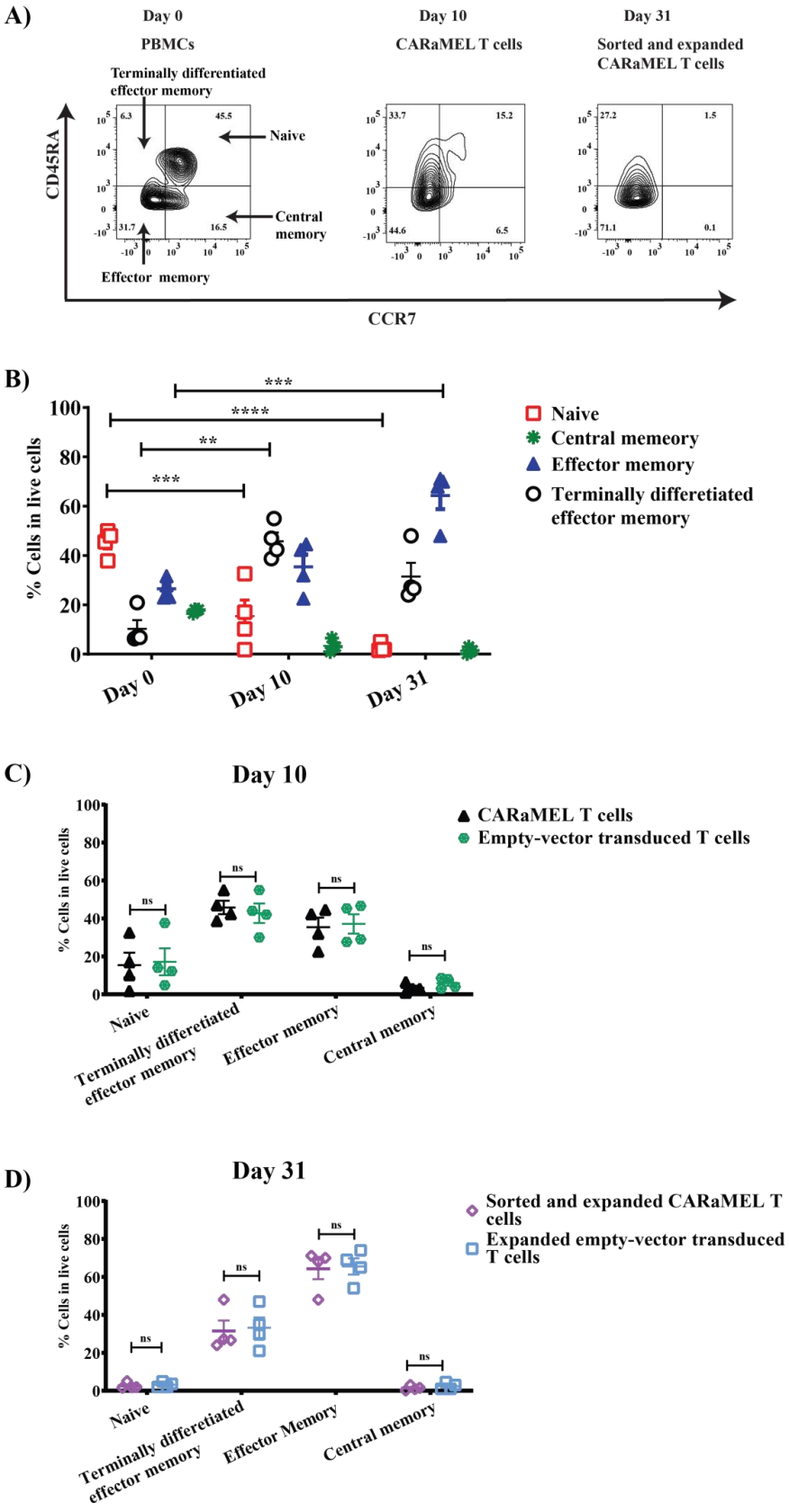


Figure 5.6. Human CARaMEL T cells were mainly effector cells

PBMCs of healthy donors or patients were activated using OKT3, then T cells were transduced with anti-Her2 CAR and gp100 TCR and cultured for 10 days, followed by sorting for anti-

Her2 CAR^+ and gp100-TCR^+ and expansion in culture for a further 21 days. Flow cytometric analysis was performed to determine the differentiation status based on CD45RA and CCR7 labelling: naïve ($CD45RA^+$, $CCR7^+$), central memory ($CD45RA^-$, $CCR7^+$), effector memory ($CD45RA^-$, $CCR7^-$) and terminally differentiated effectors ($CD45RA^+$, $CCR7^-$) on PBMCs (day 0), CARaMEL T cells (day 10), and sorted and expanded CARaMEL T cells (day 31). (**A**) Representative plots of T cells of a donor showing the differentiation status of T cell cultures at the indicated time points. (**B**) Scatter plot showing the percentage of each differentiation status in T cell cultures at the indicated time points (**C**) Scatter plot comparing the percentage of each differentiation state of CARaMEL T cells and empty-vector transduced T cells on day 10 and 31. Data are presented as mean ± SEM of four different donors or patient-derived PBMCs. Statistical analyses were performed using the Student *t*-test (ns= not significant, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Various studies have indicated that ligand binding to inhibitory receptors on immune cells is accompanied by down-regulation of the T-cell–mediated immune response and reduction of T cell killing ability. These receptors, referred to as immune checkpoints, include programmed cell death 1 (PD-1) lymphocyte activating-3 (LAG-3), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and others [536-541]. Therefore, it was important to investigate the expression levels of inhibitory receptors (PD-1, TIM-3 and LAG-3) on T cells from three healthy donors and one cancer patient using flow cytometry following staining with specific monoclonal antibodies (**Figure 5.7**). The representative plots of T cells from one donor are shown in **Figure 5.7A**. After 10 days of OKT3 stimulation, PD-1 was up-regulated on T cells and lasted for more than 30 days. LAG-3 and TIM-3 were also up-regulated on surface of T cells after OKT3 stimulation (**Figure 5.7 B**). The negative control, empty-vector transduced T cells, showed similar inhibitory receptor expression compared to CARaMEL T cells on Days 10 and 31. This indicated that this increase in the level of inhibitory receptors on T cells was not due to CAR or TCR gene transduction, but likely was a result of T cell activation (**Figure 5.7 C**).

In summary, human dual-specific CARaMEL T cells were successfully generated from donor or patient-derived PBMCs samples expressing anti-Her2 CAR and gp-100 TCR receptors. Human CARaMEL T cells were sorted and expanded on high expression of both receptors using a single round of REP. Stimulation with OKT3 resulted in similar phenotypic analysis of human CARaMEL T cells from different donors or patient-derived PBMCs: largely $CD8^+$ effector ($CD45RA^-$, $CCR7^-$) T cells with up-regulation in some inhibitory receptors including PD-1, LAG-3 and TIM-3, which lasted for more than 30 days.

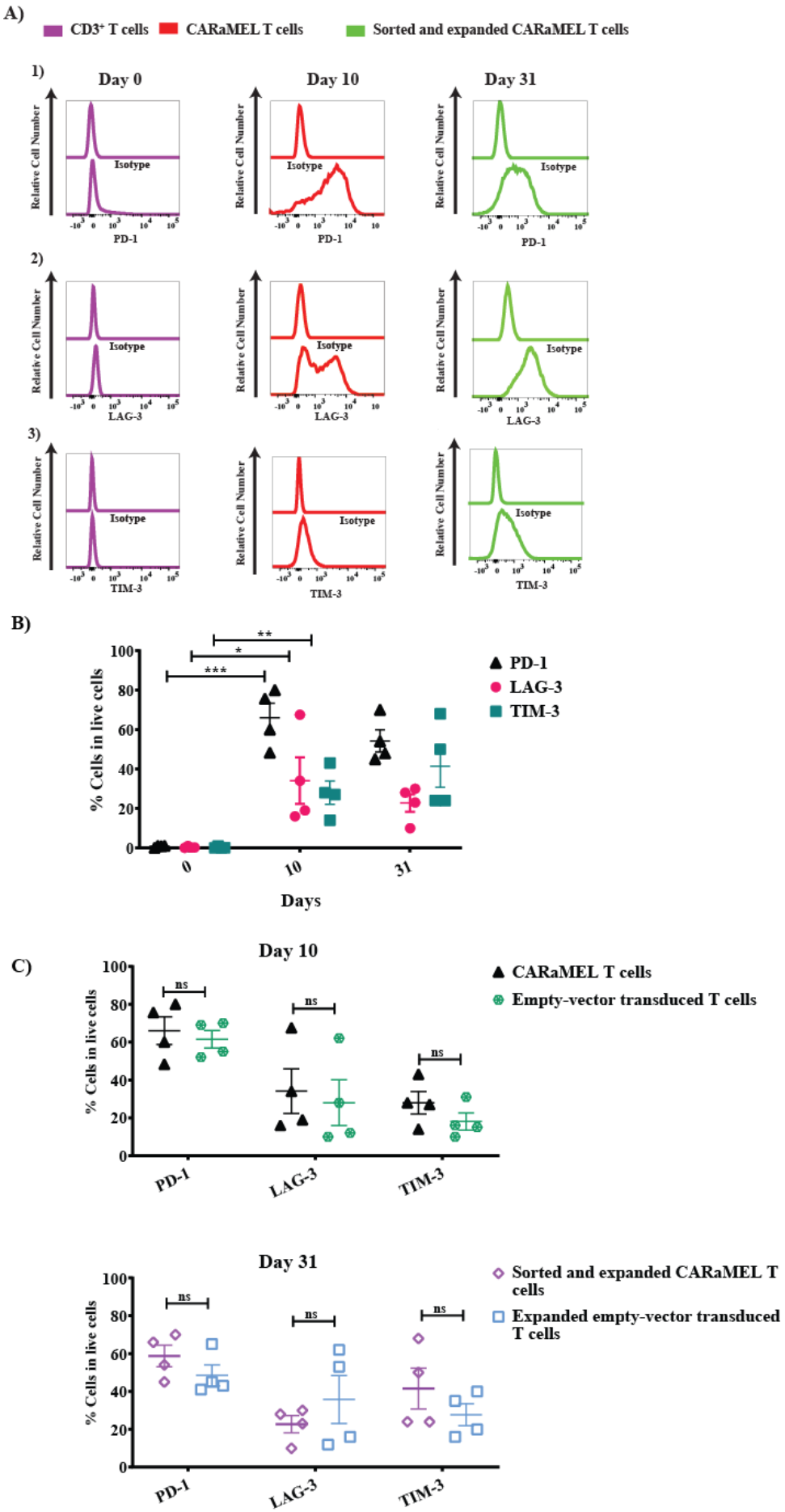

A)
CD3+ T cells
CARaMEL T cells
Sorted and expanded CARaMEL T cells
1)
Day 0
Day 10
Day 31
Relative Cell Number
Isotype
PD-1
2)
LAG-3
3)
TIM-3
B)
% Cells in live cells
PD-1
LAG-3
TIM-3
Days
C)
Day 10
CARaMEL T cells
Empty-vector transduced T cells
ns
Day 31
Sorted and expanded CARaMEL T cells
Expanded empty-vector transduced T cells

Figure 5.7. Human CARaMEL T cells express immune inhibitory receptors PD-1, LAG-3 and Tim-3

Three PBMCs of healthy donors and one cancer patient were activated using OKT3 and then transduced with anti-Her2 CAR and gp100 TCR and cultured for 10 days, followed by sorting for anti-Her2 CAR^+ and gp100-TCR^+ and expanded in culture for a further 21 days. Flow cytometric analysis was performed to determine the percentage of some immune checkpoint receptors (PD-1, LAG-3 and TIM-3) expressed on T cells before activation (day 0) and during the process of activation, transduction and REP (days 10 and 31). (**A**) Representative plots of T cells of a donor showing expression of PD-1, LAG-3 and TIM-3 on: PBMCs on day 0, human CARaMEL T cells on day 10 and sorted and expanded human CARaMEL T cells on day 31. (**B**) Scatter plot showing the percentage of PD-1, LAG-3 and TIM-3 on T cell cultures at the indicated time points (**C**) Scatter plot comparing the percentage of PD-1, LAG-3 and TIM-3 expression on CARaMEL T cells and empty-vector transduced T cells on day 10 and 31. FACS plots are gated on live cells. Data are presented as mean ± SEM of four different donors or patient-derived PBMCs. Statistical analyses were performed using the Student *t*-test (ns= not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

5.2.3. Dual-specific human CARaMEL T cells respond to both Her2 and gp100

An important measure of T-cell function is cytokine secretion, which can directly affect target cells and recruit other immune cell types [542]. Therefore, after I generated and phenotyped human CARaMEL T cells, I next evaluated the functionality of the human CARaMEL T cells for antigen-specific reactivity against human pancreatic cancer cells. The ability of the human CARaMEL T cells to secrete IFN-γ in response to anti-Her2 CAR and gp-100 TCR ligation was determined using ELISA after overnight incubation with the pancreatic tumour cell lines, MiapaCa2-Her2 and Panc1-Her2, with or without gp100 $_{(154\text{-}162)}$ peptide pulsing (**Figure 3.8**). Melanoma cell lines: MDA-MB-435 and MDA-MB-435-Her2 were used as Her2 negative and positive control cells respectively.

Human CARaMEL T cells secreted IFN-γ specifically in response to target cells expressing Her2 antigen, but not in response to Her2-negative target cells (**Figure 5.8 A**). In addition, the ability of CARaMEL T cells to respond against gp100 through their TCR was demonstrated when CARaMEL T cells were incubated with Panc1-Her2 (which is HLA-A2 A*02:01 positive cell line [543]) pulsed with gp100 $_{(154\text{-}162)}$ peptide (**Figure 5.8 A**). Control cells anti-Her2 CAR T cells secreted IFN-γ only in the presence of $Her2^+$ target cells, and control gp-100 TCR T cells secreted IFN-γ only in the presence of gp100 peptide, in agreement to expression of the responding receptor (**Figure 5.8 A**). In addition, the empty-vector transduced T cells, which lack both CAR and TCR receptors, did not secrete IFN-γ either in the presence of $Her2^+$ target cells or gp100 peptide (**Figure 5.8 A**).

The above results characterized the function of early (Day 10) T cell cultures that were composed of a mixture of transduced and non-transduced cells. To extend the investigation to later enriched populations with sufficient numbers for use in subsequent *in vivo* application, I compared IFN-γ secretion from the bulk population and pure (sorted and amplified) CARaMEL T cells. The pure population of sorted and rapidly expanded CARaMEL T cells was shown to have higher cytokine secretion against target cells compared with the bulk population, since a greater proportion of cells expressed the relevant antigen receptors (**Figure 5.8 B)**.

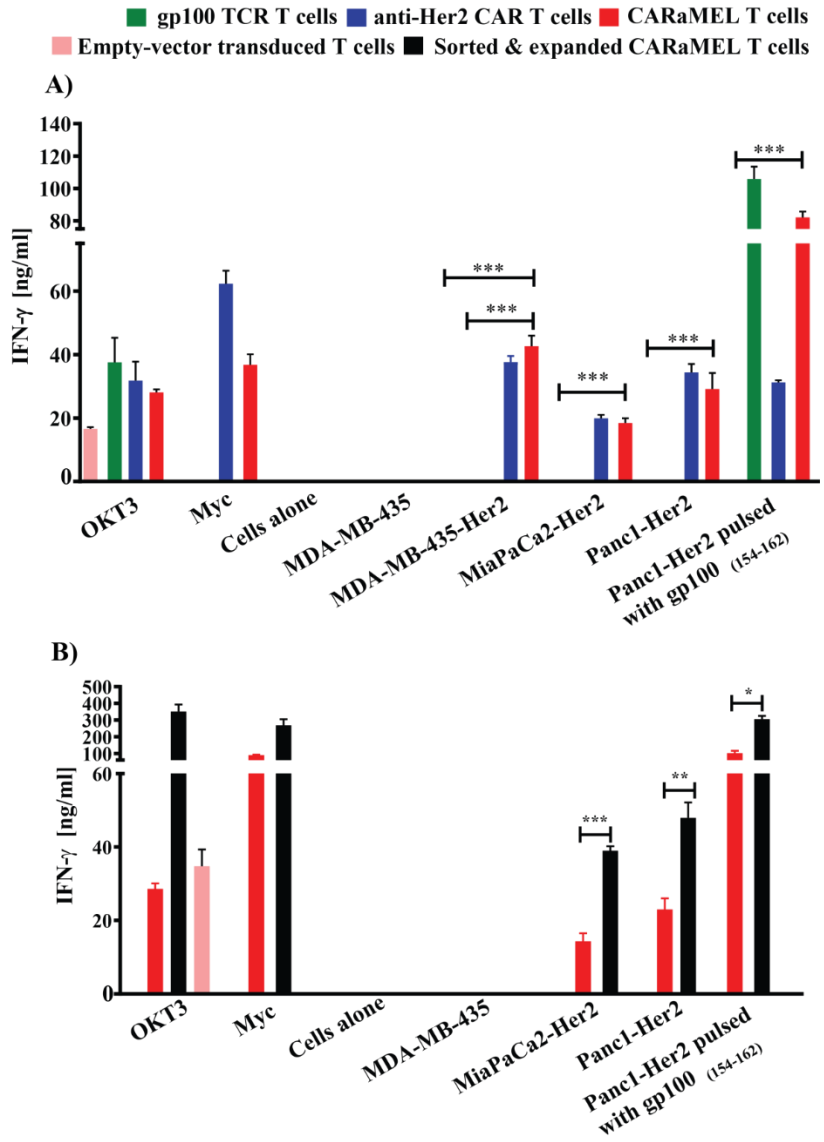


Figure 5.8. Human CARaMEL T cells secrete IFN-γ in response to Her2 and gp100 peptide

The ability of the genetically modified T cells to secrete IFN-γ in response to CAR and TCR ligation was determined by ELISA after overnight incubation with tumour cells. IFN-γ secretion was assessed in (**A**) (2×10^5 cells) empty-vector transduced T cells, gp100 TCR T cells, anti-Her2 CAR T cells or CARaMEL T cells (**B**) sorted and expanded CARaMEL T cells. Cells were co-cultured overnight at 37ºC with (1x 10^5 cells) Panc1-Her or MiapaCa2-Her2. Also, T cells were co-cultured with Panc1-Her2 cells pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide. Controls included untreated as negative control, or αCD3/αCD28-stimulated as a positive TCR control, or Myc-stimulated as a positive CAR control cells. IFN-γ levels in the culture supernatants were analysed by ELISA. Data are presented as mean ± SEM of triplicate samples; data here represent results from three independent experiments. Statistical analyses were performed using the Student *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$).

5.2.4. CARaMEL T cells recognize and specifically kill Her2$^+$ cancer cells

After confirming human CARaMEL T cells being able to secrete high levels of IFN-γ, I next confirmed that these cells have specific cytotoxicity against human pancreatic cancer cells. I co-cultured human CARaMEL T cells overnight with the ^{51}Cr labelled MiapaCa2-Her2 and Panc1-Her2 tumour cells lines, with or without gp100 $_{(154\text{-}162)}$ peptide pulsing, at various effector: target ratios. I used the melanoma cell lines MDA-MB-435 and MDA-MB-435-Her2 as Her2 negative and positive control cells respectively. Human CARaMEL T cells were able to lyse human pancreatic cancer cells (**Figure 5.9**). The specificity of the interaction was apparent from the absence of lysis of Her2$^+$ tumour cells by empty-vector transduced T cells and the lack of lysis of Her2 negative tumour cells by human CARaMEL T cells (**Figure 5.9 A**). CARaMEL T cells specifically and efficiently lysed the Her2 positive cell lines (**Figure 5.9 B and C**). The anti-Her2 CAR and gp-100 TCR mono-transduced T cells killed targeted cells only in the presence of Her2$^+$ target cells or gp100 peptide respectively, demonstrating the specificity of each corresponding receptors (**Figure 5.9 B-D**). The enriched population of sorted and rapidly expanded human CARaMEL T cells were more efficient than the bulk population at lysing Her2$^+$ pancreatic cancer cells and cells expressing gp-100 peptide (**Figure 5.9 E-G**).

In summary, human CARaMEL T cells showed that they were capable to secrete high levels of IFN-γ and kill human pancreatic cancer *in vitro* specifically and efficiently in the presence of Her2$^+$ target cells or gp100 antigen; other functional assays as proliferation assay were performed later in this Chapter for further assessment of human CARaMEL T cells.

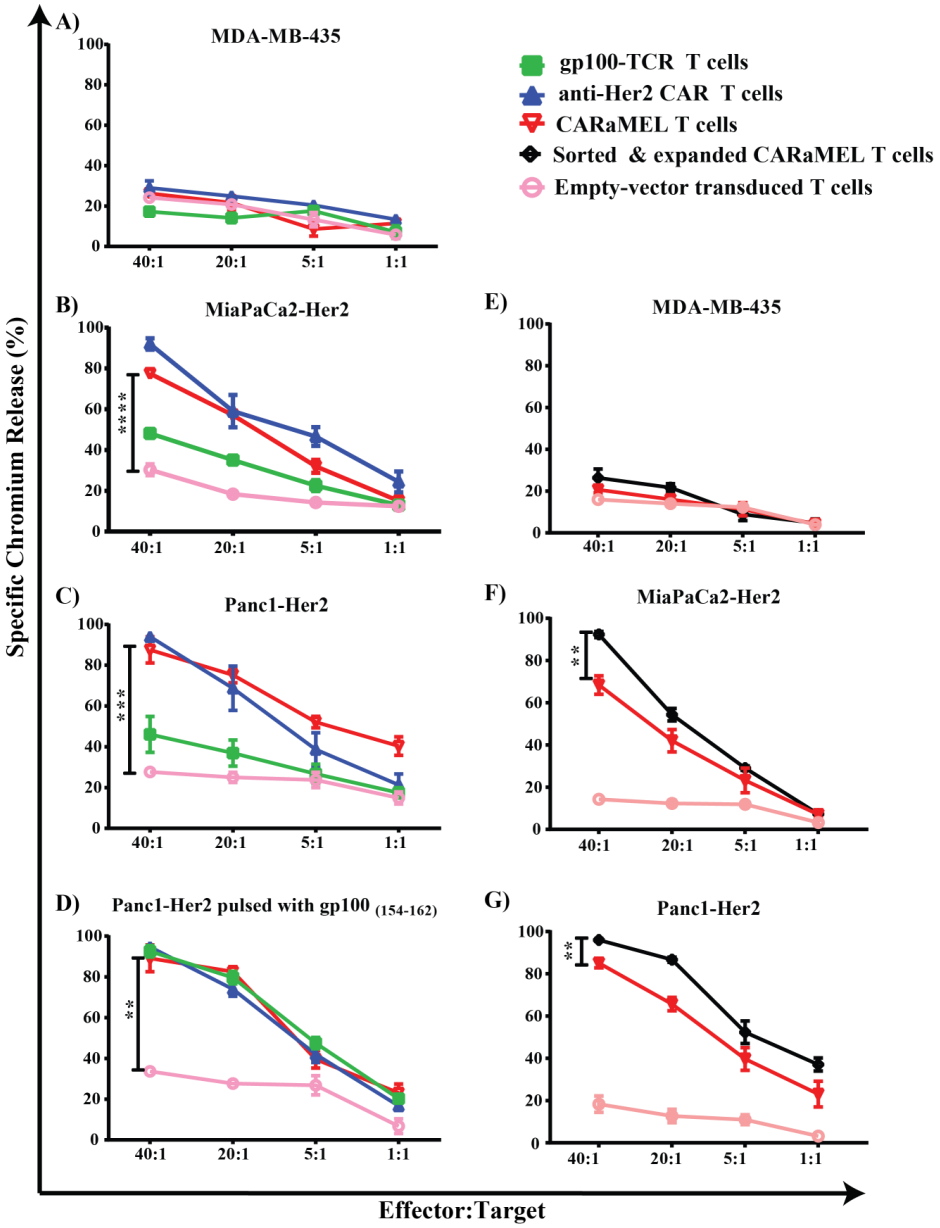


Figure 5.9. Human CARaMEL T cells specifically lyse Her2^{+} cancer cells

The antigen-specific cytolytic potential of CARaMEL T cells was determined *in vitro* using a ^{51}Cr release assay using target cells expressing Her2 or gp100 peptide, or both. (**A**) MDA-MB-435 (**B**) MiaPaCa2-Her2 (**C**) Panc1-Her2 (**D**) Panc1-Her2 cells pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide (**E**) MDA-MB-435 (**F**) MiaPaCa2-Her2 (**G**) Panc1-Her2 were co-cultured with either empty-vector transduced T cells, gp100 TCR T cells, anti-Her2 CAR T cells or CARaMEL T cells or sorted and expanded CARaMEL T cells at the indicated effector: target ratios. Following co-culture overnight, radioactivity in supernatants was measured using a gamma counter to determine percentage specific tumour lysis. Data are presented as mean ± SEM of triplicate samples. Data are representative of three independent experiments. Statistical analyses were performed using the Student *t*-test (** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.2.5. Human CARaMEL T cells against human pancreatic tumours in vivo

The principle behind using dual-specific CAR T cells to enhance adoptive immunotherapy was to use a powerful vaccine to boost T cell numbers and activity through a TCR of known specificity. In my mouse systems in **Chapter 3** and **4**, I used a vaccinia virus vaccine expressing the gp100 epitope relevant to mouse H2-D^b [348], In order to assess the *in vivo* anti-tumour activity of human CARaMEL T cells against subcutaneous $Her2^+$ human pancreatic tumours, it was necessary to use immunodeficient mice, and I could therefore not use an aggressive viral vaccine, such as vaccinia. Instead, I used three different vaccine strategies: (1) the administration of HLA-$A*02^+$ human DCs pulsed with $gp100_{(154-162)}$ peptide (which binds to human HLA-A2*02) administered to NOD-SCID (NSG) mice (**Figure 5.10 A**). (2) Murine DCs, isolated from HLA-$A*02^+$ transgenic mice, pulsed with $gp100_{(154-162)}$ peptide and used in NSG mice (**Figure 5.10 B**). (3) A highly attenuated adenoviral vaccine encoding full-length human gp100, Ad2CMV-gp100, in HLA-A2 transgenic NSG mice NSG-HLA-A2 mice (**Figure 5.10 C**).

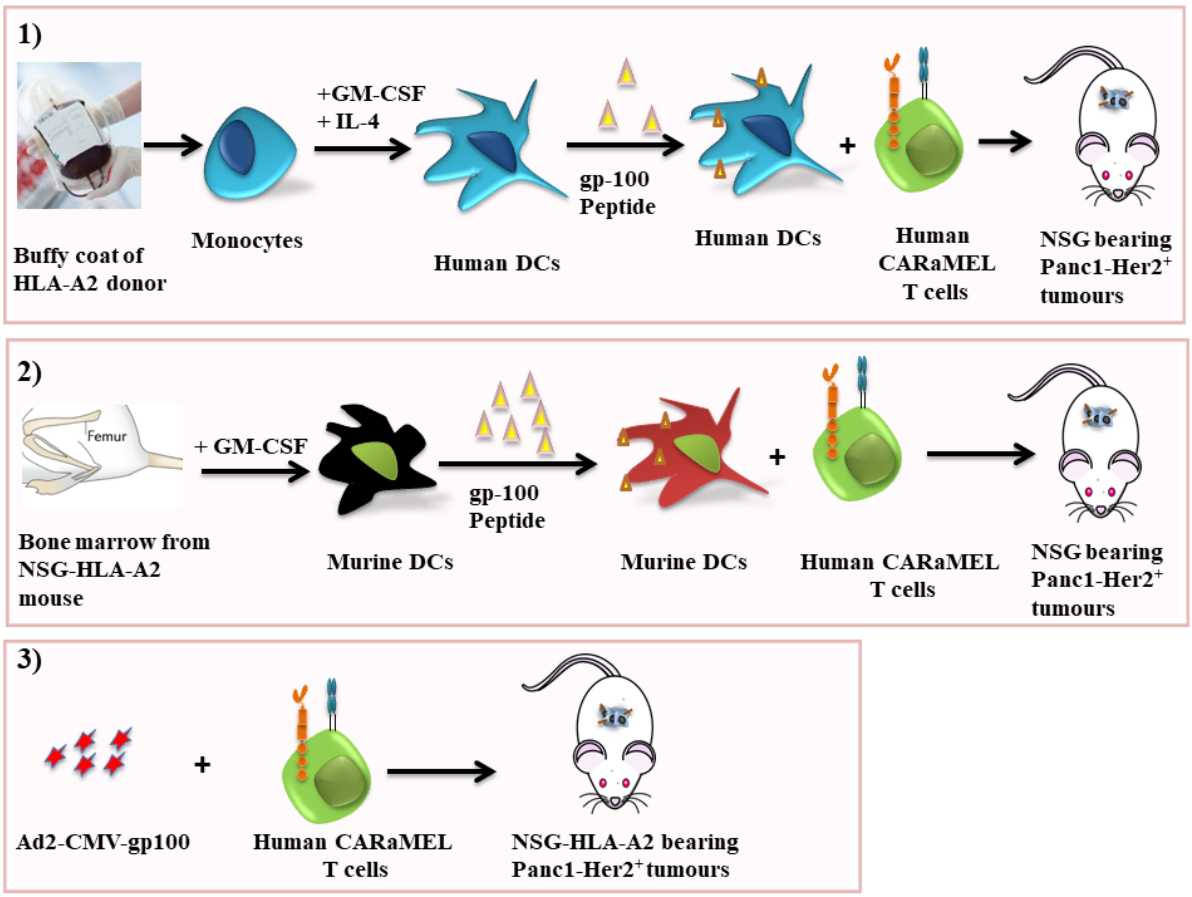


Figure 5.10. Schematic of assessing the *in vivo* anti-tumour activity of human CARaMEL T cells against subcutaneous Her2^{+} human pancreatic tumours

The *in vivo* anti-tumour activity of human CARaMEL T cells against subcutaneous Panc1-Her2 human pancreatic tumours was assessed through three different strategies: 1) administration of human CARaMEL T cells with HLA-A*02^{+} human DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide in NSG mice bearing Panc1-Her2 tumours, 2) administration of human CARaMEL T cells with HLA-A*02^{+} murine DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide in NSG mice bearing Panc1-Her2 tumours and 3) administration of human CARaMEL T cells with Ad2CMV-gp100 in NSG-HLA-A2 mice bearing Panc1-Her2 tumours.

*5.2.5.1. Phenotypic and functional characterization of human CARaMEL T cells and HLA-A*02^{+} human DCs*

Our first vaccine strategy involved HLA-A*02^{+} human DCs with pulsed with gp100 $_{(154\text{-}162)}$ peptide.

In this strategy I examined the expression of HLA-A*02 on PBMCs from different donors using flow cytometry after staining the cells with an anti-HLA-A*02 antibody (**Figure 5.11 A**). Also, I assessed the ability of these HLA-A*02^{+} PBMCs to present gp100 $_{(154\text{-}162)}$ peptide to human CARaMEL T cells and induce their response to the antigen by secretion of IFN-γ (**Figure 5.11 B)**. Human CARaMEL T cells secreted IFN-γ specifically in response to gp100 $_{(154\text{-}162)}$ peptide pulsed HLA-A*02^{+} donors (donors 466, 694 and 900) but not in response non-pulsed cells or the HLA-A*02^{-} donor (donor 473) (**Figure 5.11 A and B**). This experiment confirmed the TCR-mediated specificity of human CARaMEL T cells and the ability of HLA-A*02^{+} cells to present the gp-100 peptide. However, it should be noted that this experiment lacked a control condition involving peptide-pulsed PBMCs alone (in the absence of CARaMEL T cells). Therefore, although the frequency of endogenous T cells specific for gp100 in PBMCs is likely very low or absent, their possible contributions to IFN-γ secretion cannot be categorically ruled out in this experiment.

After I confirmed HLA-A*02 expression on PBMCs of specific donors and their abilities to present the gp100 peptide, I next generated monocyte-derived human DCs using culture of PBMCs in the presence of GM-SCF and IL-4 for five days. To get the highest proliferation and cytokine secretion capacity of T cells I also aimed to use mature DCs [544]. DC maturation was accomplished by addition of TNF-α in the culture media for another one day as described in the **Material and Method section 2.12**. On day 7, flow cytometry was performed to immunophenotype the different cell types and the expression of DC differentiation markers. Cell cultures exhibited evidence of DCs generation from monocytes manifested by downregulation of CD14 and upregulation of the surface molecules which are involved in antigen presentation and molecules that act as co-stimulators in T-cell activation such as HLA-DR, CD1a, CD80, CD86 and CD40 (**Figure 5.12**). Also, DCs exhibited indicators of maturation, manifested by upregulation of the surface molecule CD83 (**Figure 5.12**) [545, 546].

Having demonstrated that DCs could be generated from PBMCs, I next assessed the ability of these HLA-A*02^{+} human DCs to present gp100 $_{(154\text{-}162)}$ peptide to human CARaMEL T cells.

I co-cultured DCs with T cells overnight and performed ELISA to determine IFN-γ secretion by CARaMEL T cells (**Figure 5.13**). Human CARaMEL T cells secreted IFN-γ specifically in response to gp100 $_{(154\text{-}162)}$ peptide-pulsed HLA-A*02^{+} DCs but not in response to non-pulsed DCs. Specificity was confirmed since empty-vector transduced T cells that lack the CAR and TCR receptors did not secrete IFN-γ in in response to gp100$_{(154\text{-}162)}$ peptide-pulsed HLA-A*02^{+} DCs (**Figure 5.13**).

Since antigen-induced expansion of T cells is desirable in CAR T therapy against tumours, I studied the ability of the human CARaMEL T cells to proliferate in response to gp100 peptide and Her2 antigen. Proliferation of human CARaMEL T cells was detected by performing a ^{3}H-thymidine incorporation assay (**Figure 5.14).** Sorted and expanded human CARaMEL or empty-vector transduced T cells were co-cultured with Her2^{-} and Her^{+} cancer cells or with human HLA-A*02 DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide. The highest induced proliferation of human CARaMEL T cells was through gp100 TCR rather than anti-Her2 CAR. Compared with human CARaMEL T cells, empty-vector transduced T cells failed to proliferate in response to either Her2 or gp100 peptide (**Figure 5.14**). This result is consistent with my previous finding in murine CARaMEL T cells (**Figure 3.6**) and supports my hypothesis that ACTIV therapy would enable expansion of CARaMEL T cells and their activation through the gp100-TCR.

Having generated Her2^{+} pancreatic cancer cells and human CARaMEL T cells and confirmed their killing, cytokine secretion and proliferation abilities in response to Her2 antigen and gp100; I proceeded to *in vivo* to evaluate the anti-tumour activity and the efficacy of treating Panc1-Her2 tumour bearing mice with human CARaMEL T cells and gp100 $_{(154\text{-}162)}$ peptide-pulsed HLA-A*02^{+} DCs. In this experiment: I injected the Panc1-Her2 cancer cells subcutaneously in (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice or NSG (for NOD SCID Gamma) (**Material and Methods section 2.1**).

Then I allowed the tumours to grow for nine days to reach 20-45 mm^{2} size. On day 10 following tumour injection, mice received the following: sub-lethal total body irradiation preconditioning (2.5 Gy), intravenous injections of (2×10^{7}) human CARaMEL T cells and (8×10^{6}) HLA-A*02^{+} human DCs pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide. Mice also received four intraperitoneal injections of IL-2, one injection on the same day of treatment and then every 12 hours. On day 12, treatment was repeated but without total body irradiation. As controls, cohorts of mice were injected with human CARaMEL T cells without DCs, and another group

was left untreated (**Figure 5.15**). Tumour growth inhibition was significantly greater in mice that received T cells and gp100 $_{(154\text{-}162)}$ peptide-pulsed human DCs compared with mice received human CARaMEL T cells alone ($p= 0.0455$) or NT group ($p=0.0011$), leading to long-term survival (**Figure 5.15**).

In summary, I was able to generate HLA-A*02^{+} mature DCs from HLA-A*02^{+} healthy donors, and these DCs were able to present gp100 peptide to human CARaMEL T cells, which specifically secreted IFN-γ and proliferated against this antigen. Also, I demonstrated that the human CARaMEL T cells had a potent anti-tumour effect *in vivo* against Panc1-Her2 tumours when combined with gp100 $_{(154\text{-}162)}$ peptide-pulsed HLA-A*02^{+} DCs. In the next section, I aimed to determine the efficacy of this approach using HLA-A*02+ murine DCs generated from NSG-HLA-A2 mice.

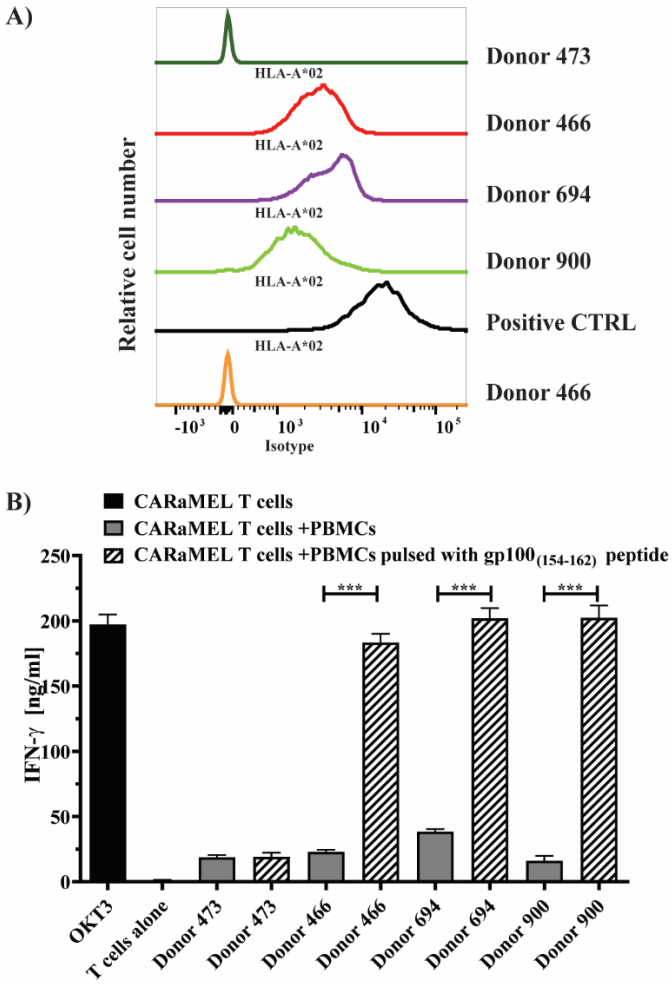


Figure 5.11. IFN-γ secretion is restricted to HLA-A*02$^+$ PBMCs donors pulsed with gp100 (154-162) peptide

(**A**) Flow cytometry was performed to assess the expression of HLA-A*02 on PBMCs of different donors. Cells were stained with anti-HLA-A*02 antibody or matched isotype control. Data are presented as histograms. FACS plots are gated on live cells. (**B**) The ability of HLA-A*02$^+$ PBMCs to present gp100 (154-162) peptide to CARaMEL T cells was investigated by IFN-γ-secretion determined by ELISA. Sorted and expanded CARaMEL T cells (1×10^5 cells) were co-cultured overnight at 37ºC with PBMCs (3×10^5 cells) from different donors pulsed with (1 µM) gp100 (154-162) peptide for one hour at room temperature and washed three times with PBS. Controls included untreated as a negative control, or αCD3/αCD28-stimulated cells as a positive control. Data are representative of two independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (*** $p \leq 0.001$).

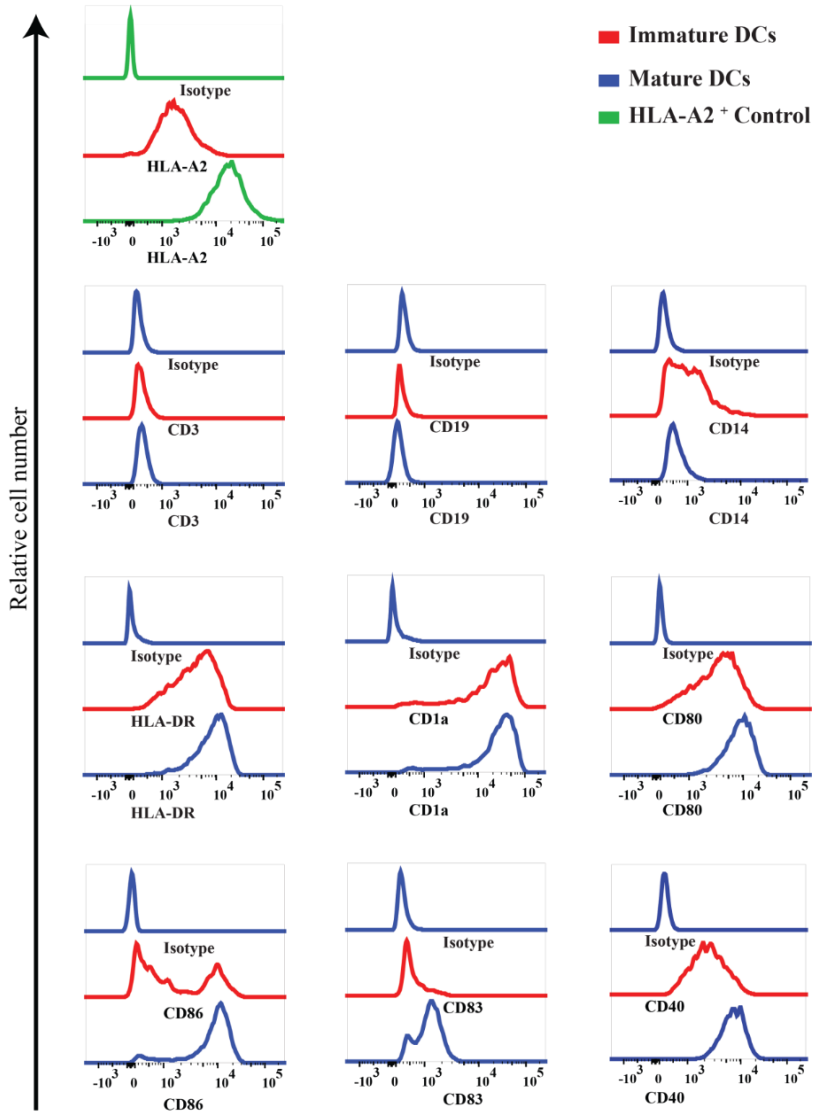


Figure 5.12. Immuno-phenotypic analyses of DCs generated from HLA-A*02 donors

Immature and mature human DCs were generated as described in material and methods. On day 7, flow cytometry was performed to determine the expression of various DC differentiation markers. Cells were stained using antibodies specific for HLA-A2, CD3, CD19, CD14, HLA-DR, CD1a, CD80, CD86, CD83 and CD40 or isotype-matched control antibodies. FACS plots are gated on live cells. Panc1-Her2 cells were used as a positive control for HLA-A2 staining. Data are representative of five independent experiments.

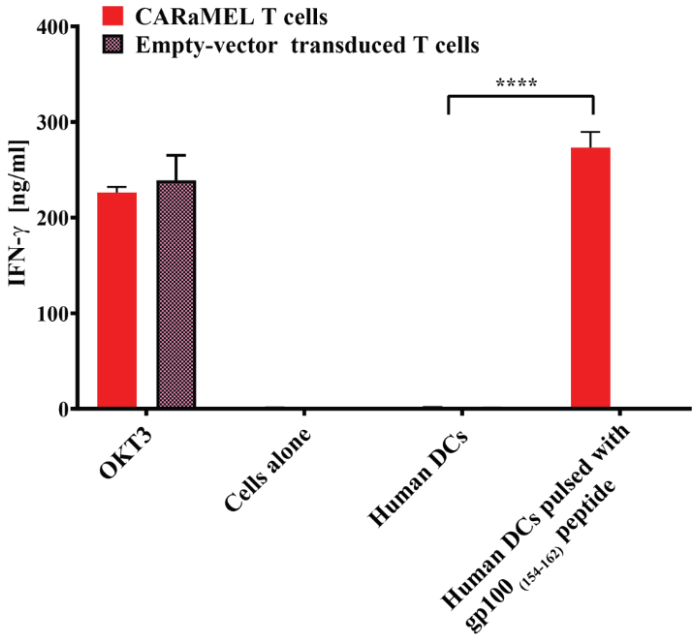


Figure 5.13. Human CARaMEL T cells secrete IFN-γ in response to HLA-A*02^{+} DCs pulsed with gp100 (154-162) peptide

The ability of HLA-A*02^{+} DCs to present gp100 (154-162) peptide to CARaMEL T cells was revealed by IFN-γ-secretion determined by ELISA. Sorted and expanded CARaMEL T cells were co-cultured overnight at 37ºC with DCs from an HLA-A*02^{+} donor pulsed with (1 µM) gp100 (154-162) peptide. Controls included non-pulsed as a negative control, or αCD3/αCD28-stimulated cells as a positive control. Data are representative of three independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (**** $p \leq 0.0001$).

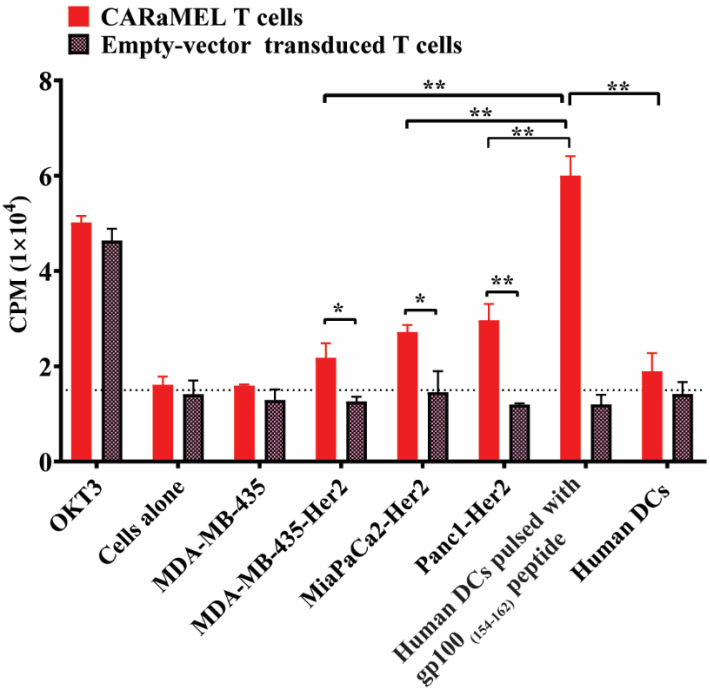


Figure 5.14. Human CARaMEL T cells demonstrate increased proliferation through their gp100-TCR

The ability of CARaMEL T cells to proliferate in response to HLA-A*02^{+} DCs presenting gp100 $_{(154\text{-}162)}$ peptide was revealed by a [^{3}H] thymidine incorporation assay. Mean proliferation ([^{3}H] thymidine incorporation) is shown for CARaMEL or empty-vector transduced T cells. T cells were co-cultured with various irradiated cancer cell lines or irradiated human DCs pulsed with or without (1 µM) gp100 $_{(154\text{-}162)}$ peptide for one hour at room temperature. Controls included T cells alone as negative control or αCD3/αCD28-stimulated cells as positive control. Data are expressed as the mean count per minute (CPM) of triplicate cultures ± SEM; data here represent results from two independent experiments. Statistical analyses were performed using the Student *t* -test (ns=not significant, * $p \leq 0.05$, ** $p \leq 0.01$).

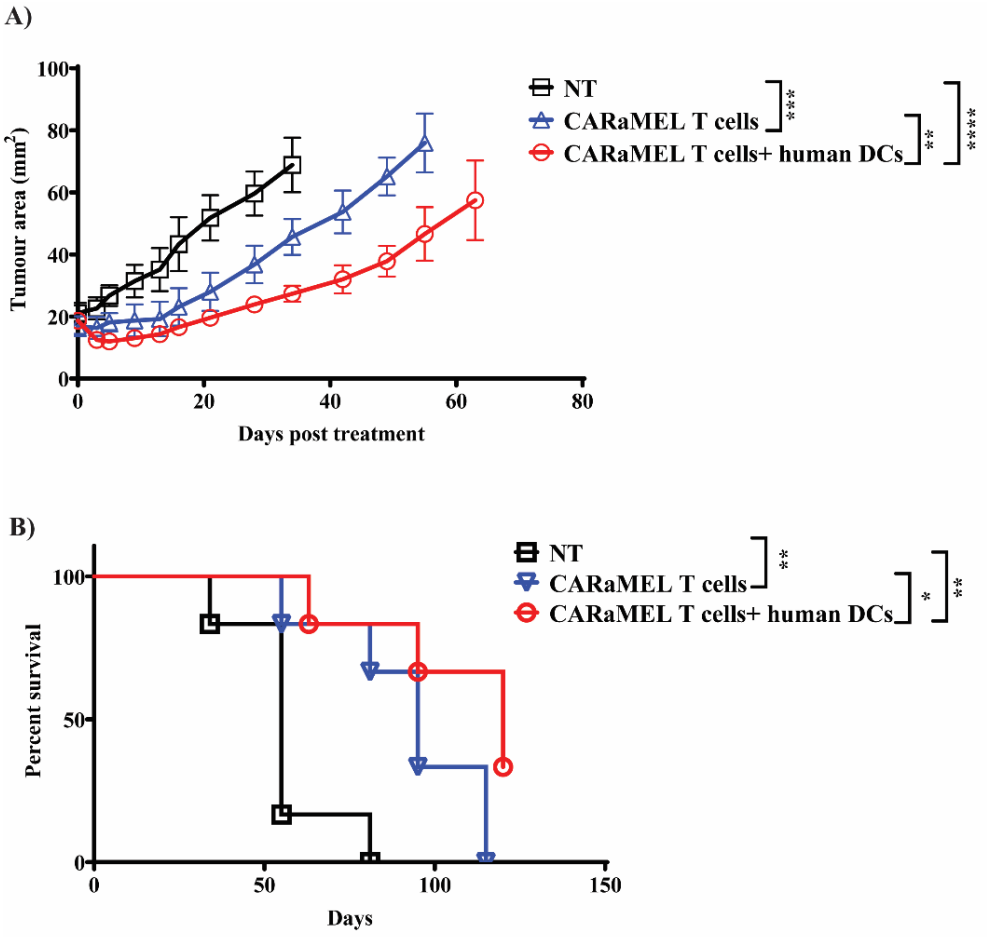


Figure 5.15. Human CARaMEL T cells together with gp100 (154-162) peptide-pulsed HLA-A*02^{+} human DCs inhibited Panc1-Her2 subcutaneous tumour growth in NSG mice and prolonged their survival

Panc1-Her2 cancer cells were injected subcutaneously into NSG mice. On day 10 following tumour injections, mice received total body radiation, intravenous injections of human CARaMEL T cells and HLA-A*02^{+} human DCs pulsed with gp100 (154-162) peptide. Mice also received intraperitoneal injection of four doses of IL-2 over the next two days. On day 12 treatment was repeated but without total body irradiation. As controls, cohorts of mice were injected with human CARaMEL T cells without DCs and another group was left non-treated (NT). (**A**) Tumour area was measured at the indicated times, and data are presented as the mean ± SEM of 5-6 mice/group, for a single experiment. (**B**) Percent survival was determined with the endpoint being tumour area reaching ≥100 mm^2. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (* $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

*5.2.5.2. A phenotypic and functional characterization of human CARaMEL T cells and HLA-A*02^{+} murine DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide against human pancreatic cancer cells*

In my second vaccine strategy I investigated the use of murine HLA-A*2^{+} DCs isolated from NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg (HLA-A/H2-D/B2M) 1Dvs/SzJ mice (abbreviated here to NSG-HLA-A2) mice. These NSG-HLA-A2 mutant mice are immunodeficient, being deficient in T cells, B cells and NK cells, and express human HLA -A2 [259]. I used the bone marrow of NSG-HLA-A2 mice to generate HLA-A*2^{+} DCs using murine GM-SCF for six days as described in the **Material and Method section 2.13**. As GM-CSF is known to induce the expansion of various myeloid lineages, I performed flow cytometry to assess expression of DC differentiation markers (**Figure 5.16**). On day 7, flow cytometry was performed to immunophenotype the different cell types and the expression of DC differentiation markers. Cells exhibited evidence of DCs generation from bone marrow and the cells were CD11c^{+}, F4/80^{+}, CD11b^{+}, CD80^{+}, CD86^{+}, CD40^{low} DC8α^{-} which is consistent with previous descriptions of immature DCs generated from murine bone marrow [547-549].

Furthermore I evaluated the ability of HLA-A*02^{+} murine DCs to present gp100 $_{(154\text{-}162)}$ peptide to human CARaMEL T cells and the ability of the T cells to respond to the antigen and secrete IFN-γ following overnight co-culture (**Figure 5.17)**. Human CARaMEL T cells secreted IFN-γ specifically in response to gp100 $_{(154\text{-}162)}$ peptide pulsed HLA-A*02^{+} but not in response to non-pulsed cells DCs. These results indicate the specificity of human CARaMEL T cells and the ability of HLA-A*02^{+} DCs derived from NSG-HLA-A2 mice to present the peptide (**Figure 5.17)**.

In addition, I studied the ability of CARaMEL to proliferate in the presence of gp-100 peptide using a ^{3}H thymidine incorporation assay (**Figure 5.18).** Sorted and expanded human CARaMEL or empty-vector transduced T cells were co-cultured with or without gp100 $_{(154\text{-}162)}$ peptide-pulsed murine HLA-A2^{+} murine DCs. gp100 TCR triggering resulted in robust T-cell proliferation. These results demonstrated that murine DCs generated from NSG-HLA-A2 mice can efficiently present gp100 peptide to induce cytokine secretion and T-cell proliferation. This result is consistent with my previous finding in human DCs and CARaMEL T cells (**Figures 5.13 and 5.14**).

Having determined that HLA-A2^{+} murine DCs can efficiently present gp100 peptide to human CARaMEL T cells; I progressed to *in vivo* studies to assess the efficacy of human CARaMEL

T cells with HLA-A*02^{+} murine DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide against a human pancreatic cancer xenograft model in NSG mice. In this experiment I injected Panc1-Her2 cancer cells subcutaneously in NSG mice and allowed the tumours to grow for nine days to reach 20-45 mm^{2} size. On day 10 following tumour injection, mice received the following: sub-lethal total body irradiation, intravenous injections of human CARaMEL T cells and HLA-A*02^{+} murine DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide and IL-2. On day 12, treatment was repeated without total body irradiation. As controls, cohorts of mice were injected with human CARaMEL T cells without DCs and another group was left non-treated (NT) (**Figure 5.19**). Administration of human CARaMEL T cells with gp100 $_{(154\text{-}162)}$ peptide pulsed HLA-A*02^{+} murine DCs into mice bearing Panc02-Her2 tumours produced significant tumour growth inhibition compared with mice that received human CARaMEL T cells alone or the non-treated group (**Figure 5.19 A**). Furthermore, mice treated with human CARaMEL T cells and gp100 $_{(154\text{-}162)}$ peptide-pulsed HLA-A*02^{+} murine DCs survived significantly longer than all other groups (**Figure 5.19 B**). This result is consistent with my previous finding in the previous section using human HLA-A*2^{+} DCs and human CARaMEL T cells (**Figure 5.15**), which confirms my overall hypothesis.

A central reason for investigating the use of HLA-A2^{+} murine DCs as a vaccine was the ready availability of large numbers of HLA-A2^{+} DCs that could be generated from NSG-HLA-A2 mice. In contrast, it was difficult to identify consistent sources of human HLA-A2^{+} DCs. Thus, it was not my primary aim to compare human and murine DCs, but rather to confirm my findings from human DCs by using plentiful murine DCs expressing the vaccine restriction element, HLA-A2.

In summary, I was able to generate HLA-A*02^{+} mature DCs from NSG-HLA-A2 mutant mice and these murine DCs were able to present gp100 peptide to human CARaMEL T cells which specifically secreted IFN-γ and proliferated against gp100. Moreover, I demonstrated that human CARaMEL T cells had a potent anti-tumour effect *in vivo* against Panc1-Her2 tumours once combined with gp100 $_{(154\text{-}162)}$ peptide pulsed HLA-A*02^{+} murine DCs. The next step of this project was to apply the concept of ACTIV therapy in Panc1-Her2 tumour bearing mice and treat them with human CARaMEL T cells and a vaccine.

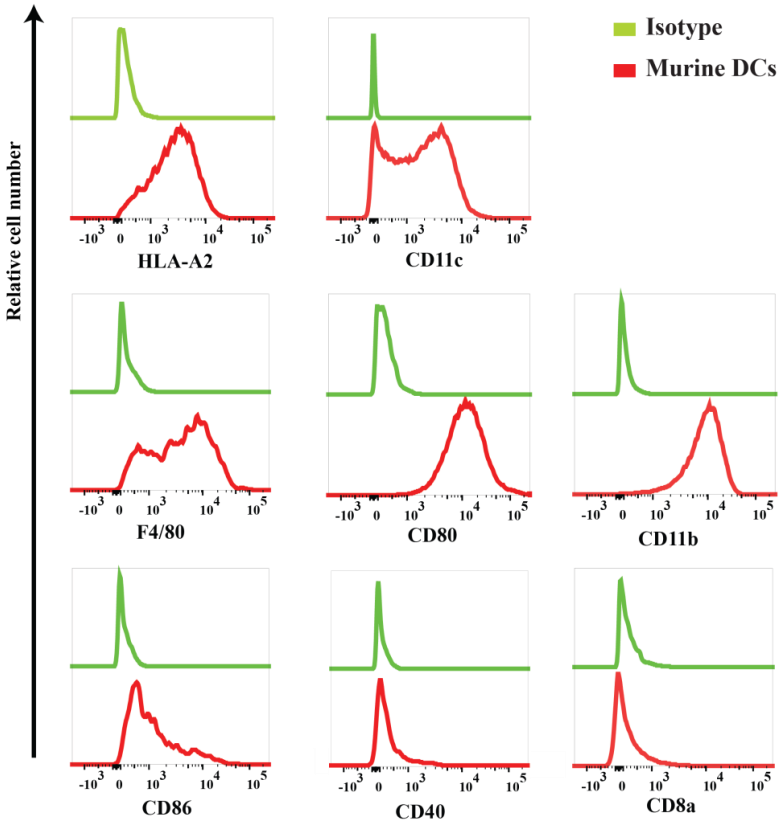


Figure 5.16. Immuno-phenotypic analyses of murine DCs generated from NSG-HLA-A2 mice

Murine DCs were generated as described in **Material and Methods section 2.13**. Flow cytometry was performed to determine the expression of DC differentiation markers. Murine DCs were stained using antibodies specific for HLA-A2, CD11b, CD11c, CD80, F4/80, CD40 and CD8a or isotype-matched control antibodies. Data are presented as histograms. FACS plots are gated on live cells. Data are representative of six independent experiments.

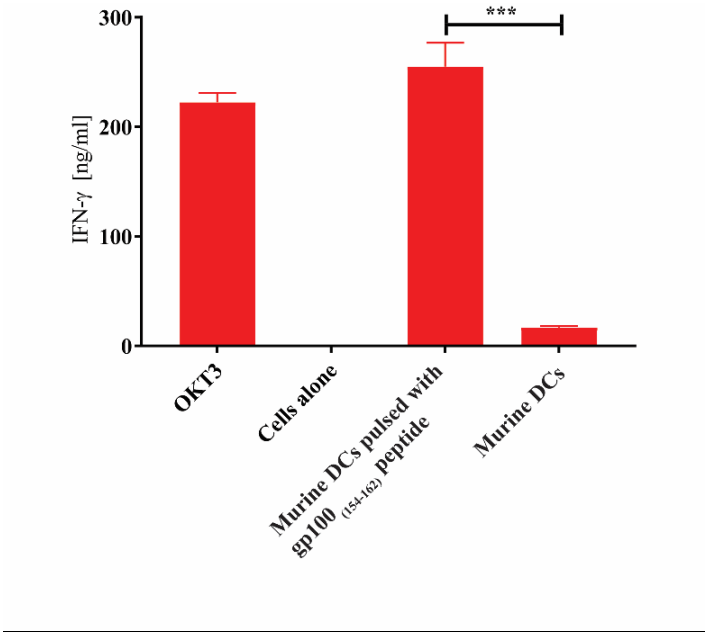


Figure 5.17. CARaMEL T cells secreted IFN-γ in response to HLA-A*2^{+} murine DCs pulsed with gp100 (154-162) peptide

The ability of HLA-A*02^{+} murine DCs to present gp100 (154-162) peptide to CARaMEL T cells was revealed by IFN-γ-secretion determined by ELISA. Sorted and expanded CARaMEL T cells co-cultured overnight with murine DCs (generated from NSG-HLA-A*02^{+} mice as described as per **Material and Methods section 2.13**) pulsed with or without gp100 (154-162) peptide. Controls included untreated as negative control, or αCD3/αCD28-stimulated cells as positive control. Data are representative of three independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (*** $p \leq 0.001$).

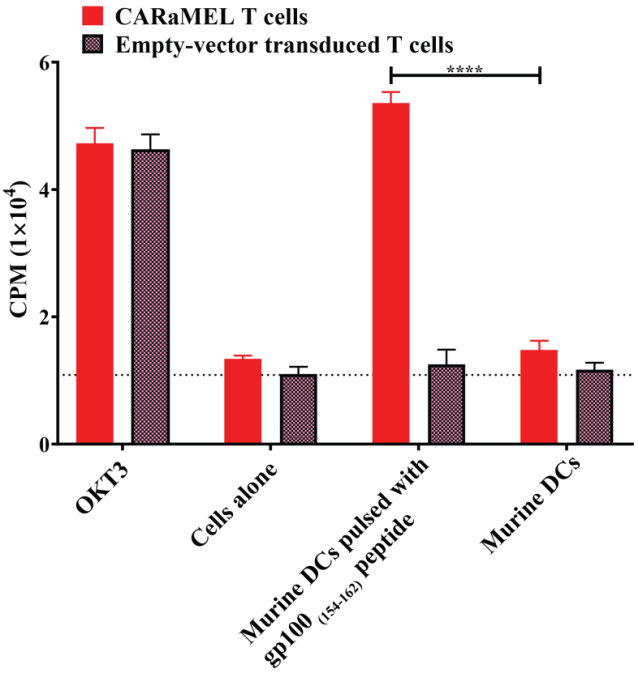


Figure 5.18. CARaMEL T cells proliferate in response to HLA-A*2^{+} murine DCs pulsed with gp100 $_{(154\text{-}162)}$ peptide

The ability of peptide-pulsed HLA-A*02^{+} murine DCs to induce proliferation of CARaMEL T cells was revealed by a [^{3}H] thymidine incorporation assay. Mean proliferation ([^{3}H] thymidine incorporation) is shown for human CARaMEL T cells or empty-vector transduced T cells. T cells were co-cultured with murine DCs pulsed with or without (1 µM) gp100 $_{(154\text{-}162)}$ peptide. Controls included T cells alone as negative control or αCD3/αCD28-stimulated cells as positive control. Data are expressed as the mean count per minute (CPM) of triplicate samples ± SEM; data here represent results of two independent experiments. Statistical analyses were performed using the Student *t* -test (**** $p \leq 0.0001$).

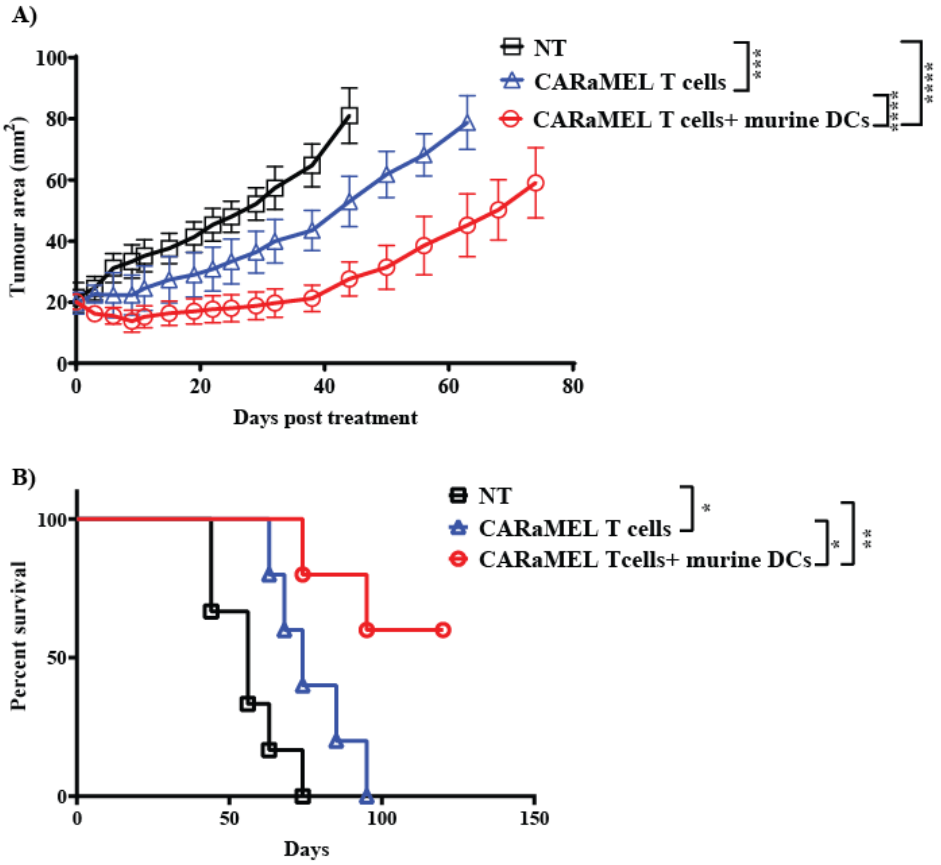


Figure 5.19. Human CARaMEL T cells combined with gp100 (154-162) peptide-pulsed HLA-A*02^{+} murine DCs inhibited Panc1-Her2 subcutaneous tumour growth in NSG mice and prolonged their survival

Panc1-Her2 cancer cells (1×10^6 cells/mouse) were injected subcutaneously into NSG mice. On day eight following tumour injections, mice received total body irradiation, intravenous injections of sorted and expanded human CARaMEL T cells and HLA-A*02^{+} murine DCs pulsed with gp100 (154-162) peptide. Mice also received intraperitoneal injections of IL-2. On day 10 treatment was repeated without total body irradiation. As controls, cohorts of mice were injected with human CARaMEL T cells without DCs and another group was left non-treated (NT). (**A**) Tumour area was measured at the indicated times, and data are presented as the mean ± SEM of 5-6 mice/group, for a single experiment. (**B**) Percent survival was determined with the endpoint being tumour area reaching ≥100 mm^2; however, tumours of three mice that received CARaMEL T cells and murine DCs did not exceed 100 mm^2 but were culled after 120 days to terminate the experiment. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.2.5.3. ACTIV therapy using human CARaMEL T cells in a human pancreatic cancer xenograft model in mice

In the third combination vaccination strategy, I sought to utilize a live viral vaccine, similar to my studies in **Chapters 3** and **4** in mice. However, NSG mice are immunodeficient and severe disease can develop using viruses which could be lethal. Therefore, I used a highly attenuated replication-defective recombinant adenovirus Ad2CMV-gp100 (**Material and Method section 2.4**) in NSG-HLA-A2 mice, which expressed the relevant restriction element for presentation of gp100 to T cells expressing the gp100-specific TCR.

First, in order to confirm whether the recombinant adenovirus vector contained the gpl00 antigen and whether viral-pulsed HLA-$A2^+$ murine DCs could present the gp-100 antigen to human CARaMEL T cells, IFN-γ ELISA and ^3H thymidine incorporation assays were performed.

For the IFN-γ ELISA assay, I first infected HLA-$A*02^+$ murine DCs, which were generated from the bone marrow of NSG-HLA-A2 mice as per **Material and Methods section 2.13**, with the Ad2CMV-gp100 virus overnight. On the next day these murine DCs were washed to remove any excess virus, and then these murine DCs were co-cultured with human CARaMEL T cells overnight. The supernatant was harvested and then analysed for IFN-γ by ELISA (**Figure 5.20)**. Human CARaMEL T cells secreted IFN-γ specifically in response to Ad2CMV-gp100 infected HLA-$A*02^+$ murine DCs but not in response to non-infected murine DCs which confirms the presence of gp-l00 antigen in the adenovirus vector. Also, this finding confirmed the ability of HLA-$A*02^+$ murine DCs to present the gp-100 antigen derived from the Ad2CMV-gp100 virus and the reactivity and specificity of human CARaMEL T cells ($p \leq 0.0001$). In contrast, empty-vector transduced T cells did not secrete IFN-γ in response to Ad2CMV-gp100 infected HLA-$A*02^+$ murine DCs (**Figure 5.20)**.

In further experiments, I studied the ability of human CARaMEL T cells to proliferate in response to Ad2CMV-gp100 infected HLA-$A*02^+$ murine DCs, using a [^3H] thymidine incorporation assay. I co-cultured human CARaMEL T cells or empty-vector transduced T cells with irradiated and Ad2CMV-gp100 infected HLA-$A*02^+$ murine DCs (**Figure 5.21**). Human CARaMEL T cells proliferated through their gp100 TCR in response to Ad2CMV-gp100 infected HLA-$A*02^+$ murine DCs but not in response non-infected murine DCs. In contrast to, CARaMEL T cells, empty-vector transduced T cells failed to proliferate in response to gp100 antigen. This result again confirms the following: 1) the presence of gpl00 antigen in

the adenovirus vectors 2) the ability of HLA-A*02^{+} murine DCs to present the gp100 antigen in the Ad2CMV-gp100 virus and 3) the reactivity and specificity and of human CARaMEL T cells (**Figure 5.21**).

Having demonstrated that Ad2CMV-gp100 infected HLA-A*02^{+} murine DCs can efficiently present gp100 antigen to human CARaMEL T cells to induce T cell function *in vitro*, I progressed to *in vivo* studies. In this experiment I injected Panc1-Her2 cancer cells subcutaneously in NSG-HLA-A2 mice and allowed the tumours to grow for nine days. I anticipated that endogenous HLA-A2^{+} murine DCs in these mice would be able to present gp100 to T cells following administration of Ad2CMV-gp100 virus. On day 10 following tumour injections, mice received the following: total body irradiation, intravenous injections of human CARaMEL T cells and Ad2CMV-gp100. Mice also received intraperitoneal injection of IL-2. As controls, cohorts of mice were injected with human CARaMEL T cells without virus or Ad2CMV-gp100 alone or left untreated. Also, the T cell-treated mice received another intravenous injection of human CARaMEL T cells on day 12 after tumour inoculation (**Figure 5.22**). Administration of human CARaMEL T cells with Ad2CMV-gp100 into mice bearing Panc02-Her2 tumours produced significant tumour growth inhibition compared with mice that received human CARaMEL T cells without virus. Furthermore, mice treated with human CARaMEL T cells and Ad2CMV-gp100 survived significantly longer than all other groups (**Figure 5.22**). This result is consistent with my previous findings in **sections 5.2.5.1** and **5.2.5.2** that the anti-tumour activity of CARaMEL T cells was boosted through gp100-TCR.

In summary, I utilized Ad2CMV-gp100 as a vaccine to stimulate human CARaMEL T cells through their gp-100 TCR. Ad2CMV-gp100 stimulated human CARaMEL T cells and induced their proliferation and lead to high levels of IFN-γ secretion *in vitro*. Also, the combination of Ad2CMV-gp100 and human CARaMEL T cells demonstrated significantly higher anti-tumour activity against Panc1-Her2 tumours compared with mice received human CARaMEL T cells alone.

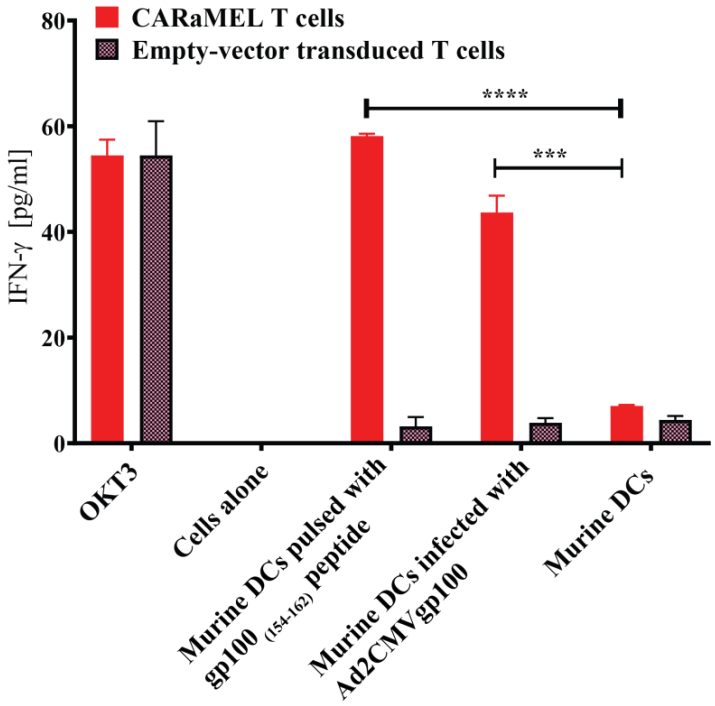


Figure 5.20. CARaMEL T cells secrete IFN-γ in response to Ad2CMV-gp100 infected HLA-A*02^{+} murine DCs

The ability of to Ad2CMV-gp100 infected HLA-A*02^{+} murine DCs to present gp100 antigen to CARaMEL T cells was revealed by IFN-γ-secretion determined by ELISA. CARaMEL T cells were co-cultured overnight with or without HLA-A*02^{+} murine DCs that were infected with Ad2CMV-gp100. Controls included untreated as a negative control, or αCD3/αCD28-stimulated cells or HLA-A*02^{+} murine DCs pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide-stimulated cells as positive controls. Data are representative of four independent experiments and shown as mean ± SEM of triplicate samples. Statistical analyses were performed using the Student *t*-test (*** $p \leq 0.001$, **** $p \leq 0.0001$).

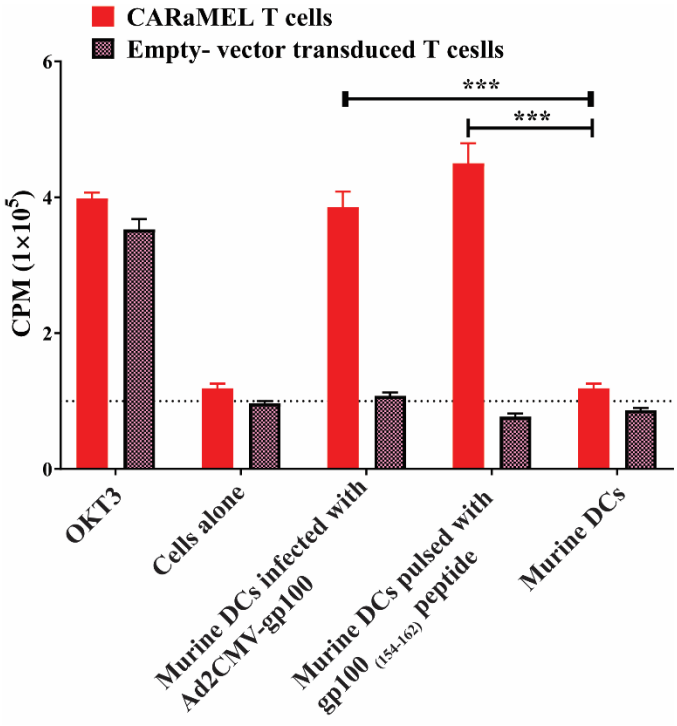


Figure 5.21. Human CARaMEL T cells proliferate in response to Ad2CMV-gp100 infected HLA-A*02^{+} murine DCs

The ability of human CARaMEL T cells to proliferate in response to Ad2CMV-gp100 infected HLA-A*02^{+} murine DCs was revealed using a [^{3}H] thymidine incorporation assay. Mean proliferation ([^{3}H] thymidine incorporation) is shown for human CARaMEL or empty-vector transduced T cells. T cells were co-cultured with 50 Gy irradiated HLA-A*02^{+} murine DCs infected with Ad2CMV-gp100. Controls included untreated as negative control, or αCD3/αCD28-stimulated cells or HLA-A*02^{+} murine DCs pulsed with (1 µM) gp100 $_{(154\text{-}162)}$ peptide -stimulated cells as positive control. Data are expressed as the mean count per minute (CPM) of triplicate cultures ± SEM; data here represent results of two independent experiments. Statistical analyses were performed using the Student *t* -test (*** $p \leq 0.001$).

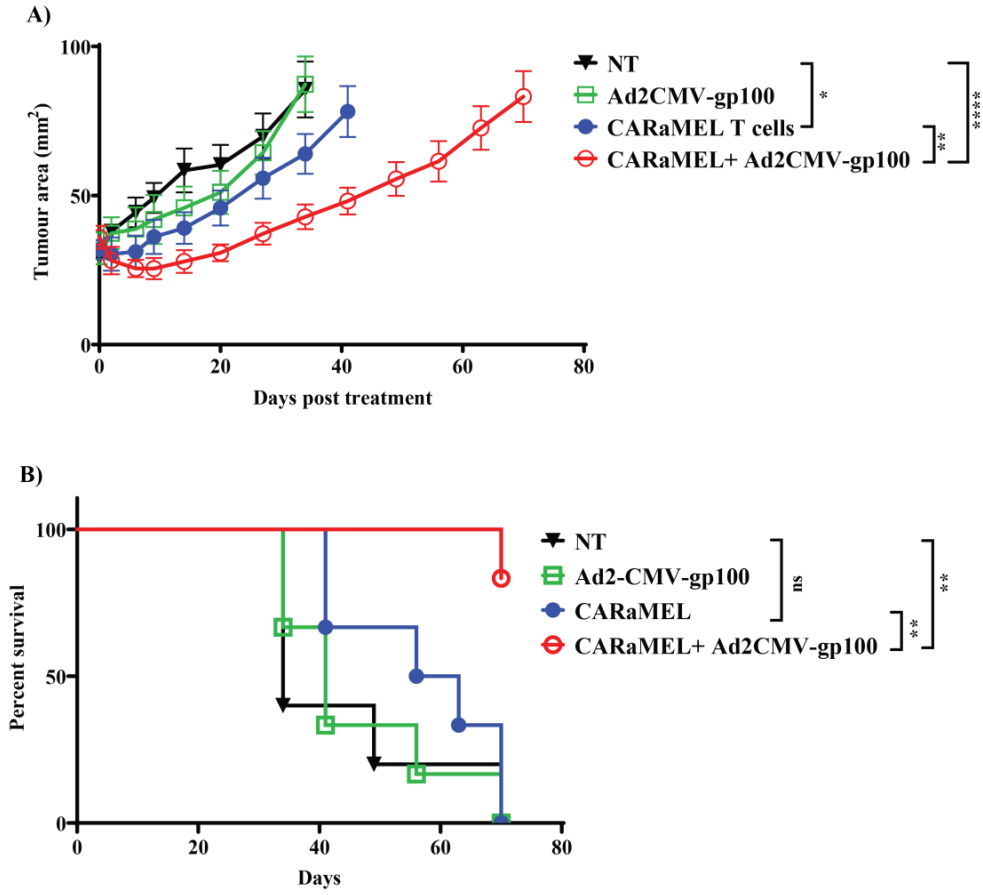


Figure 5.22. Human CARaMEL T cells combined with Ad2CMV-gp100 inhibited Panc1-Her2 subcutaneous tumour growth

Panc1-Her2 cancer cells were injected subcutaneously into NSG-HLA-A2 mice. On day nine following tumour injections, mice received the following: total body radiation, intravenous injections of human CARaMEL T cells and Ad2CMV-gp100. Mice also received intraperitoneal injection of IL-2. As controls, cohorts of mice were injected with human CARaMEL T cells or Ad2CMV-gp100 or left untreated. Also, the human CARaMEL T cell-treated mice groups received a repeat intravenous injection of human CARaMEL T cells on day 12 after tumour inoculation. (**A**) Tumour area was measured at the indicated times, and data are presented as the mean ± SEM of 5-6 mice/group, representative of two independent experiments. (**B**) Percent survival was determined with the endpoint being tumour area reaching ≥100 mm^2; however, tumours of five mice that received CARaMEL T cells and Ad2CMV-gp100 did not exceed 100 mm^2 but were culled after 70 days to terminate the experiment. Statistical analyses were performed using two-way ANOVA for tumour size and the Log-rank (Mantel-Cox) test was used for Kaplan-Meier survival curves (ns=not significant, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$).

5.3. Discussion

In this chapter I generated dual-specific human CARaMEL T cells which express gp100 TCR and anti-Her2 CAR from healthy donors or patient-derived PBMCs. Functional analyses showed that these human CARaMEL T cells have potent cytotoxic activity, specific IFN-γ secretion and proliferation abilities against Her2 and gp100 antigens. Human CARaMEL T cells also showed more potent anti-tumour activity against pancreatic cancer *in vivo* when combined with vaccine compared with mice that received human CARaMEL T cells alone. These results support the translational aspect of the ACTIV therapy.

Antibodies against CD3 have been used in T cell activation and proliferation protocols. Both anti-CD3/CD28 beads and soluble anti-CD3 (OKT3) promoted extensive expansion of T cells. However, anti-CD3/CD28 beads have been shown to stimulate superior $CD4^+$ expansion and OKT3 greater $CD8^+$ T cells expansion with younger phenotype [550-552]. In my project I used anti-CD3 (OKT3) to stimulate and activate T cells and showed >60% of T cells were $CD8^+$, which is a favourable phenotype that I would like to use *in vivo.* However, in this project I co-transferred a mixture of $CD4^+$ and $CD8^+$ T cells in (1:3.5) ratio and not $CD8^+$ alone, as some studies showed that survival of tumour bearing mice was significantly prolonged when a combination of $CD4^+$ and $CD8^+$ T cells were transferred compared to $CD8^+$ T cells alone [553-556].

Several studies have shown adoptively transfer of T cells having differentiation phenotype of naïve and central memory T cell subsets have longer persistence and stronger anti-tumour activity compared with more-differentiated effector memory and terminally differentiated effector cells [533-535]. In my project, human CARaMEL T cells demonstrate a shift in the phenotypic maturation demonstrated as more effector memory T cells were developed from naïve T cells after activation and REP using soluble anti-CD3 and IL-2. However, some studies showed that using some pharmacological compounds such as glycogen synthase-3β inhibitor (TWS119) or cytokines such as IL-7 and IL-15 can generate effector memory phenotype or even T stem cell memory cells, which have self-renewal and anti-tumour capacities [557-562]. It would be interesting to evaluate the effect of using these compounds on human CARaMEL T cells differentiation status and anti-tumour activity in future experiments.

Tumours cells have different mechanisms to evade immune system and progress for instance upregulating immunoinhibitory molecules [563-565]. These molecules can inhibit of CAR-T cell therapies by engaging immune inhibitory receptors [566, 567]. Repetitive T cell activation

can lead the cells to a dysfunctional state and exhaustion which has been observed during cancer progression or chronic viral infection [568-571]. Upon interacting with the cognate ligands; exhausted T cells start to lose their functional capacities such as killing, proliferation, production of different cytokines as IFN-γ, IL2 and TNF and they will die upon repetitive antigen exposure [572, 573]. These receptors or immune checkpoints are PD-1, LAG-3, TIM-3, CD160 and CTLA4 [536-541, 574]. In my project I found that human CARaMEL T cells express different types of the inhibitory receptors and it would be interesting to study inhibitory ligands that maybe expressed on pancreatic cancer cells and their effects on CARaMEL T cells.

In addition, several studies demonstrated that blocking checkpoint receptors enhanced the anti-tumour $CD8^+$ T cell activity and slowed tumour growth [575-577]. Administration of a checkpoint inhibitor blocking antibodies such as anti-PD1, anti-TIM-3 and anti-LAG-3 around the time of adoptive T cell transfer showed an enhanced proliferation and cytokine production [578-583]. Different checkpoint inhibitors have been approved by FDA to be used in some solid tumours including antibodies that block PD-1 (nivolumab, pidilizumab and pembrolizumab) CTLA-4 (tremelimumab and ipilimumab), and PD-L1 (MPDL3280A and MDX-1105) [112] and different clinical trials combining CAR T cells with checkpoint blockade have been initiated in several countries [565, 584]. Another strategy to overcome these inhibitory checkpoint receptors and enhanced the functionality of CAR T cells is by using genetically engineered switch receptor constructs, that contain the extracellular domain of the inhibitory receptors linked to the transmembrane and cytoplasmic domains of co-stimulatory molecules into CARaMEL T cells to augment their efficacy [585]. It would be of interest to determine if the use of checkpoint inhibition could be combined with ACTIV therapy regimens used in this thesis to enhance the anti-tumour responses.

Also, my results revealed that human CARaMEL T cells have effective cytotoxic activity, specific IFN-γ secretion and proliferative abilities when encountering Her2 and gp100 antigens. Since T cell expansion is critical for CAR-T functionality, the main function and aim of gp100 TCR in my project is to induce proliferation and expansion of T cells. ^{3}H-thymidine incorporation assay confirmed my hypothesis and showed human CARaMEL T cells proliferated more when stimulated through TCR than Her2-CAR (**Figure 5.14**).

In my previous experiments ACTIV against breast, sarcoma, liver tumours and pancreatic tumours which were investigated in this project; I used the oncolytic vaccinia virus (VV-gp100). Hence using the oncolytic vaccinia virus in NSG immunodeficient mice can cause

severe or even lethal disease; I used a recombinant adenovirus Ad2CMV-gp100 which encode gp100 antigen as a viral vaccine in this model system. Ad2CMV-gp100 is a replication defective type II adenovirus because El region was replaced with the gp100 gene under the control of a CMV immediate early enhancer-promoter element. This promoter is known for its potent transcriptional signals with little species specificity [586].

Infection of HLA-$A2^+$ human or murine DCs with Ad2CMV-gp100 resulted in recognition by CARaMEL T cells as demonstrated by specific release of IFN-γ cytokine and proliferation of T cells. It is likely that Ad2CMVgp-100 likely contributed to the anti-tumour activity through the activation and expansion of CARaMEL T cells. However, it would be of interest in future to perform *in vivo* experiments to assess if Ad2CMVgp-100 resulted in CARaMEL T cell proliferation and longer persistence in the circulation of mice or tumours, compared with mice that received both CARaMEL T cells alone. I hypothesise that activation of CARaMEL T cells in mice was mediated by HLA-$A2^+$ murine DCs that were infected with Ad2CMVgp-100. However, I cannot rule out the role of other APCs such as macrophages and B cells in gp100 presentation to CARaMEL T cells. Therefore, it would be of interest to perform further experiments to study these mechanisms. Also, further experiments are required to assess if Ad2CMV gp-100 can infect and induce tumour cell death of Panc1 Her2 cells. In the clinical setting, other recombinant viruses, particularly oncolytic ones, could feasibly be used for intravenous administration, which may include vaccinia virus, reovirus, herpes virus or adenovirus [349].

In this chapter I used two types of vaccinations to activate CARaMEL T cells *in vivo*: 1) *ex-vivo* gp100 antigen-loaded DC-based vaccine (murine or human); 2) viral-based vaccine using adenovirus expressing the gp100 antigen. As tumour cells secrete various immunosuppressive molecules and cytokines to support tumour progression such as IL-10 and TGF-β, which can impair the function of DCs and T cells, the use of immunostimulatory cytokines or TLR ligands as DC vaccine adjuvants can overcome these immunosuppressive molecules and cytokines [587]. Therefore using an adjuvant with DC vaccination may achieve greater anti-tumour responses [588, 589]. In addition, another type of vaccination that is also used in the clinic is tumour-associated antigenic peptides in adjuvants [590-592]. While both DC vaccines and peptide-adjuvant vaccines support CTL expansion, cytokine production, and cytotoxic activity, there is some evidence to suggest that DC vaccines can induce immune-mediated tissue destruction to a greater extent than a peptide-adjuvant vaccine [593].

DC-based vaccine strategies have been shown to be safe and effective in inducing tumour-specific immune responses in patients in several clinical trials. However, ex-vivo antigen-loaded DC-based vaccines are considered as a customized treatment for each patient, and therefore it is complicated, costly and time-consuming and these obstacles limit its clinical application on a large scale [594]. On the other hand, the recombinant viruses can be described as an "off the shelf" vaccine and can be produced more easily and cheaper than DC-based vaccines. Therefore, viral vaccines have an advantage to be used in clinical trials [594, 595]. However, one disadvantage can limit the use of some viral vaccines is the production of neutralizing antibodies against these viral vectors [595].

In this project, I evaluated human CARaMEL T cells *in vivo* using the non-obese diabetic severe combined immunodeficiency (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mouse strain. This mouse strain is highly immunocompromised which have the severe combined immunodeficiency mutation (scid), a spontaneous mutation in the Prkdc gene [596], and another mutation removes common γ chain gene expression important for several cytokine receptors. The *scid* mutation is in the DNA repair complex protein *Prkdc* and blocks the somatic V(D)J gene segment recombination resulting in lack of mature B and T cells in mice [596, 597]. The deletion of the common γ abrogates murine signalling for the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 cytokine receptors [598-601]. Therefore, these mice considered as a valuable model for studying human cancer as they offer a lymphopenic environment that can enhance the survival of human cells and can be xenografted with different human tissues [602-606]. However, in my project this mouse strain will not allow us to determine the CARaMEL T cell-mediated anti-tumour response in the presence of a normal complement of immune regulatory mechanisms and will not show us any potential contributions from the endogenous immune system including immune cells or their cytokines production [339-341].

Moreover, to achieve HLA-restricted human immune response, I utilized NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg (HLA-A/H2-D/B2M) 1Dvs/SzJ which known as NSG-HLA-A2 mice which are immunodeficient mice and express human HLA class I [259]. This mouse strain was generated using the HLA class I transgene onto the NSG background covalently bound to human β2-microglobulin [259, 607]. The availability of these HLA-A2- transgenic immunodeficient mice allowed us to study HLA-A*2-restricted human CARaMEL T cells response to the vaccine Ad2CMV-gp100 in human pancreatic cancer xenograft model mice and to generate murine HLA-A*2^{+} DCs**.** The engagement of adoptively transferred human CARaMEL CD8^{+} T cells with HLA-A2^{+} mouse cells can support both recognition and survival

of CARaMEL T cells [608]. However, humanized mice have some limitations including the difference in cytokines and growth factors, which are needed for immune system development, and species-specificity of homing molecules may obstruct human immune cells' trafficking and mechanisms of viral pathogenesis regulation.

In ACTIV therapy I supported the adoptively transfused human CARaMEL T cell growth, activation and functions by injecting the mice with exogenous IL-2 [229, 352]. Because the high-dose of IL-2 can cause significant side effects, it can be substituted in patients with less toxic alternate γ(c) cytokines such as IL-7, IL-15, and IL-21 which can enhance CARaMEL T cell persistence and improve their anti-tumour effects [354, 356-361]. Also, to enhance anti-tumour efficacy of ACTIV therapy; I preconditioned the mice using total body irradiation to support of CARaMEL T cells engraftment [362, 363]. However in the clinic setting total body irradiation may have some oncogenic effects in patients and it can be substituted with systemic chemotherapy which is more convenient and less invasive [376, 377]. Therefore, it would be of interest to test alternate cytokines and cyclophosphamide as a preconditioning regimen with human CARaMEL T cells *in vivo* and determine its efficacy.

In this project I used a model system for T cells expressing both anti-Her2 CAR and gp100-TCR. These antigens were used to provide a proof of principle that adoptively transfused CAR T cells would expand through TCR and kill tumours through CAR. Her2 was my targeted antigen but other overexpressed pancreatic cancer TAA can be used such as CEA [319], MUC1 [320, 321] and MSLN [317]. Also, TCRs specific for foreign antigens such as influenza virus can be used as a safer option.

In the clinical setting, ACTIV therapy must be reproducible and safe. Mice received ACTIV therapy showed some transient toxicity for few days, evident as lethargy. The potential explanation for this toxicity is pro-inflammatory cytokines that was released by human CARaMEL T cells; however, this would need to be confirmed in further experiments.

In conclusion, the functional characterization that I performed on human dual-specific CARaMEL T cells may provide support for the clinical investigation of ACTIV therapy against pancreatic and other cancer types. Dual-transduced human CARaMEL T cells showed potent cytotoxic activity and killing ability, IFN-γ secretion and proliferation against Her2 and gp100 antigens. Also, human CARaMEL T cells showed potent anti-tumour activity against pancreatic cancer *in vivo*. In addition, the combination of Ad2CMV-gp100 and human CARaMEL T cells demonstrated significantly higher anti-tumour activity against Panc1-Her2

tumours compared with mice that received human CARaMEL T cells alone, which support the concept of ACTIV therapy in which dual-specific T cells are activated and proliferate through their TCR and kill cancer cells through their CAR.

CHAPTER 6: Discussion

6.1. Summary and discussion

Pancreatic cancer is one of the most aggressive and lethal common malignancies. It is associated with an overall 5-year survival rate of <7% and only 20% of patients are alive at the first year [3-5, 269, 270]. This poor survival rate of pancreatic cancer patients is due to many reasons including the late diagnosis of the disease in patients which is usually at an advanced stage; its highly resistant nature to different therapies including chemotherapy because of its remarkable desmoplastic reaction with dense stroma and an immunosuppressive tumour TME [274-276] and its inherent genetic instability [273]. Even with aggressive chemotherapy, there is no prolonged enhancement in overall survival [3]. While pancreaticoduodenectomy or Whipple procedure offers the best opportunity for cure with the longest survival rate in pancreatic cancer patients, this surgery is complex and associated with postoperative complications [17], and most patients arrive in the clinic with distant metastases which excludes surgery from treatment options [4, 18, 271].

Therefore, other effective and innovative therapeutic options for this deadly cancer are highly required. Adoptive cell transfer (ACT) using CAR T cells is a new promising form of cancer immunotherapy which has demonstrated impressive results in different cancer types, including haematological malignancies, with response rates up to 90% [240-244]. In ACT, T cells are isolated from cancer patients, re-programmed to express tumour-specific CAR, expanded to therapeutic levels and reinfused back to patients, where they traffic to tumours and mediate its regression [129, 229, 282-284].

ACT using CAR T-cell therapy has shown significant progress in malignant haematological diseases with significant clinical results [240-244]. However, clinical trials using CAR-based ACT have only shown low response rates in solid cancer patients including those with pancreatic tumours [129, 245, 246, 285]. This unsatisfactory outcome of CAR T-cell therapy in solid tumours is due to the unique challenges and obstacles that present in solid tumours and which are not seen in haematological malignancies. This includes inefficient trafficking of CAR-T cells to tumour sites, inefficient penetration of CAR T cells into tumour lesions, poor T cell persistence, and the aggressive and immunosuppressive TME of solid tumours, which can result in T cell exhaustion and inhibit CAR T cell activity [245, 249-255, 286]. Furthermore, the difficulties in selecting the ideal TAA, due to higher antigen heterogeneity in solid tumours [609], and the risk of on-target/off-tumour toxicity, as the targeted antigen can

be expressed in other organs [609-612], are other obstacles that limit the success of CAR T-cell therapy in solid tumours.

To address some of these limitations, our laboratory provided CAR T cells with a strategy for better expansion and persistence in tumour tissues. This novel approach termed ACTIV therapy included total-body irradiation preconditioning followed by administration of CARaMEL T cells, an indirect vaccine (VV-gp100) and IL-2. CARaMEL T cells are dual-specific T cells expressing both a TCR specific for gp100 and an anti-Her2 CAR. The recombinant vaccinia virus vaccine that incorporates a minigene encoding the gp100 antigen (VV-gp100) provided CARaMEL T cells with activation and expansion signals through their gp100-specific TCR. The anti-Her2 CAR provided CARaMEL T cells with the ability to kill $Her2^+$ tumours. In ACTIV therapy, the preconditioning step using total body irradiation aimed to enhance the anti-tumour efficacy [362, 363] of CARaMEL T cells through the removal of cellular cytokine sinks [294]. In addition, I aimed to support the growth, activation and function of CARaMEL T cells through the administration of exogenous IL-2 [229, 261, 352]. In our previous research, ACTIV therapy was effective in eradicating large $Her2^+$ tumours in immunocompetent mice expressing human Her2. Tumour types included E0771-Her2 breast tumours, 24JK-Her2 subcutaneous sarcoma, and MC38-Her2 colon carcinoma present as large liver masses [287]. In this PhD project, I sought to extend ACTIV therapy against pancreatic cancers.

In **Chapter 3**, I determined the efficacy of ACTIV therapy in a mouse pancreatic cancer model. First, I generated $Her2^+$ murine pancreatic cancer cell lines and used them to investigate CAR T cell activity against pancreatic cancer cells. In addition, I characterized murine CARaMEL T cells and confirmed their specificity for both Her2 and gp100 through secretion of IFN-γ and induction of cell lysis in response to murine pancreatic cancer cells. CARaMEL T cells also achieved more proliferation when stimulated through their gp100 TCR than the Her2-CAR. Interestingly, relatively poor proliferation was observed when dual-specific T cells were stimulated through their CAR, compared to stimulation through their TCR. This is consistent with our hypothesis in using a vaccine to stimulate CAR T cells, which can be understood when considering the requirement of several costimulatory signals for optimal T cell expansion. These several signals can be delivered by interaction with APCs.

In addition, I addressed the role of the VV-gp100 vaccine in ACTIV therapy and found that it works through the activation, expansion and accumulation of CARaMEL T cells in tumours, but not through the potential infection and oncolysis of Panc02-Her2. In subsequent parts of

Chapter 3, I demonstrated that ACTIV therapy inhibited KPC-Her2 and Panc02-Her2 subcutaneous tumour growth in mice and prolonged their survival. However, both types of pancreatic tumours usually relapsed approximately two weeks after ACTIV therapy. This relapse was not due to Her2 antigen loss on the tumour cells but could have been due to the loss of function of CARaMEL T cells mediated by immunosuppressive mechanisms within the TME.

Therefore, in **Chapter 4** I aimed to improve the anti-tumour activity of ACTIV therapy and CARaMEL T cells by the administration of either the pan-HDACi (Panobinostat) or αCD40 monoclonal antibody.

My study revealed that *in vitro* treatment with Panobinostat significantly suppressed the growth of Panc02-Her2 cells which was associated with increased apoptosis and cell cycle arrest at the G1/S stage at nanomolar concentrations and in a dose-dependent manner. Also, I found that the addition of Panobinostat significantly increased the apoptosis mediated by CARaMEL T cells against various cancer cells *in vitro* and significantly increased the suppression of Panc02-Her2 cell growth compared with the control groups. This powerful anti-tumour activity synergistic effect was also observed *in vivo*. The combined (Panobinostat and ACTIV therapy) treatment significantly reduced the growth of Panc02-Her2 tumours and prolonged the survival of mice compared with control treated groups. I also demonstrated that IFN-γ was important for this combined treatment of Panobinostat and ACTIV therapy, but it was mainly secreted by other endogenous immune cells rather than CARaMEL T cells (**Table 6.1**).

Table 6.1. Potential mechanisms underlying the enhancement of the anti-tumour activity when Panobinostat is administered with ACTIV therapy

ACTIV+ Panobinostat	
Tumour cells:	**CARaMEL T cells**
VVgp-100 infection =	Cell growth ↓
TAA expression =	IFN-γ secretion ↓
Cell growth ↓	Infiltration in tumours ↑
Apoptosis ↑	Cytotoxicity through sensitizing tumour cells ↑
Cell cycle arrest ↑	
Tumour growth ↓	

HDACi have been previously used to improve the efficacy of several immunotherapy studies [451-453]. HDACi including Panobinostat have been reported to increase histone acetylation and regulate the expression of different tumour suppressor proteins [487] leading to an intracellular pro-apoptotic milieu in various cancer cells [175, 487-491]. Panobinostat is a potent pan-HDACi against multiple HDAC enzymes associated with cancer progression [177], and has been used at low nanomolar concentrations for different hematologic malignancies [174-178] and is considered as the third-line treatment of multiple myeloma [179] with limited toxicity [174, 423-425].

In my study Panobinostat induced apoptosis in pancreatic cancer cells and the combination of CARaMEL T cells and Panobinostat significantly increased tumour apoptosis *in vitro.* HDACi including Panobinostat have been previously shown to have potent anti-proliferative and pro-apoptotic effects both *in vitro* and *in vivo* leading to reduction of tumour growth and improved survival of tumour-bearing mice [442, 492], including pancreatic cancer [169, 494-510]. HDACi can induce death of tumour cells through activation of the extrinsic (death receptor) or intrinsic (mitochondria) pathways [498, 613, 614]. Extrinsic apoptotic pathways in cancer cells by HDACi may be mediated through various mechanisms such as downregulation of FLICE-like inhibitory protein (c-FLIP), induction of cell surface death receptors or ligands (TNF-TNF receptors, FAS/FASL, tumour-necrosis factor-related apoptosis-inducing ligand TRAIL-TRAIL receptors) and death inducing signal complex (DISC) [421, 491, 498, 502-510, 615]. In addition, HDACi can exert intrinsic apoptosis through decreasing the expression of the anti-apoptotic proteins (for example: Bcl-2, Bcl-xl and Bcl-w) and increasing the expression of pro-apoptotic proteins (for example: Bax, and Bim) [491, 613, 616-618]. It would be of interest in future studies to determine the effect of Panobinostat on these molecular pathways in my pancreatic tumour models.

In addition, I found that Panobinostat induced cycle arrest at the G1/S-checkpoint in Panc02-Her2 cells. This is consistent with previous studies demonstrating that HDACi to have anti-proliferative effects on cells by induction of cell-cycle arrest in both normal and transformed cells [399, 619]. Panobinostat has been shown to induce cell-cycle arrest at both G1/S and G2/M stages in a variety of cell lines including pancreatic cancer [425, 480, 482, 500, 511]. HDACi were shown to induce G1 or G2 cell cycle arrest and/or apoptosis to a variable extent both *in vitro* and *in vivo,* depending on the cancer cell line, the specific HDACi used, the dose and time of exposure [415, 483, 521, 620-622]. In dividing cells the G1/S checkpoint is

considered as the first line of defence against any genomic stress as the cells will not enter the S-phase of DNA replication [623].

Interestingly, in my study, I found that Panobinostat did not increase Her2 expression on tumour cells *in vitro* or *in vivo*. Since Her2 expression in the tumour cell lines is under the control of a strong viral promoter, the epigenetic effects of HDACi might differ from those on endogenous TAA. Previous studies have found the effect of HDACi on tumour antigen expression to be variable. In some studies, HDACi have been shown to control the immunogenicity and modulate TAA expression on tumour cells, leading to increased tumour recognition by T cells and improved anti-tumour immune responses [182, 183, 422, 436, 438, 624, 625]. For example, in one study, Panobinostat increased melanoma immunogenicity through increasing expression of melanoma differentiation antigens in several human and a murine melanoma cell line *in vitro,* which resulted in greater antigen-specific T cell activation and a significant increase in the survival of tumour-bearing mice [435].

Therefore, the choice of tumour antigens to be targeted may be critical when considering combining HDACi and immunotherapy, as HDACi can either increase or decrease TAA-expression on tumour cells. For example, the HDACi Valproic acid, downregulated the expression of the tumour antigen MUC-1 and its recognition by MUC1-specific $CD8^+$ T cells, however it upregulated New York oesophageal squamous cell carcinoma-1 (NY-ESO-1) in mesothelioma cells *in vitro* [437]. Interestingly, in my pancreatic cancer model, the enhancement of ACTIV therapy mediated by Panobinostat was not through increasing Her2 expression. However, I cannot rule out the participation of endogenous T cells that may have responded to changes in the expression of other tumour-associated antigens.

Moreover, in this study I found that Panobinostat increased CARaMEL T cell-mediated suppression of Panc02-Her2 cells *in vitro* and increased the frequency of tumour-infiltrating $CD8^+$ CARaMEL T cells in Panc02-Her2 tumours. I also found that Panobinostat decreased IFN-γ secretion by CARaMEL T cells *in vitro.* However, despite this, IFN-γ was still found to play an important role in therapy (Panobinostat + ACTIV therapy) *in vivo*. Experiments using neutralizing antibodies and IFN-γ knock out CARaMEL T cells indicated that the main source of IFN-γ was not CARaMEL T cells, but could come from endogenous immune cells, such as CD4 Th1, $CD8^+$ cytotoxic T cells, NK cells, NKT or macrophages [464, 465], in which Panobinostat may have epigenetically affected IFN-γ production in these immune cells [466]. Therefore, it would be interesting to further investigate the source and requirements of IFN-γ

in this combined therapy. In contrast to this finding, our previous study of ACTIV therapy alone, against cancer types other than pancreatic cancer, demonstrated that the anti-tumour activity of ACTIV therapy was not dependent on IFN-γ [287] but was dependent on CXCR3, which is also in contrast to what I found in this combined therapy. These contradictory results suggest that different molecular contributors played roles when ACTIV therapy is administrated alone or combined with other drugs in different cancer types. Further investigations would be necessary to gain more insight into these and other molecular contributors in the combined Panobinostat + ACTIV therapy against pancreatic tumours.

In my study, I demonstrated that Panobinostat could enhance the cytolytic activity of T cells against pancreatic cancer cells. The contribution from other T cell mechanisms is unclear at present, but various previous studies have indicated that pan-HDACi, including Panobinostat, can enhance $CD8^+$ T cell activation and function through various mechanisms, including increasing cytotoxicity, increasing memory function and restoring immune function of exhausted $CD8^+$ T cells. On the other hand, several studies showed that HDACi can have some inhibitory effects on T cells, in certain circumstances, reducing their viability and function leading to weak anti-tumour T cell responses [431, 626-628]. A previous clinical trial showed that the percentage of CD8 T cells in patient's blood were decreased by around half after Romidepsin administration in cutaneous T-cell lymphoma patients [629].

There are important factors that should be considered in immunocombination therapies in which can either enhance or inhibit $CD8^+$ T cell therapy including: 1) type of HDAC inhibitor used 2) the timing of administration 3) the activation status of $CD8^+$ T cells. In ACTIV therapy, the timing of Panobinostat administration was a critical factor. I found that administration of Panobinostat during ACTIV therapy was more effective in inhibiting pancreatic tumour growth than administration of Panobinostat before ACTIV therapy. Various other studies from different groups have shown the importance of the timing of administration when using HDACi combined with immunotherapy [431-434]. In contrast to my finding, some studies showed that the inhibitory effect of HDACi seems to be less effective during the activation of immune cells and better before or after the activation of immune cells [431-434]. Therefore, the timing of HDACi administration when combined with ACTIV therapy or other immunotherapies should be carefully considered.

In summary, Panobinostat enhanced the efficacy and boosted the anti-tumour activity of ACTIV therapy to significantly reduce tumour growth and prolong the survival of mice treated

with the combined therapy through various mechanisms of action including direct cytotoxicity of cancer cells and their sensitization to CTL.

In the second part of **Chapter 4**, I showed that ACTIV therapy could be significantly enhanced by the administration of an agonistic αCD40 antibody. Mice treated with the combined αCD40 antibody and ACTIV therapy showed significantly greater reduction in tumour growth and significantly prolonged survival rate compared with mice treated with ACTIV therapy alone. The mechanism underlying this enhancement was found to include a greater influx of T cells into tumours and a higher production of IFN-γ secreted from the CARaMEL T cells compared with other groups (**Table 6.2**). In addition, I demonstrated a significant reduction in macrophage numbers in mice receiving the combined treatment of CD40 agonist antibody + ACTIV therapy. Interestingly, I did not demonstrate M2-pro-tumour to M1-anti-tumour macrophage phenotype switching in tumours of mice receiving CD40 agonist antibody + ACTIV therapy. Depletion studies confirmed that anti-tumour activity of theCD40-agonist antibody and ACTIV therapy was through a T-cell-dependent mechanism. In contrast, macrophages did not seem to play a significant role in the enhanced therapy, although these results need to be confirmed, perhaps by macrophage depletion using an anti-F4/80 antibody.

Table 6.2. Potential mechanisms underlying the enhancement of anti-tumour activity when anti-CD40 agonist is administered with ACTIV therapy

ACTIV+ CD40 agonist	
Tumours:	**CARaMEL T cells:**
Tumour growth ↓	IFN-γ secretion ↑
Macrophages infiltration ↓	Cytotoxicity ↑
Monocytes infiltration ↑	Accumulation ↑
T cells ($CD4^+$ and $CD8^+$) ↑	Infiltration in tumours ↑

Previous studies have identified roles for T cells and macrophages, which seem to depend on the tumour type and treatment. Anti-CD40 can induce both enhanced macrophage-mediated anti-tumour effects with stromal remodelling, and $CD8^+$ T cell-dependent anti-tumour immunity [630]. CD40 is a member of TNFR superfamily, and it has an important role in

several cell-mediated immune responses [133-135]. The CD40-CD40L interaction can license DCs to mediate CD4-independent $CD8^+$ T cell responses [142, 144-148], and mediate the modulation of myeloid populations in tumours [631]. Various studies demonstrated that using agonistic anti-CD40 monoclonal antibody [142-144, 146, 632-634] can activate tumour -specific immune responses and reduce tumour growth. Although CAR T cells are MHC independent, I cannot exclude a possible role for endogenous T cells, which may enhance the anti-tumour activity after CD40-CD40L interaction. Therefore, it would be interesting to investigate this possible mechanism in further experiments.

In pancreatic cancer, CD40 agonists have been reported to be a promising approach in combination with gemcitabine [635] and/or nab-paclitaxel [139]. CD40 agonists were demonstrated to mediate tumour regression through either a T-cell-dependent [139] or T-cell-independent/macrophages-mediated [132] immune mechanisms. Beatty and colleagues showed that anti-CD40 recalibrated macrophages to become tumoricidal and facilitate the depletion of tumour stroma [141]. This reprogramming of macrophages happened via inhibition of the NF-κB pathway [155] or via the involvement of IFN-γ and CCL2 [156]. Reprogramming of macrophages in tumours from a suppressive state to an anti-tumour state is a promising alternative to macrophage depletion for cancer therapy. A recent study showed that macrophage depletion diminished the anti-tumour activity of anti-mesothelin TCR-engineered T cells, whereas macrophage programming enhanced the accumulation and longevity of these cells [636]. However, in my study I did not identify macrophage reprogramming, which may due to the differences in therapy, where I used adoptively transferred CARaMEL T cells and other components of ACTIV therapy such as the indirect vaccine, VV-gp100.

In summary, my results demonstrated an increase in tumour-infiltrating T cells ($CD4^+$, $CD8^+$ T cells and $Thy1.1^+$ $CD8^+$ CARaMEL T cells) and a reduction in macrophage frequency in tumours of mice receiving combined treatment of αCD40 antibody and ACTIV therapy. In addition, an αCD40 agonist antibody improved IFN-γ production by T cells. These results suggest that enhancement of ACTIV therapy by αCD40 antibody was not macrophage-mediated but through a T-cell-dependent mechanism.

Compared with our previous study of ACTIV therapy against breast or colon cancers or sarcoma, in my current study I showed a novel tumour inhibitory capacity of ACTIV therapy combined with either Panobinostat or a CD40 agonist. The addition of either Panobinostat or

a CD40 agonist enhanced the efficacy of ACTIV therapy to significantly reduce tumour growth and prolong the survival of mice treated with the combined therapy. Panobinostat significantly enhanced ACTIV therapy through a mechanism involving direct cytotoxic effects against cancer cells and their sensitization to CTL with an increase in the percentage of tumour-infiltrating $CD8^+$ CARaMEL T cells in Panc02-Her2 tumours. Also, combining αCD40 antibody with ACTIV therapy was associated with a massive influx in percentage of T cells into Panc02-Her2 tumours with an increase in IFN-γ production, which was not observed in ACTIV-treated tumours. Compared with our previous study, it seems that different molecular contributors operated the combined therapy of ACTIV therapy and Panobinostat in the pancreatic cancer setting. In contrast to our previous study of ACTIV therapy alone against other cancer types, in my study I showed that the anti-tumour activity of ACTIV therapy combined with Panobinostat was dependent on IFN-γ but not CXCR3 [287]. Also, my data suggest that CXCR3 and type I/type II interferons played crucial roles in mediating tumour regression in mice treated with the ACTIV therapy and a CD40 agonist. Further studies would be essential to gain insight into these different molecular contributors.

In **chapter 5**, I performed preclinical studies in mice, using human-derived CAR T cells and tumour cells, to investigate the translational potential for using ACTIV therapy in the clinic. ACTIV therapy of cancer patients would involve the isolation of their T cells and engineering them to express both a CAR specific for a TAA and a TCR specific for a strong immunogen. The resulting dual-specific T cells would then be expanded and then reinfused back to the cancer patient with a specific antigen-expressing viral vaccine. In my system, I re-engineered T cells with an anti-Her2 CAR and a pMEL-specific TCR for modelling purposes, to generate CARaMEL T cells.

Functional assays indicated that these human CARaMEL T cells mediate powerful and antigen-specific killing, and cytokine secretion against Her2, and demonstrate strong proliferative ability in response to pMEL (also known as gp100). In addition, I showed that the administration of both human dual-specific CARaMEL T cells and Ad2CMV-gp100 vaccine had more potent anti-tumour activity against subcutaneous human Panc1-Her2 tumours in NSG-HLA-A2/HHD mice compared with the control group (human dual-specific CARaMEL T cells alone).

The majority of human CARaMEL T cells showed >60% were $CD8^+$ due to the use of OKT3 for activation as various studies have shown that OKT3 stimulates superior $CD8^+$ expansion

[552]. Moreover, human CARaMEL T cells demonstrated a phenotypic maturation of more effector memory T cells following activation with OKT3, sorting and REP. In ACT, the transfer of naïve and central memory T cells subsets have shown better anti-tumour activity compared with more-differentiated effector cells with long-term persisting memory T cells in patients [533-535]. Therefore, it would be interesting to investigate alternate means of T cell activation/expansion, including the use of some drugs such as TWS119 which has been reported to result in an increased frequency of stem cell-like memory cells with great abilities for both self-renewal and anti-tumour activity [557-562].

Continuous T cell activation during cancer progression can exhaust T cells and upregulate immune inhibitory receptors. In the tumour microenvironment, the expression of these markers by intra-tumoural lymphocytes can lead to hypo-responsiveness or immune exhaustion. When these receptors interact with their ligands, which are expressed on tumour cells and other stromal cell types [563-565], T cells can lose their immune functions such as killing and cytokine production [566, 567, 572, 573]. In my study, the expansion protocol involving stimulation with OKT3 and IL-2 that resulted in up-regulation of some immune inhibitory receptors including PD-1, LAG-3 and TIM-3 on the surface of CARaMEL T cells.

These immune inhibitory receptors have previously been found to be expressed on CAR T cells [637] and the use of checkpoint blockade antibodies is a potentially attractive strategy to overcome inhibition, which may enhance the functionality of CAR T cells. A number of groups have shown that checkpoint blockade can augment the anti-tumour activity, cytokine production and proliferation of $CD8^+$ T cells [576, 578-582, 638-641]. Several studies showed that the combination of immune checkpoint blockade and ACT can boost the efficacy of ACT and result in superior anti-tumour responses [576, 638, 642, 643]. For example, in adoptive transfer studies, Darcy's group showed increased tumour regression of various murine $Her2^+$ tumours treated with anti-Her2 CAR T cells combined with anti-PD-1 monoclonal antibody [640]. Similarly, another group showed that using anti-PD1 enhanced the anti-tumour effects of human mesothelin-directed CARs in an immunodeficient animal tumour model [637]. Moreover, antibodies against CTLA4, TIM-3 and LAG-3 have also been shown to augment adoptive T cell transfer [578-582, 644] and can be more effective if used in combination with PD1 blockade [103].

Although antibody-mediated checkpoint blockade is the most common approach used, other alternate strategies can block these inhibitory pathways and enhance CAR T cell function. One

study demonstrated that PD-1/PD-1 ligand pathway interference, through a PD-1 dominant negative receptor, or through cell-intrinsic PD-1 shRNA blockade, restored the effector function of CAR T cells and improved survival in an orthotopic mouse model of pleural mesothelioma [645]. Another strategy involves insertion of a switch receptor construct, comprised of a truncated extracellular domain of PD1 and the transmembrane and cytoplasmic signalling domains of CD28, into CAR T cells (PD1CD28 CAR) [585]. In another approach, Rupp *et al.* generated PD-1 deficient anti-CD19 CAR T cells through CRISPR-Cas9–mediated deletion of the PD-1 gene. CRISPR-Cas9–mediated PD-1 disruption increased CAR T cell-mediated tumour cell killing *in vitro* and improved clearance of PD-L1^{+} tumour xenografts *in vivo* [646]. Interestingly, although, gene-editing technologies have been applied to other immune checkpoints, including LAG-3 and CTLA-4, increases to CAR T cell efficacy have not been observed [647]. Several checkpoint blockade therapies have achieved FDA approval, including PD-1, PD-L1 and CTLA-4 antibodies, against various solid and haematological tumours, and they have been found to be effective clinically and demonstrated tumour regression and overall survival benefit [112, 648-654]. Preclinical models have showed enhancement of the anti-tumour activity of CAR T cells when combined with checkpoint blockade [565, 584], and this combination is now being tested in the clinic [565]. It would be interesting, in future experiments, to assess the combined treatment of ACTIV therapy and checkpoint blockade.

In my project I used two alternate types of vaccination to activate CARaMEL T cells *in vivo*: 1) a viral-based vaccine, and 2) an *ex-vivo* antigen-loaded DC-based vaccine. Another form of vaccination that is also used in the clinic involves TAA peptide with adjuvant [590-592, 655]. DC vaccines can mediate greater immune-mediated tumour tissue destruction than peptide with adjuvant [593]. However, the DC-based vaccine strategy is expensive, time-consuming and complex and its application on a large scale clinical setting is difficult [594]. On the other hand, recombinant viruses are relatively cheap and easily produced, and can potentially be used as an “off the shelf” vaccine in large scale clinical trials [594, 595]. In any event, it will be important to consider the type of vaccine and the target antigen with respect to potential toxicity against normal tissues.

6.2. Safety of ACTIV therapy

In ACTIV therapy I used immunocompetent mice that express human Her2 as a self-antigen in normal tissues which allowed us to assess potential on-target/off-tumour toxicity [260]. In my current study I observed some transient toxicity in mice that received ACTIV therapy that began on the 5^{th} day following treatment and lasted for three days. Toxicity included: unsteady movement, weight loss and lethargy. This transient toxicity, in the pancreatic cancer model setting, was similar to what we had observed in a previous study of ACTIV therapy against breast or colon cancers or sarcoma [287]. We previously demonstrated that this toxicity was due to CARaMEL T cells reacting against $Her2^{+}$ expressed in normal cerebellum [287], and this is likely the case in the pancreatic tumour model involving the same symptoms.

Moreover, my current study found that this transient toxicity was not further increased in severity when ACTIV therapy was combined with Panobinostat or αCD40 antibody, although it lasted longer, up to five days. The expression of Her2 in the brain of mice provides a stringent model for testing side effects, but it should be noted that not all CAR antigens are expressed in the brain. Nevertheless, it will be important if combining Panobinostat or an αCD40 antibody with ACTIV therapy in any future clinical setting, to start with Phase I dose escalations studies.

Among various types of HDACi, Panobinostat is an oral agent and has a better safety profile than traditional chemotherapeutic medications [515] and favourable clinical responses [423, 424, 516, 517]. Panobinostat showed some side effects in patients and mice, including thrombocytopenia, fatigue, nausea and vomiting, however these conditions were manageable [518-521].

Multiple phase I studies of CD40 agonists revealed dose dependent side effects in patients including cytokine release syndrome [132, 157, 524], liver injury and reduction in numbers of monocytes, B cells, and platelets [265, 266, 525]. As I did not assess these particular toxicities in the current study, it would be necessary to evaluate liver function, platelet activation and cytokine levels in mice to get the full toxicity profile following the combined therapy of ACTIV and α CD40 agonist antibody prior to proceeding to clinical trials.

Mice receiving human CARaMEL T cells and Ad2CMV-gp100 exhibited some signs of transient toxicity, such as lethargy, after three days of treatment, which lasted for 2-3 days. This transient toxicity could have been due to pro-inflammatory cytokines produced by human

CARaMEL T cells, and further experiments in the future would be required to investigate this aspect.

Serious adverse events have been reported after administration of CAR T cells into cancer patients. These adverse events have been related to toxicity resulting from cytokine release syndrome, mediated by pro-inflammatory cytokines released by the adoptively transferred T cells [328, 367, 656-658]. Toxicity can also be due to on-target/off-tumour responses against TAAs expressed on normal tissues [328-333]. Symptoms of cytokine release syndrome include febrile neutropenia, transient neurologic toxicity and multiple organ failure, leading to death in some cases [244, 333, 659-661]. However, using corticosteroids may manage these toxicities; also IL-6 blockade (tocilizumab) can result in rapid reversal of life-threatening of severe cytokine release syndrome [385, 662-664].

Moreover, the safety of ACT therapies can be enhanced through new approaches that can rapidly eliminate the gene-engineered T cells in case of toxicity or adverse reaction. Several technologies are under investigation to increase the specificity and safety of ACT therapy. A variety of novel cutting-edge strategies have been developed including the insertion of suicide genes, or systems that can be pharmacologically triggered to eradicate gene-engineered T cells in case toxicity [337, 338]. For example the insertion of herpes simplex virus thymidine kinase, or inducible human caspase nine could eliminate gene-engineered T cells in presence of ganciclovir or FK506 binding protein respectively [337, 665-667]. However, this technique limits the therapeutic effect of transferred T cells [668].

Targeting various TAAs expressed on tumour cells or the use of dual CAR-T recognition antigen is a promising strategy to reduce adverse events of CAR T cell therapy [669, 670]. There are two main strategies being explored:

1) The first strategy uses Boolean and logic gated CAR T cell approaches, which allows target selectivity by recognition of two different TAAs expressed on the same tumour cells and is only activated in the presence of both targets. In this strategy dual CAR-T cells have one CAR that transmits only a primary signal and another CAR against another TAA that transmits co-stimulation. Examples of these dual CARs were generated against $HER2^{+}$ MUC1 and mesothelin+ α folate receptor in which both became fully activated and only killed specific target cells that express both antigens [671, 672]. Kloss and colleagues showed that dual CAR T cells destroyed tumours that express both antigens without any effect on tumours expressing

one antigen in a prostate tumour model using both antigens PSCA and prostate-specific membrane antigen (PSMA) [336].

Another Boolean or logic gated CAR T cell approach targets two or more TAAs with a single CAR. Although, the first or second antigen is sufficient for some activation, effector function is synergistically improved when tandem CAR T cells recognize both targeted TAAs. Examples of these tandem CARs have been generated against $HER2^{+}$IL13Ra2 and $HER2^{+}$ CD19 [673, 674]. One study reported that tandem CAR prevented antigen escape, improved anti-tumour efficacy and enhanced survival in a glioblastoma mouse model [675]. To date, no clinical trials using tandem CAR have been initiated against solid tumours, but a clinical trial has used tandem CAR-20/19-T Cells in patients with relapsed refractory B cells malignancies (NCT03019055) [676].

2) The second strategy uses inhibitory CARs (iCARs), in which an extracellular scFv specific for a normal self-antigen is fused to inhibitory signalling domains. These iCARs specifically inhibit CAR T cell activation when encountering antigens expressed on normal tissue [335, 677, 678]. A previous study used iCARs T cells and reported that PD-1- or CTLA-4-based iCARs selectively limited on target/off tumour toxicity through controlling cytotoxicity and cytokine production [335].

Another strategy is to control the activation of CAR T cells to respond only in the presence of an exogenous activator (on-switch). Removal of the exogenous molecule will not lead to the loss of the transferred T cells, as is the case with the suicide gene approach [679]. Thus, a range of strategies exist that could be incorporated into the ACTIV therapy regimens described in this thesis, which could reduce toxicity and enhance the safety of the approach.

6.3. Future directions

In this thesis, I used targeted Her2 as the TAA. High level expression of Her2 is not common in pancreatic cancer but was chosen as a target in this study due to the ready availability of reagents to use in the model systems, which served as proof-of-principle. Possible future directions for this project involve evaluating other TAAs that are highly expressed on pancreatic cancers including: MSLN [317], MUC1 [320, 321], CEA [319] and PSCA [318].

Similarly, I used an anti-gp100 TCR to provide the second specificity for dual-specific CAR T cells in my model, however, other TCRs specific for foreign antigens such as influenza virus could be used instead of gp100 that may represent a better and safer option in the clinic.

It would be of interest to evaluate other recombinant viruses that are used in the clinic such as adenovirus, reovirus and herpes virus [349]. Some of these viruses have greater oncolytic potential and specific tumour tropism, which could provide direct anti-tumour activity in addition to stimulating dual-specific CAR T cells.

In my study, I used whole body irradiation as the preconditioning regimen to induce a state of lymphopenia, which enhances the engraftment of adoptively transferred T cells. However, immunosuppressive drugs, such as cyclophosphamide, are generally used in the clinic as a preconditioning regimen. Although both methods induce lymphopenia, it would be of interest to explore the use of cyclophosphamide as a preconditioning step prior to ACTIV therapy. This form of preconditioning has desirable properties given it more convenient and less invasive than total body irradiation, and also mediates oncogenic effects [376, 377].

In this thesis, I used subcutaneous models of pancreatic cancer in mice, due to the simplicity of use and ease of monitoring, which provided relatively rapid assessment of treatments towards proof-of-principle. However, pancreatic cancer in patients does not occur as subcutaneous disease, but frequently metastasizes to liver. Subcutaneous tumours can differ in their composition and responses to immunotherapy [680], and further studies are needed to investigate the efficacy of ACTIV therapy in orthotopic and metastatic pancreatic tumour models.

Pancreatic cancer exhibits a desmoplastic stromal reaction that generates a physical barrier for T cell and drug penetration [129]. However, I did not evaluate the stromal microenvironment in my pancreatic cancer models. Further studies in future can evaluate this limitation.

Our current study has showed that ACTIV therapy efficacy in pancreatic cancer can be significantly enhanced when administered with HDACi. HDACi can alter the immunogenicity and enhance anti-tumour immune responses, but they also have the ability to upregulate check point receptor and ligand expression [183, 681-684]. It would be of interest to explore the regulation of check point receptor and ligand expression in pancreatic cancer by Panobinostat and combine ACTIV therapy and HDACi with check point inhibitors.

6.4. Feasibility of ACTIV therapy

ACT represents a proven treatment option especially against some haematological malignancies, and has demonstrated promise against some solid tumours, including melanoma and prostate tumours. ACTIV therapy, as described in the current study, is a novel strategy which combines two main components (dual-specific T cells and a vaccine) together with a preconditioning step and administration of exogenous IL-2. ACTIV showed promising results in these pre-clinical studies. However, the complexity of preparing customized cells, patient preparation and the high cost of ACT are serious hurdles that make ACT less applicable. In addition, the use of a live viral vaccine further complicates matters and raises safety concerns. Another challenging limitation of ACTIV therapy that needs consideration is the MHC-restricted TCR signalling requirement. This would require developing a range of TCRs to use for patients of various HLA types.

Translation of ACTIV therapy would require further preclinical work, as described above, with respect to safety. Identification of the optimal antigen, CAR and vaccine would also be necessary. Although, CAR T cells and viral vaccines have been used separately, clinical translation would initially involve Phase I studies of each reagent in isolation followed by dose escalation studies of the full combination therapy.

ACT therapy is an expensive undertaking, but the potential benefit of long-term immune memory responses, which can last for many years, makes ACT more acceptable [685]. Moreover, to reduce manufacturing time and costs several companies are trying to expand the use of ACT through manufacturing allogeneic universal off-the-shelf CAR T cell products, which may make CAR T cell therapy more applicable to multiple patients with high levels of safety and efficacy [686]. In addition, using new gene-editing technology such as CRISPR may promote the development of universal CAR T cell therapy to extend the clinical use of these cells [687, 688].

6.5. Concluding remarks

Pancreatic cancer is a lethal disease often resistant to conventional therapies. In my PhD thesis, I investigated an effective approach to treat this difficult cancer in mice with optimal utility of ACTIV therapy combined with HDACi or an αCD40 monoclonal antibody. My work indicated that these combinatorial approaches show much promise for enhancing CAR T cell therapy to

overcome the immunosuppressive TME of pancreatic cancer. In addition, my work in this project demonstrated that ACTIV therapy using human dual-specific CARaMEL T cells together with an adenoviral vaccine could inhibit human pancreatic cancer in mice. The insights gained from my thesis represent a significant improvement for ACTIV therapy. Increasing the specificity, efficacy and safety of ACTIV therapy may accelerate the application of this novel and promising therapy in the clinic and may lead to improved therapeutic options for patients with pancreatic cancer.

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