Harnessing The Immune System To Reject Cancers Through Genetic Modifications Of Tumour Cells

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Contents

(i) Abstract

The immune system, which defends the body against a wide array of threats, is gaining a growing role in the fight against cancer. For an immunotherapy to be successful, it needs to overcome intrinsically weak tumour-specific immune responses. There are two broad approaches to achieving this goal: targeting the various arms of the immune system or targeting the cancer and its microenvironment. The experiments discussed in this thesis adopt the second approach. Tumours were transduced with a combination of costimulatory molecules: CD48, CD54, CD70 & CD86, the chemokine CX3CL1 and the cytokines: IFNγ, GM-CSF and IL-12. Transduction of costimulatory molecules enhances priming in-vitro and cause tumour rejection and delayed tumour growth in-vivo. This effect is demonstrated with single costimulatory molecules but is more pronounced when multiple costimulatory molecules are transduced. Addition of the cytokines and chemokine enhanced tumour rejection, and also resulted in partial rejection of contralateral parental tumours. Attempts to enhance anti-tumour memory by fusing IL-2 and IL-15 to their respective receptors are also discussed. Work in a human/mouse chimeric PD-1 mouse model shows that transduction of multiple costimulatory molecules is able to overcome intrinsic anti-PD-1 resistance. Radiation is known to result in upregulation of several costimulatory molecules within tumours or their infiltrating dendritic cells. The experiments presented here suggest that radiation therapy may be useful in overcoming anti-PD-1 therapy resistance. In human trials, approximately three quarters of cancers fail to respond to anti-PD-1 therapies. Understanding and potentially overcoming anti-PD-1 therapy resistance is therefore of great interest.

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(ii) Declaration

I declare that this thesis is entirely my own work, and except where otherwise stated, describes my own research.

(iii) Acknowledgements

The work presented in this thesis would not have been possible without the following assistance, which I gratefully acknowledge. The lentviral constructs described in these experiments were designed and made in the laboratory of Professor Simon Davis by David Sleep, Rachel Knox and Simon Davis (CD48, CD54, CD70, CD86, IFNγ, IL-12, GM-CSF & CX3CL1) and Rachel Knox and Simon Davis (IL-2 & IL-15 constructs). The CFP lentivirus was made in the Cerundolo lab by Dr Madeline Hipp. C57Bl/6 Rag Knock Out and BALB/c common gamma chain knock out mice were provided by Dr Andrew Bushell, and the C57Bl/6-GFP mice, by Professor Richard Cornall. The B16-F10, CT26, EG7 and CTLL cell-lines were obtained from Dr Jonathan Silk. Lung and tumour slides were processed by Richard Stillion with help in analysis from Oxford University Hospitals Pathologists Dr Daniel Royston and Dr Ruth Asher. Many thanks also to the invaluable input of Dr Uzi Gileadi, senior post-doc in the Cerundolo lab and to my two supervisors, Professors Vincenzo Cerundolo and Ruth Muschel throughout my time in the laboratory. I am very grateful to the Oxford Cancer Imaging Centre, the Medical Research Council and Cancer Research UK for funding this work. Finally I would like to thank my wife, Dikla for all her help and support.

(iv) Dedication

I dedicate this thesis to my two greatest accomplishments, my daughter Talia & son Jonathan, who were born during the period of this research and also to my father Serge who is currently fighting a personal battle with cancer.

(v) Abbreviations

2-ME: 2-mercaptoethanol
APC: antigen presenting cells
B16-CD54: B16-F10 transduced with CD54
B16-CD70: B16-F10 transduced with CD70
B16-CD86: B16-F10 transduced with CD86
B16-Costimulatory: B16-F10 transduced with CD54, CD70 & CD86
B16-dsR: B16-F10 transduced with dsRed
B16-Multiple Costimulatory: B16-F10 transduced with CD54, CD70 & CD86
B7-2: Alternative nomenclature for CD86, Cluster of Differentiation 86
BALB/c DKO: BALB/c Gamma chain Rag knock out mice
Bcl-2: B-cell lymphoma 2
Bcl-XL: B-cell lymphoma-extra large
CD: Cluster of differentiation
CEA: Carcinoembryonic antigen
CFSE: Carboxyfluorescein succinimidyl ester fluorescent cell staining dye
CT26-CD48: CT26 transduced with CD48
CT26-CD54: CT26 transduced with CD54
CT26-CD70: CT26 transduced with CD70
CT26-CD86: CT26 transduced with CD86
CT26-Costimulatory, Cytokine & Chemokine: CT26 transduced with CD48, CD54, CD70, CD86, GMCSF, IFNγ, IL-2 & CX3CL1
CT26-Cytokines & Chemokine: CT26 transduced with GMCSF, IFNγ, IL-2 & CX3CL1
CT26-Multiple Costimulatory: CT26 transduced with CD48, CD54, CD70 & CD86
CTLA-4: cytotoxic T-lymphocyte associated molecule-4
CTO: Cell Tracker Orange fluorescent cell staining dye
CX3CL1: Chemokine (C-X3-C motif) ligand 1
Cy7: Cyanine 7
DCs: Dendritic Cells
DKO: See BALB/c DKO
DMEM: Dulbecco's Modified Eagle's Medium
dsRed: Discosoma Red Fluorescent Protein
FBS: Foetal Bovine Serum
FDA: United States Food and Drug Administration
FITC: Fluorescein isothiocyanate
Fox-3: Forkhead box p3
GCN2: General control nonderepressible 2
G-CSF: Granulocyte colony stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
Gy: Gray (SI unit of ionizing radiation)
HMGB1: High-mobility group box 1
ICAM-1: Intercellular Adhesion Molecule 1
IDO: Indoleamine 2,3-dioxygenase
IFNγ: Interferon gamma
IL: Interleukin (eg. IL-12)
iNOS: inducible nitric oxide synthase
IPEX: Immune dysregulation, Polyendocrinopathy, Enetropathy, X-linked syndrome
MCA: 3-Methylcholanthrene
MDSCs: Myeloid-Derived Suppressor Cells

MHC: Major Histocompatibility Complex
NF-κβ: Nuclear factor kappa-light-chain-enhancer of activated B cells
NK: Natural Killer
NKT: Natural Killer T cells
NNMU: N-nitroso-N-methylurethane
PE: Phycoerythrin
PD-1: Programmed Cell Death -1
PD-L: Programmed Cell Death Ligand -1
PD-L2: Programmed Cell Death Ligand -2
PI3K: Phosphoinositide 3-kinase
PFU (pfu): Plaque forming units
PSA: Prostate-Specific Antigen
R0: RPMI 1640 without supplementation
R10: RPMI 1640 supplemented with 10% FBS
SEM: Standard error of the mean
SNPs: Single nucleotide polymorphisms
TCR: T Cell Receptor
TNFR: Tumor necrosis factor receptor
TLR: Toll-like receptor

1. Introduction

1.1 Overview

There is much current interest in targeting the immune system as an anti-cancer therapy. The link between the immune system and the development of cancer was first proposed by Rudolf Virchow, the father of modern pathology who observed a "lymphoreticular infiltrate" within tumours in 1863.[1,2] One hundred and fifty years later, successful anti-cancer immunotherapy treatments are for the first time entering routine clinical practice and are making a real difference to the lives of patients.

The immune system is the body's main defence against an array of threats. Although its primary role is to differentiate self from non-self and to defend the body against an array of foreign threats, it is also involved in identifying the body's own cells that have undergone changes to become cancerous. During this process of carcinogenesis, whereby normal cells undergo series of mutations to become cancer cells, there is an evolutionary pressure on developing cancers to evade immune surveillance. Amongst changes described are the loss of MHC-I expression and β-2 microglobulin[3-7], the presence of regulatory T Cells[5,8-14] and myeloid derived suppressor cells (MDSCs)[5,15,16] in the tumour microenvironment along with the secretion of immunosuppressive cytokines.[17] The net result of these changes is that tumour-specific immune responses are weak. Broadly, there are two approaches that can be taken towards reversing this situation. It is possible to target either the various arms of the immune system or target the cancer and its microenvironment. The experiments discussed in this thesis adopt the second approach, through genetic modification of tumour cells, with the aim of simultaneously activating multiple arms of the immune system.

1.2 Immune recognition of cancers

1.2.1 Tumour sensing and recognition by the adaptive and innate immune systems

The immune system is continually exposed to dead and dying cells that arise from physiological processes such as cell turn over and repair following injury and pathological processes such as infection or malignancy. In some instances it is appropriate for dead and dying cells to activate the immune system, whilst in other situations this would lead to autoimmunity. It therefore stands to reason that mechanisms exist to differentiate these various triggers. With regard to infected cells, the presence of pathogen-associated molecular patterns (PAMPs)[18] that include components of the bacterial cell wall and viral DNA, are able to trigger an immune response. The immune system is also able to recognise dying cells through damage-associated molecular patterns (DAMPs). This is a more general process that relies on various immunogenic signals arising from the dying cells.

Anti-cancer therapies such as chemotherapy, radiotherapy or biological therapies that result in tumour cell death have the ability to trigger an immune response. The term "cell death" encompasses a number of physiological processes including apoptosis, autophagic death, necrosis and mitotic catastrophe (reviewed in Galluzzi et al[19] and Green et al[20]). Whilst dying cells share a common fate, their mode of death can result in divergent effects on the immune system, resulting in either immunogenic cell death or conversely induction of immune tolerance. In addition to the mode of tumour cell death, the immunological outcome of the encounter between dying tumour cells and the immune system is also dependent upon the intrinsic antigenicity of the tumours and the presence of immune cells in the tumour microenvironment. Radiation therapy, and some classes of chemotherapeutic drugs, eg anthrocyclines along with the platinum agent oxaliplatin have been shown to be able to

activate the adaptive arm of the immune system. Tumour derived peptide epitopes, which are discussed in greater detail in section 1.2.3 below, can be presented to CD8 T cells in a process that involves the calreticulin pathway, Toll-Like Receptor 4 (TLR4) and High-Mobility Group Box 1 (HMGB1).[21-25] This however, is not true of all chemotherapeutic agents. Alkylating agents such as cyclophosphamide and other chemotherapeutic agents including the platinum agent cisplatin cause tumour cell death without activating these pathways. Rather than activating the immune system this second class of chemotherapeutic agents have instead been shown to contribute to immune tolerance.[25,26]

1.2.2 Immunoediting and Immune Surveillance

Immunoediting and immune surveillance are host protection processes involved in both eliminating nascent tumours and sculpting the immunogenic phenotypes of tumours that eventually form in immunocompetent hosts. They can be broken down into three distinct steps: elimination, equilibrium and escape.[27] Several immune effector cells and secreted cytokines play a critical role in pursuing each process. Nascent transformed cells can initially be eliminated by an innate immune response by NK or NKT cells.[28-30] If the elimination stage is fully successful, all tumour cells are removed with no sequelae. If there is only partial elimination of the tumour however, the developing tumour and immune system enter an equilibrium phase.[31] Whilst in equilibrium, tumour cells can either remain dormant or continue to evolve, with the immune system exerting a counter-selective pressure, immune editing, which eliminates susceptible tumour clones. Escape occurs at such a time that the tumours evolve a mechanism of escaping immune control.[32] A variety of tumour-derived soluble factors contribute to the emergence of complex local and regional immunosuppressive networks, including vascular endothelial growth factor (VEGF)[33], IL-10[34], TGF-β[35], prostaglandin E2[36], soluble Fas[37] and soluble FasL100[38]. These secreted factors are able to exert their effects both

locally within the tumour, and also in local lymph nodes, thereby promoting both local invasion and distant metastases.[32-38]

1.2.3 Tumour Antigens

Tumour antigens are a term used to describe antigen preferentially expressed by tumour cells compared to normal tissues. They can be derived from any protein or glycoprotein synthesised by the tumours. They can be primarily found in any of the subcellular compartments and can be membrane-bound, cytoplasmic, nuclear-localised, or secreted.[39] They are categorised as follows:

- Oncofoetal Antigen: These are defined as antigens expressed only in foetal tissues and in cancerous somatic cells. Carcinoembryonic antigen (CEA) is the most important antigen in this class and is reviewed by Shively et al.[40] and discussed further in section 3.1.6.[40-42]
- Oncoviral Antigen: These are tumour antigens encoded by tumorigenic transforming viruses. The main clinical relevance of this relates to Human papillomavirus (HPV) induced squamous cell carcimonas of the cervix, anus and oropharynx.[43]
- Overexpressed Antigen: Tumour antigens of this class are present in normal tissues, but are found at increased frequency in tumours. The Her-2/neu oncogene is the most clinically important tumour antigen in this class and is reviewed in references [43] & [44].[43,44]
- Cancer-Testis Antigen: These are expressed in cancer cells along with reproductive tissues, particularly testis and placenta e.g. MAGE[45] and NY-ESO-1[46-48]
- Lineage-Restricted Antigen: expressed principally by one cancer histotype. This subtype includes Prostate-specific antigen (PSA)[49] along with the melanoma antigen Melan-A/MART-1[50], Gp100/pmel17[51], TRP-1[52] and TRP-2[53,54]
- Mutated Antigen: This class of antigen is expressed by cancer cells as a result of either a genetic mutation, or alteration in transcription. The melanoma antigen MART-2 is one such

example.[55]

- Posttranslationally Altered Antigen: This class of antigen arises through alterations in glycosylation. One such example of this antigen class is the breast antigen MUC-1[56,57]
- Idiotypic Antigen: These are highly polymorphic genes where a tumour cell expresses a specific "clonotype". This type of antigen is more pertinent to haematological malignancies than solid tumours and is particularly relevant in B cell, T cell lymphoma and leukaemia which result from clonal aberrancies.[58,59]

1.3 The Tumour Microenvironment

In addition to the neoplastic cells themselves, the tumour microenvironment comprises stromal cells epithelial cells, blood vessels, lymphatic vessels, soluble factors including cytokines, chemokines and several different infiltrating immune cells. Tumours are able to manipulate the microenvironment to the advantage of the tumour and detriment of the host. This process involves key cell types including Regulatory T Cells and Myeloid Derived Suppressor Cells. Understanding the mechanisms by which they facilitate tumour growth opens potential therapeutic options to disrupt these processes.

1.3.1 Regulatory T Cells

Regulatory T Cells evolved to maintain a balance between the occasionally conflicting goals of the immune system: targeting harmful stimuli whilst avoiding autoimmunity. Regulatory T cells were initially defined on the basis of CD4 and CD25 expression[8], however subsequent attempts to further refine this population have led to the current definition on the basis of forkhead family transcription factor, forkhead box p3 (Foxp3) positivity.[60] In addition to being CD4 and

CD25 positive, this population of cells usually express high levels of cytotoxic T-lymphocyte associated molecule-4 (CTLA-4) and low levels of the IL-7 receptor (CD127). Congenital absence of the human Foxp3 gene gives rise to Immune dysregulation, Polyendocrinopathy, Enetropathy, X-linked (IPEX) syndrome which is fatal within the first year of life.[61,62] The transgenic Scurfy mouse which also has mutation in the Foxp3 gene has a similar phenotype.[61,62] When regulatory T cells are removed from adult mice in an in-vivo tumour model, it is possible to inhibit tumour growth in B16 and other animal tumour models.[8]. A new class of drugs targeting regulatory T cells such as Ipilumumab, a fully humanised monoclonal antibody against CTLA-4, have now entered clinical use. These agents will be discussed in greater detail in section 1.4.1 below.

1.3.2 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs arise from immature myeloid cells of granulocytic and monocytic origin that fail to differentiate into mature myeloid cells. Instead they expand and are activated within the tumour microenvironment where they suppress both innate and adaptive immune functions.[15,63-65] They have been characterised in both mice and humans. Compared to normal controls, high levels have also been identified in the blood of patients with a variety of tumour types including non small cell lung cancer, breast cancer, head and neck cancers and renal cell cancer.[66-68] Their presence correlates with worst clinical outcomes.[69-71] The majority of MDSCs are granulocytic and exert their immunosuppressive effects predominantly via production of reactive oxygen species[64,72] Their monocytic counterparts, though less in number are more potent in their immunosuppressive function and act via production of inducible nitric oxide synthase (iNOS) and arginase-1.[64,73-76] The ratio of tumour derived granulocyte colony stimulating factor (G-CSF) to granulocyte-macrophage colony stimulating factor (GM-CSF) is a

determinant of the relative size of the respective populations, with G-CSF driving granulocytic MDSC differentiation.[77] Adoptive transfer of MDSCs promotes tumour growth.[78] Monocytic MDSCs have been implicated in tumourigenesis via inducible nitric oxide production, with abolishment of MDSC proliferation in tumour bearing iNOS deficient mice.[79] Conversely, it is possible to enhance anti-tumour response by elimination of MDSCs in pre-clinical models.[80,81] Several chemotherapeutic agents including 5-Fluorouracil[82], docetaxel[83], paclitaxel[84] and gemcitabine[85] are believed to owe some of their anti-cancer activity to their ability to suppress MDSCs.

1.3.3 Indoleamine 2,3-dioxygenase (IDO)

IDO is the rate-limiting enzyme of tryptophan catabolism in the kynurenine pathway. It is a negative regulator of T cells via several complimentary pathways. It is expressed in the placenta where it plays a physiological role in pregnancy, protecting the growing foetus from maternal T cells.[86] It is also expressed by many human tumours leading to non-physiological immunosuppression, which correlates with aggressive, advanced disease and worst prognosis.[87-95] This is mediated by several complimentary pathways, it activates the GCN2 (general control nonderepressible 2) kinase pathway leading to arrest of T cell proliferation and anergy.[96] Some tryptophan metabolites also mediate lymphocyte apoptosis.[97] In addition, IDO regulated decrease in tryptophan induces a regulatory T Cell phenotype in naïve T Cells within the tumour[98-101] and draining lymph nodes[102]. Attempts to inhibit IDO using the small molecule inhibitor 1-methyl-L-tryptophan (1-MT) are under current evaluation, showing delayed tumour growth in murine tumour models[87,89,103] and IDO inhibitors are also undergoing evaluation in several early phase clinical trials[104-106]

1.3.4 Tumour Associated Macrophages (TAMs)

Tumour Associated Macrophages (TAMs) are derived from circulating monocytes or resident tissue macrophages and have been identified within the tumour microenvironment of many different tumour types.[107-110] TAMs are a heterogeneous population comprising M1 and M2 macrophages. M1 macrophages are characterised by LPS and IFNγ mediated activation and secretion of high levels of IL-12 and low levels of IL-10. They are also termed "classically activated" or "killer" macrophages. In contrast M2 phenotype macrophages, also termed "alternatively activated" or "repair" macrophages are characterised by IL-4 mediated activation and secretions of high levels of IL-10 and TGFβ and low levels of IL-12.[111] In several tumour types, TAMs appear to undergo intratumoural differentiation such that there becomes a preponderance of M2 Macrophages. This in turn promotes tumour growth and is associated with worse clinical outcomes.[112]

1.3.5 Experimental animal tumour models

Several experimental animal tumour models are available for investigating tumour growth and response to treatment in a preclinical setting. The benefits and disadvantages of genetically engineered tumour models, humanised mouse tumour models and transplantable tumour models are discussed below:

Genetically engineered tumour model: many mouse strains with mutations in immune response genes have a susceptibility to the development of cancer. This predisposition can be used either using a temporal model where mice are allowed to age and frequency and nature of tumours studied. Alternatively the genetic predisposition can be combined with a further insult such as exposure to carcinogens (eg. 3-Methylcholanthrene, MCA) akin to the Knudson two-hit theory for tumour development[113]. The benefit of this model is its ability to reproduce the

multistage pathogenesis of cancer along with imitating the interaction between cancer cells and their microenvironment. This model has also proven useful in developing an understanding of the mechanisms involved in immunoediting and immune surveillance[114-116], which were discussed in section 1.2.2. Because of the considerable variability in onset and nature of tumours that develop, this model does not lend itself well to comparative studies of different interventions for which in one would aim to have several animals developing comparable tumours starting at the same point of time.

Humanised mouse models of cancer: This model utilises human tumours xenografted into immunodeficient mice. Using actual human tumours has certain advantages over other experimental models, particularly in the investigation of novel biological and cytotoxic agents. From an immunological perspective, whilst it is technically possible to immunologically reconstitute these mice[117], when it comes to studying the interaction between tumour and cells of the immune system, other tumour models have clear advantages.

Transplantable tumour model: Tumour cell lines including B16, CT26 and EL4 are injected subcutaneously, orthotopically (e.g. injection of breast cancer cell line into mammary fat pad) or intravenously. Benefits of this model include the speed and reproducibility of tumour growth. The technique also lends itself well to expression of transduced model antigens, including ovalbumin, which coupled with the availability of mice expressing transgenic T cell receptors specific for such antigen, allows investigation of T cell - tumour interactions.[118] Transduced foreign antigens, such as ovalbumin, are more immunogenic than intrinsic antigen and as such may result in T cell tolerance. This issue can be overcome by using endogenous tumour antigens, recognised by CD4 or CD8 T cells.[119-121] Possible disadvantages to using transplantable tumour models include that both local injection and sudden delivery of a large number of tumour cells at a site will induce a degree of local inflammation that may affect

therapeutic responses. This would be predicted to be particularly problematic with tumour lines, such as EL4, that are intrinsically immunogenic as compared to those, such as B16, which are poorly immunogenic.[122] Most importantly, as discussed further in sections 1.4, 3.1, 4.1 and 5.1 of this thesis, experimental transplantation tumour models have in several cases successfully identified active immunotherapy targets that have subsequently been shown to also have activity against human tumours in the clinical setting. In other instances, also discussed in sections 3.1, 4.1 and 5.1, successful immunotherapeutic interventions in transplantation tumour models have failed to translate to the clinic or have failed to predict toxicities in man and further research to better predict both is therefore required.

Whilst none of the experimental tumour models provide a perfect model for human tumours, the experiments described in this thesis utilise the transplantable tumour model in view of its considerable advantages over the other models discussed.

1.4 Immunological anti-cancer therapies

Immunotherapies, together with other biological therapies are gaining a role in cancer treatment alongside the established treatment modalities of surgery, radiotherapy and chemotherapy.

There are three main classes of immunotherapies that are of clinical interest, and which will be discussed in further detail below. These approaches are not mutually exclusive and in fact compliment one another.

1. nonspecific stimulation of the immune system
2. active immunization using cancer vaccines
3. adoptive cell transfer immunotherapy

1.4.1 Non-specific stimulation of the immune system

These are immunological therapies that do not target a specific cancer antigen but rather represent therapies that are able to change the existing immune homeostasis in favour of enhanced anti-tumour responses.

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

CTLA-4, also known as CD152, is a member of the immunoglobulin superfamily which also includes the structurally related molecule CD28. The genes for both molecules are located adjacent to one another in both humans (chromosome 2) and mice (chromosome 1).[123,124] Both molecules form homodimers and both bind the ligands CD80 and CD86 which are expressed on antigen presenting cells.[125] The binding affinity is different as are the downstream signals with CD28 enhancing T Cell activation and CTLA-4 inhibiting T Cell activation through the TCR.[126-131] Inhibition of CTLA-4 has been demonstrated to cause tumour rejection in several pre-clinical murine tumour models.[132-136] Although classified as non-specific, the tumour rejection seen is mediated through enhanced response to tumour antigen.[137] Ipilimumab, a fully humanised CTLA-4 blocking monoclonal antibody, has been licensed by the FDA for the treatment of unresectable or metastatic melanoma. Several phase 2 and phase 3 clinical trials demonstrated its efficacy in the treatment of advanced melanoma.[138-141] It also appears to have activity in the treatment of prostate cancer and pancreatic cancer.[142,143] Tremelimumab, a second monoclonal antibody targetting CTLA-4 has also shown activity in patients with advanced melanoma, but to date no improvement in overall survival in a randomised controlled trial setting.[144-147] It appears to have little anti-tumour activity in several other tumour types investigated.[145,148-150]

1.4.1.2 Programmed Cell Death -1 (PD-1) and its ligands

PD-1 is another immunoregulatory molecule. Naïve T cells do not express PD-1, but T cell activation leads to its expression in both humans and mice.[151,152] Activated B cells and monocytes also express PD-1 as do several other cell types including NK cells and DCs.[152] PD-1 has two known ligands, PD-L1, alternatively named CD274 or B7-H1[153,154] and PD-L2, alternatively named CD273 or B7-DC[155,156]. PD-L2 has a higher affinity for PD-1 than PD-L1.[157] Whilst mRNA encoding for PD-L1 is found in a large number of normal tissues in both mice and humans[153,154,158], the molecule itself is normally expressed only on cells of macrophage lineage. This is not the case with tumours where it is found to be expressed on many murine tumour cell lines along with most human tumours.[158-162] Manipulation of the PD-1 / PD-L1 axis is showing some promise in several clinical settings. Recent trials show improved survival in metastatic melanoma.[163-167] Patients with non-small cell lung cancers recruited into two separate phase I clinical trials targeting PD-1[66] and PD-L1[67] have also shown good responses to inhibition of the PD-1/PD-L1 pathway, with overall response rates in approximately one quarter of heavily pretreated patients. In patients whose tumours expressed high levels of PD-L1 however, the response rate was considerably higher with more than two thirds of such patients responding to blockade of the PD-1/PD-L1 pathway.[168,169] The pathway has also been targeted in patients with relapsed follicular lymphoma. A Phase II clinical trial using combined blockade of PD-1 and CD20 appears to improve survival compared to previously published data for blockade of CD20 alone.[170]

1.4.1.3 Interleukin-2 (IL-2)

Anti-tumoural effects of IL-2 have been recognised for over 30 years and result from expansion and activation of specific T cells. [171,172] It owes its anti-tumour properties to its ability to expand

and activate specific T cells. In prospective and retrospective trials in patients with melanoma, whilst the treatment is associated with considerable toxicities, 15-20 per cent of patients with metastatic melanoma demonstrate an objective response to high-dose IL-2 therapy with approximately 5% achieving a complete response.[172-175]

1.4.2 Active immunization using cancer vaccines

The last few years have been an exciting time for anti-cancer vaccines, as exemplified by the following two examples. FDA approval has recently been given to the first anti-cancer vaccine shown to prolong survival in a phase III randomised controlled trial. Provenge (Sipuleucel-T) is a personalised vaccine against prostate acid phosphatase (PAP), a tissue antigen expressed by most prostate cancers. Autologous peripheral blood mononuclear cells (PBMCs) obtained by leukapheresis were incubated with the fusion protein PA2024, which combines recombinant PAP with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). Antigen-presenting cells within the autologous PBMCs were activated by the GM-CSF leading to activation and induced replication of PAP-specific immune T cells able to recognize and kill PAP-positive prostate cancer cells. This therapy has been shown to improve 2 year survival in patients with metastatic castration resistant prostate cancer by 10% compared to placebo (31.7% versus 21.7%) and prolongs mean survival by just over 4 months.[176,177]

Another cancer vaccine which is generating some interest in late phase trials, albeit with mixed results, is the gp100 melanoma antigen vaccine. In one phase III trial, patients were randomised to receive high dose IL-2 in the control arm or IL-2 with gp100 peptide administered with incomplete Freund's adjuvant, in the trial arm. Patients randomised to receive the gp100 arm had significantly better response rates of 16% compared to only 6% in

the control arm. There was also a 6.7 month improvement in median survival to 17.8 months in favour of the trial medication.[178] This trial is slightly difficult to interpret in view of the low overall survival of 6% in the control IL-2 arm being considerably lower than the historical predicted rate of 15-20%.[172-175] Furthermore in a phase III trial comparing gp100 alone, ipilumumab alone or ipilumumab with gp100, the gp100 vaccine appears to have little single agent activity and does not enhance the effects of ipilumumab.[179]

A related vaccine approach involves using irradiated autologous tumour cells transduced to secrete GM-CSF and injected intradermally as a vaccine. This approach which has to date been assessed in phase I and phase I/II trials appears to have therapeutic activity in patients with a wide spectrum of malignancies including melanoma[180-182], renal cell carcinoma[183], prostate cancer[184], pancreatic cancer[185], bronchioalveolar and other non-small cell lung cancers[186,187], but has not yet been assessed in a randomised phase 3 trial.

1.4.3 Adoptive cell transfer immunotherapy

A third therapeutic approach involves adoptive transfer of immune cells. This approach was touched upon briefly in the context of the Provenge (Sipuleucel-T) personalised vaccine, but does not by necessity rely upon co-administration of exogenous vaccine. Tumour infiltrating lymphocytes (TILs), which can be isolated from tumours and expanded and activated in-vitro and then returned to patients is an approach which is showing promise in patients with melanoma. In three separate small phase II trials including 68 patients with metastatic melanoma approximately half of treated patients consistently have measurable responses with approximately one in ten having a complete response.[188-190]

1.5 Thesis Aims

As outlined above, there are several hurdles that need to be overcome for cancer immunotherapy to be successful. Genetic modification of tumour cells, with the aim of simultaneously activating multiple arms of the immune system, has the potential to overcome many of them and this approach is investigated in the following chapters.

2. Materials and Methods

2.1 Tissue Culture

All tissue culture work was carried out using sterile technique in a HEPA-filtered, laminar-flow microbiological safety cabinet. All cell lines were incubated at 37°C and 5% CO2. Sterile, disposable plastic pipettes, flasks and tubes were used.

2.1.1 Tumour Cell lines

2.1.1.1 B16-F10 Cell Line

B16-F10 is a murine melanoma tumour cell line on C57Bl/6 background which is maintained in DMEM media supplemented with 10% FBS, L-glutamine, 1% non-essential amino acids (v/v), 50U/ml penicillin, 50mg/ml streptomycin. This cell line was transduced with CD54, CD70 and CD86 individually and in combination, using lentiviral vectors and sorted for the relevant molecule(s) as described in section 2.3.1 below. Despite several attempts, it was not technically possible to transduce this cell line with either CD48 or a lentiviral vector cassette containing CX3CL1, IFNγ, IL-12 and GM-CSF.

2.1.1.2 CT-26 Cell Line

CT-26 is an murine colorectal tumour on the BALB/c background originally generated by intra-rectal N-nitroso-N-methylurethane-(NNMU) injection, maintained in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, 100µg/ml streptomycin, 100U/ml penicillin. This cell line was transduced with CD48, CD54, CD70 and CD86 both separately and all four in combination using lentiviral vectors. The cell line was also transduced using a lentiviral cassette containing 4 genes encoding CX3CL1, IFNγ, IL-12 and GM-CSF in combination. In addition this cell line

was also transduced with all eight of the above genes in combination. Cells were sorted for their respective molecule(s) as indicated in below.

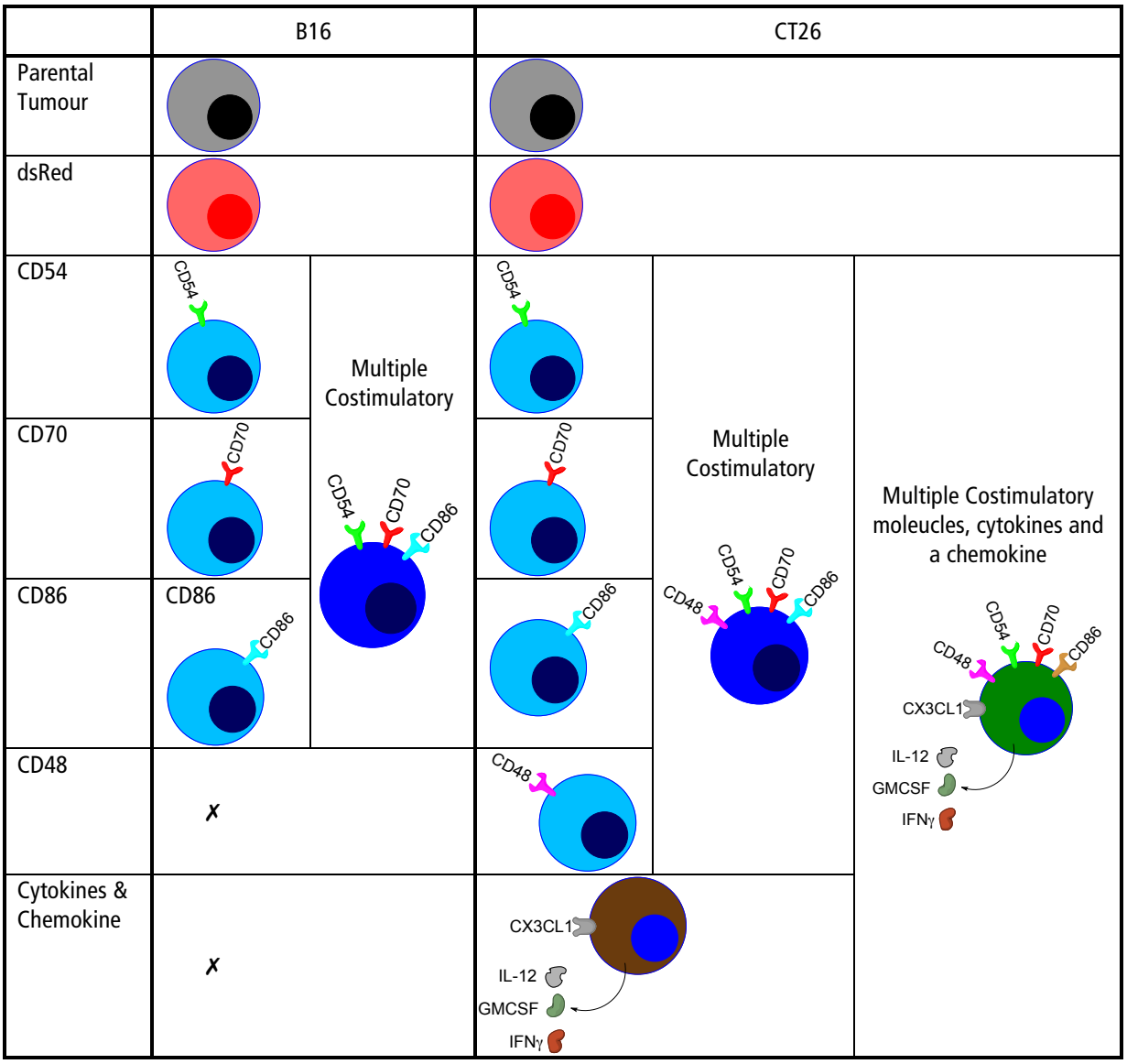


Figure 2.1: Cartoon illustration of lentiviral constructs transduced onto B16-F10 & CT26 tumour cells lines

2.1.1.3 EG7 Cell line

The EG7-OVA cell line was obtained from the American Type Culture Collection and is a mouse T lymphoma line transfected with an ovalbumin encoding vector conferring aminoglycoside resistance.[191] EG7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, L-

glutamine, 1% nonessential amino acids (v/v), 50 U/ml penicillin, 50 mg/ml streptomycin and 2-ME. Geneticin® (G418, #11811-031, Invitrogen) 0.4 mg/ml was added to maintain ovalbumin expression. This cell line was transduced with CFP and the EG7-CFP tumours transduced with IL-2 / IL2R and/or IL-15 / IL-15R lentiviral constructs as described in section 2.3.1and summarized in figure 2.2 below.

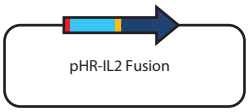
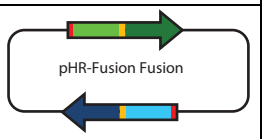
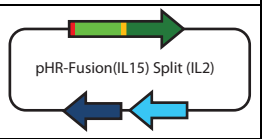
Abbreviation	Definition	Lentiviral Construct
EG7-CFP	EG7 tumours transduced with Cyan Fluorescent Protein but not with any IL-2 or IL-15 constructs	
EG7-CFP-IL-2	EG7-CFP cells transduced with IL-2 fused to the α-subunit of the IL-2 Receptor	
EG7-CFP-IL15	EG7-CFP cells transduced with IL15 fused to the α-subunit of the IL-15 Receptor	
EG7-CFP-FF	EG7-CFP cells transduced with both IL-2 and IL-15 fused to the α-subunits of their respective receptors	
EG7-CFP-FS	EG7-CFP cells transduced with IL-15 fused to the α-subunit of its receptor, along with IL-2 and the α-subunit of its s receptor which are not fused (split)	

Figure 2.2: Cartoon illustration of lentiviral constructs transduced onto EG7 cells line

2.1.1.4 EL4 Cell line

The EL4 tumour cell line was originally derived from ascetic fluid of a C57BL/6N mouse lymphoma and are therefore typed as lymphoblastic. They are maintained in a similar manner to EG7-OVA, with the exception that G418 is not added to the media.

2.1.2 Cell Sorting Efficiency

Cells Sorting by FACS Aria at the Weatherall Institute of Molecular Medicine FACS sorting facility. Cells were sorted to the following percentage positivity: B16-CD54 98.0%, B16-CD70 96%, B16-CD86 93%, B16-multiple costimulatory molecules 93.3% triple positivity (individual molecules sorted to 97.3-98.0% positivity), B16-dsRed 92%, CT26-CD48 99.3%, CT26-CD54 98.4%, CT26-CD70 99.9%, CT26-CD86 100%, CT26-dsRed 58.9%, CT26-multiple costimulatory molecules 91.5% quadruple positivity (individual molecules 97.0-98.6% positivity), CT26 tumours expressing the cytokines and chemokine were sorted on the basis of surface CX3CL1 to 62.3% positivity. Multiple costimulatory molecules, cytokines and chemokine were sorted to CX3CL1 58% positivity and costimulatory molecules to 96.9% for all four (individual molecules 98.0-99.8% positivity).

2.1.3 Cell Counting

Adherent cells were lifted from the flask in PBS supplemented with 1% EDTA. Non-adherent cells were removed from the flask and counted directly. 20μL of cellular suspension and equal quantity of trypan blue were mixed and counted manually using a haemocytometer with assessment of cell viability on the basis of uptake of the trypan blue through compromised membranes.

2.1.4 Freezing, Thawing and Storage of Cells

Following the successful transfection of cells, aliquots were frozen to preserve the lines. To freeze, adherent cells were initially lifted from the surface of the flask in PBS supplemented with 1% EDTA. Cells were counted and $10x10^6$ cells were spun at 1500rpm for 3 minutes and

the pellet resuspended in 1ml freezing solution (10% dimethyl sulphoxide in foetal calf serum). The suspension was transferred to cryovials and frozen at -80°C in a freezing chamber that allows for a gradual decrease in temperature. Cryovials were then transferred to liquid nitrogen. To resuscitate cells, vials were removed from the liquid nitrogen and defrosted at 37°C and the cell suspension washed in 30mls of warm tissue culture medium. Cells were spun out at 1500rpm for 3 minutes and the pellet resuspended in 10mls of fresh tissue culture medium before transferring to a 25cm2 flask. The viability of the cells was assessed microscopically and incubated prior to experimental use.

2.1.5 Mycoplasma Testing

Mycoplasma testing of all cell lines was undertaken at regular intervals and prior to in vivo injection.

2.1.6 Cell irradiation

Where indicated, cells were irradiated with Caesium-137 irradiator located in the Biomedical Service Unit of the John Radcliffe Hospital. Dose to cells quoted in Gray, Gy, as calculated on the most recent calibration in 2007 and known rate of Caesium 137 decay.

2.2 Mice

All mouse experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under approved personal and project licenses issued by the United Kingdom Home Office. All mice were aged six to twelve weeks and sex matched at commencement of experiments. Mice were housed in a specific pathogen free animal facility unit of the

Biomedical Services Unit, John Radcliffe Hospital, Oxford, UK. To maintain high standards of animal welfare and minimise the risk of any suffering, all mice were assessed daily with regular monitoring of tumour burden, and assessment for the presence of tumour ulceration, weight loss and other signs of distress. Animals with weight loss of 20% of body weight, tumour burden of 1000 mm^3, tumour ulceration or signs of distress including subdued or unresponsive behaviour, hyperactivity, piloerection, hunching and/or abnormal gait for more than 24 hour, diarrhoea or dehydration (skin tenting) or neurological symptoms such as peripheral neuropathy were culled immediately using an approved home office schedule 1 method.

2.2.1 C57Bl/6

C57Bl/6 mice were bred and housed in the Biomedical Services Unit of the John Radcliffe Hospital.

2.2.2 C57Bl/6 RAG1$^{-/-}$

C57Bl/6 RAG-1 knock out mice were kindly provided by Dr Andrew Bushell. These mice are deficient in RAG-1, Recombination activating gene 1, which is involved in activation of immunoglobulin V-D-J recombination. They therefore lack mature B or T lymphocytes.[192]

2.2.3 BALB/c

BALB/c mice bred and housed in the Biomedical Services Unit of the John Radcliffe Hospital.

2.2.4 BALB/c Common Gamma Chain RAG Knockout Mice

BALB/c recombination–activating gene 2 knockout and common γ chain knockout (BALB/c Rag2$^{-/-}$/γ$^{-/-}$) is a double knock out transgenic mouse. These mice lack both the RAG-2, Recombination Activating Gene 2 and the interleukin-2 receptor subunit gamma (CD132). This gene is also known as the common gamma chain as it is common sub-unit to several cytokine receptors: IL-2, IL-4, IL-7, IL-9, IL-15 and interleukin-21 receptor. Functionally, these mice lack T cells, B cells and NK cells.[193] The strain was originally obtained from Charles River Laboratories, bred and housed in the Biomedical Service Unit, John Radcliffe Hospital and were a gift from Dr Andrew Bushell.

2.2.5 F5 T Cell Receptor Transgenic mouse

The F5 T Cell Receptor transgenic mouse expresses a T-cell receptor isolated from the cytotoxic T Cell clone F5 on most (>80%) of thymocytes and peripheral T cells.[194] This T Cell clone is CD8 positive and recognizes αα366-374 of the nucleoprotein (NP 366-374) of influenza virus (A/NT/60/68), in the context of Class, MHC D^{b}.[195] Mice were bred and housed in the Biomedical Service Unit, John Radcliffe Hospital.

2.2.6 FELIX Chimeric PD-1 mouse

The FELIX mouse is a transgenic knock-in mouse strain, on the C57Bl/6 background, which expresses human/mouse chimeric PD-1. The exon encoding the ligand binding V-set IgSF domain of the endogenous murine pdcd1 gene has been swapped for the equivalent exon from the human PDCD1 gene.[196] Mice were bred and housed in the Biomedical Service Unit, John Radcliffe Hospital and were a gift from Professor Simon Davis.

2.2.7 GFP mice

The GFP mouse strain used in these experiments is UBI-GFP/BL6. GFP is expressed on all tissues examined with the highest levels present in hematopoetic cells. B cells, T cells, and dendritic cells express distinct levels of GFP fluorescence.[197] The strain was originally obtained from the Jackson Laboratories, bred and housed in the Biomedical Service Unit, John Radcliffe Hospital and were a gift from Professor Richard Cornall.

2.2.8 OT-1 OVA T Cell Receptor Transgenic mouse

CD8+ T cell specific for ovalbumin 257-264 in the context of Kb

OT-I mice express the Va2, V65 T cell receptor derived from the Ovalbumin restricted peptide OVA257-254-specific cytotoxic T cell clone 149.42. This T cell uses rearranged Va2Ja26 and Vf35-D82-Jf32.6 T cell receptor α- and β-chains, respectively.[198-200] This strain is bred and housed in the Biomedical Service Unit, John Radcliffe Hospital

2.2.9 GFP-OT1

These mice are the first generation offspring of UBI-GFP/BL6 transgenic and OT-1 OVA T Cell Receptor Transgenic strains described above. These mice were bred, characterised and maintained by me in the Biomedical Service Unit, John Radcliffe Hospital.

2.3. Techniques

2.3.1 Lentiviral Transduction and Cell Sorting

10,000 tumour cells were plated in 96 well flat bottom plates, and left to adhere at 37°C for 2

hours. Media removed and replaced with 100μL lentivirus. After a further 2 hour incubation at 37°C, 100μL media added and cells returned to incubator overnight. The following morning, supernatant removed and replaced with fresh media. Cells bulked and then sorted on the WIMM FACS Aria machine for molecule(s) selected.

2.3.2 Tumour injection

All tumours were washed twice in RPMI media and resuspended in sterile PBS prior to injection. B16-F10 tumours with or without transduced molecules were injected in the subcutaneous tissues of the loin with $1x10^6$ cells in 100μL PBS per mouse using a 29 gauge needle. In the intravenous lung metastasis model, cells were injected in 200μL PBS into the tail vein. For C57Bl/6 mice $5x10^5$ cells were injected and for FELIX mice $1x10^6$ cells. CT26 tumours with or without transduced molecules were injected subcutaneously, $1x10^5$ cells in 100μL PBS per mouse. For experiments involving two separate cell types, $0.5x10^5$ of each cell type were injected per mouse. Single tumour type controls were also injected with $0.5x10^5$ cells. EG7 tumours with or without transduced molecules were injected in the subcutaneous tissue of the loin at concentrations of 0.5 – $5x10^6$ cells in 100μL PBS. Mice were monitored daily throughout.

2.3.3 T-Cell Adoptive transfer

T Cells obtained from the generated GFP-OT-1 transgenic mouse described above, were adoptively transferred into C57Bl/6 mice with the aim of detecting them in tissues obtained from mice injected with Ovalbumin expressing tumours

2.3.4 Tumour Infiltrating Lymphocyte (TIL) extraction

Tumours were removed under sterile technique and placed in petri dish and diced into small pieces with scalpels and then forced through a 70μM filter using sterile 2ml syringe plunger. Flowthrough was collected in 10ml cold FACS wash and spun for 5 minutes at 1200rpm and then resuspended in 5ml cold PBS, which was layered above 3ml cold lymphoprep. This was then spun for 10 minutes at 2000rpm with slow acceleration and deceleration. The lymphocytes at the interface between the PBS and lymphoprep were collected by pastette and placed in FACS tubes. Lymph nodes were extracted as positive controls and were filtered through 70μM, washed and spun at 1200rpm for 2 minutes and pellet retained. Preliminary FACS analysis performed to assess percentage live cells along with TcRβ, CD4 and CD8 positive populations. In-vitro parental and transduced tumours were plated at $1x10^5$ cells per well in 96 U-bottomed well plates and irradiated to an approximate dose of 20Gy using the BMSU Cs-137 irradiator. Wells containing no tumours (labelled as no APCs) were also included. 50μL supernatant removed for ELISA at 24 and 48 hours and wells replenished with fresh mR10. At day 7 fresh irradiated (20Gy) parental or transduced tumour cells were added to the appropriate wells and the cells were returned to incubator and cultured at 37°C for 14 days in total. After this time cells were removed and FACS analysis performed with staining for live cells, TcRβ, CD4 and CD8 positive cell populations.

2.3.5 Splenocyte harvesting

Fresh spleens were individually mashed up in warm R10, passed through a cell strainer and spun .The pellet was resuspended in 2ml of RBC lysis buffer and left at room temperature for 1 min prior to the addition of 20ml R10. Following an additional spin, the resulting pellet was resuspended in 20ml of R10 and cells counted.

2.4. Assays

2.4.1 Fluorescence-activated cell sorting (FACS)

All preparation was performed on ice with cold reagents protected from direct light. Centrifuges were refrigerated to 4°C. Plates were initially washed with PBS twice prior to a 20 minute incubation with 50µl/well with the mastermix of vital (LIVE/DEAD® Fixable Aqua Dead Cell Stain, Invitrogen #L34957, used at a 1:400 dilution) and surface stains for the particular experiment. Samples were analysed using CyAN™ ADP 9 Colour Analyzer by Beckman Coulter. A minimum of 10^4 relevant events were collected, stored ungated and the data analysed with TreeStar FlowJo software v8.7 for Mac. Gating strategy unless otherwise indicated involved exlusion of dead cells and removal of pulse width, forward scatter and side scatter outliers. Statistical analysis and graph plotting was undertaken using Graphpad PRISM v6.0c for Mac.

2.4.1.1 Antibodies

The cell surface antibodies listed in table 3.1 on the next page were used as part of the experiments undertaken for this thesis. Unless otherwise indicated all are conjugated anti-mouse antibodies.

2.4.2 Carboxyfluorescein succinimidyl ester (CFSE) Proliferation Assay

Parental B16-F10 tumours and B16-F10 tumours transduced with the costimulatory molecules CD54, CD70 or CD86 individually and in combination were plated on 12 well plates. Tumours were either pulsed for 1 hour at 37°C with 10^{-7}M FluNP peptide, 10^{-10}M FluNP peptide or unpulsed. Cells were washed twice, placed in incubator at 37°C for two hours and then irradiated in a CS-137 irradiator for 2 minutes at a dose rate of approximately 10Gy/min. Cells

were then combined at a 1:1 ratio with splenocytes harvested from 3 female F5 transgenic mice and labelled with 0.5μM CFSE. Cells were incubated at 37°C for 48 hours and then analysed using a FACSCalibur with CellQuest & Flowjo software, with gating on CD8+ cells excluding dead cells and surviving tumours cells. Experiments were performed in triplicate with results presented as FACS plots, CFSE histogram and proliferation index. The proliferation index is calculated mathematically, using the inverse of the mean CFSE level.

Antibody	Fluorochrome	Clone	Manufacturer	Catalogue Number
CD11b	APC	M1/70	eBioscience	17-0112
CD152 (CTLA-4)	PE	UC10-4B9	eBioscience	12-1522
CD25	Alexa Fluor®488	PC61.5	eBioscience	53-0251
CD25	eFluor®780	PC61.5	eBioscience	47-0251
CD25	FITC	PC61.5	eBioscience	53-0251
CD25	PE	PC61.5	eBioscience	12-0251
CD273 (PD-L2)	PE	TY25	eBioscience	12-5986
CD274 (PD-L1)	PE	M1H5	eBioscience	12-5982
CD4	APC	GK1.5	eBioscience	17-0041
CD4	eFluor®450	GK1.5	eBioscience	48-0041
CD4	PE-Cy7	GK1.5	eBioscience	25-0041
CD4	FITC	RM4-5	eBioscience	11-0042
CD4	APC-eFluor®780	RM4-5	eBioscience	47-0042
CD45R (B220)	FITC	RA3-6B2	eBioscience	11-0452
CD45R (B220)	PE	RA3-6B2	eBioscience	12-0452
CD45R (B220)	eF450®	RA3-6B2	eBioscience	48-0452
CD48	Pacific Blue	HM48-1	BioLegened	103417
CD54	Alexa Fluor®647	YN1/1.7.4	BioLegend	116113
CD69	PE-Cy7	H1.2F3	eBioscience	25-0691
CD70	PE	FR70	eBioscience	12-0701
CD8a	PE-Cy7	53-6.7	eBioscience	25-0081
CD8a	PerCP	53-6.7	BD Biosciences	553036
CD8b	APC	H35-17.2	eBiosicence	17-0083
CD8b	FITC	H35-17.2	eBiosicence	11-0083
CD86	Pacific Blue	GL-1	BioLegend	105022
IL-15Ra	PE	DNT15Ra	eBioscience	12-7149
F4/80	APC	BM8	eBiosicence	17-4801
FoxP3	Alexa Fluor®647	MF23	BD Pharmingen	560401
Live/Dead	Aqua	N/A	Invitrogen	L34957
Live/Dead	Near-IR	N/A	Invitrogen	L10119
Live/Dead	Far Read	N/A	Invitrogen	L10120
NK1.1	APC	PK136	eBiosicence	17-5941
NK1.1	PE-Cy7	PK136	eBiosicence	25-2941
F(ab')2 IgG	PE	Polyclonal	eBiosicence	12-4010
{ mCX3CL1 IgG2A { anti-Rat IgG2A	Purified Rat FITC	1265315 MRG2a-83	BioLegend BioLegend	MAB571 407505

PD-1	FITC	J43	eBioscience	11-9985
PD-1	PE	RMP1-30	eBioscience	12-9981
TcRβ	APC-eFluor® 780	H57-597	eBioscience	47-5961
TcRβ	PE	H57-597	eBioscience	12-5961

Figure 2.1: Antibodies used in Flow Cytometry experiments

2.4.3 VITAL (*in-vivo*) cell killing assay

VITAL – (*in vivo* technique for assessing lysis) Cell killing assay: This technique was initially described by Hermanss et al.[201] Spleens were harvested from male, strain-matched donor mice (one spleen per three recipients), combined and then separated into four tubes suspended in mR10 media in volume of 1ml per tube. Two candidate peptides along with an irrelevant peptide (negative control) were added to 3 tubes at a concentration of 10μM. The fourth tube was not pulsed with peptide as an additional negative control. Cells were incubated at 37°C for 2 hours and then washed. For CFSE Stained splenocytes, cells were washed in PBS and resuspended at concentration of $2x10^7$/ml. CFSE was prepared in PBS at the following concentrations: 2.5μM, 0.5μM and 0.1μM and resuspended with cells in equal volume to achieve a final concentration of $1x10^7$ cells/ml. Cells were then incubated at room temperature for 8 minutes after which an equal volume of FCS was added to quench stain and sharpen population intensity. Cells were then spun immediately, washed twice in mR10 media, and transferred to new tube after each wash. Cells were then resuspended in RPMI media for injection. For Cell Tracker Orange (CTO) stained splenocytes, cells were washed in pre-warmed mR10 and suspended at a concentration of $1x10^7$ cells/ml. CTO was added for final concentration of 10μM and cells were vortexed. Cells were then incubated at 37°C for 15 minutes, spun and then resuspended in pre-warmed mR10 media for a further 20 minutes at 37°C. Cells were then washed twice in RPMI media and resuspended in RPMI for injection. Finally, target cells were mixed together in equal proportions (at a ratio of 1:1:1:1) and $1x10^7$ cells/mouse injected intravenously via the tail vein. Mice were tail bled 24 hours later and the presence and quantification of fluorescent cells was performed by FACS analysis. The percentage survival and killing of candidate peptide-pulsed targets relative to those pulsed with irrelevant FluNP peptide was calculated and analysed by unpaired two-sided t-test using GraphPad Prism statistical analysis software.

2.4.4 Assessment of in-vivo tumour growth

Mice were assessed daily and subcutaneous tumours measured along the longest and shortest axis every 24-72 hours with callipers. Surface area was then calculated by multiplying the two results. In the intravenous lung metastasis model, assessment of tumour volume was made at post mortem, using the same lung lobe, photographed in the same orientation to enable comparison. Lung surface area occupied by metastases as a percentage of total lung surface area was calculated, in a blinded manner. The calculation was automated using either Adobe Photoshop® or ImageJ® with the assistance of the WIMM Computational Biology Group.

2.4.5 Quantifying IgG antibodies against tumours

Serum isolated from mice that survived a CT26 tumour challenge was incubated with $0.5x10^6$ fresh CT26 tumours at a ratio of 1:25 for one hour at room temperature. Cells were washed twice with cold PBS and Goat anti-mouse IgG was added for 20 minutes and then washed off with cold PBS. FACS analysis was performed with dead cells excluded by live staining and percentage IgG positive cells quantified as previously described by Simon et al.[202] Serum from C57Bl/6 mice immunised with intraperitoneal CT26 tumours was used as a positive control and from naïve BALB/c mice as negative controls.

2.4.5 IL-2 / IL-15 Biological Activity Assay

To confirm that the IL-2 and IL-15 genes transduced into EG7-CFP tumours have biological activity, cells were cultured with CTLL-2, an IL-2 and IL-15 dependent cell line.[203,204] CTLL-2 survival was titrated using exogenous human IL-2 demonstrates that than 50 U/ml of human IL-2 is required for normal CTLL-2 survival. Viability of CTLL-2 cells was assessed using a live/dead stain to calculate percentage survival by FACS.

2.5. Statistical Analysis

2.5.1 Unpaired Student's t test

The Unpaired Student's t test was used to calculate statistical significance of transduced tumours as compared to parental tumour controls.

2.5.2 The Log-Rank Test

Log-Rank (Mantel Cox) test was used to asses the statistical significance of differences observed in Kaplan Meir survival curves.

3. Results Chapter: Transduction of costimulatory molecules on to B16 Tumours

3.1 Introduction

Co-signalling molecules play a crucial role in regulating T cell activation during the presentation of antigen by MHC molecules to T cell receptors. These co-signalling molecules, which can be either costimulatory or co-inhibitory receptors, often co-localise with the T cell receptor at the immunological synapse. As their name suggests, costimulatory molecules are cell surface receptors that transduce signals into T cells to positively modulate T cell receptor signalling. By converse, co-inhibitory receptors are involved in negative modulation of T cell signalling.[205-207] In this chapter, the focus will be on the following three costimulatory molecules CD86, CD54 and CD70.

3.1.1 CD86

CD86, alternatively known as B7-2, is a costimulatory molecule normally found on antigen presenting cells (APCs) which binds to CD28 on the T-Cell surface.[208-210] Although T Cell priming is initiated when tumour antigens are processed within APCs and presented to the T Cell via the MHC molecules, this alone is not sufficient for priming an immune response. For this to occur, either CD80 (B7-1) or CD86 (B7-2) must also be present on the antigen presenting cell and bind to CD28 on the T-Cell surface. This dual signalling is then able to unlock downstream signalling via the PI3K/Akt pathway, upregulating transcription factor NF-$\kappa\beta$ together with other pro-survival signals including Bcl-2 and BCL-XL.[211] The expression of both CD80 and CD86 are modulated by the activation state of antigen presenting cells. CD86 is constitutively

expressed at low levels on antigen presenting cells with transcription, translation and transportation of both CD80 and CD86 to the cell surface occurring in response to various stimuli including infection, cellular stress and damage.[209,212]. Under normal conditions CD80 is more immunogenic than CD86, however in tumour cells where MHC Class I expression is frequently low, the converse has been shown to be the case.[3,213] When transduced onto MC38 murine colorectal tumours both CD80 and CD86 result in tumour rejection and protection against subsequent parental tumour challenge.[214] However, in a related experiment using the A20 B cell lymphoma cell line, transducing CD86 prevented tumour growth whereas transduced CD80 did not.[215] CD86 transduction onto B16 tumours together with MHC class II has also been shown to partially protect against development of lung metastases in an IV tumour challenge model.[216] The presence of CD86 expressing cells within human nasopharengeal carcinoma is a good prognostic sign[217], and upregulation of CD86 along with CD70 on tumour infiltrating DCs following irradiation has been suggested as contributing to the beneficial effects of radiotherapy.[218,219] Presence of CD86 on tumour cells has been shown to prevent anergy of activated T cells.[220] In human lymphomas, loss of CD86 expression is associated with reduced number of tumour infiltrating lymphocytes[221]. This particular study did not characterise the nature of the tumour infiltrating lymphocytes, and further interpretation is therefore not possible. By expressing CD86 on a tumour cell in these experiments it is theorised that MHC expressing cells within the tumour will be able to present tumour antigen to tumour infiltrating lymphocytes and also prevent anergy of activated T cells.

3.1.2 CD54

CD54 is the murine analogue of human intercellular cell adhesion molecule-1 (ICAM-1). CD54 and ICAM-1 are cell-surface glycoproteins which are ligands for Lymphocyte Function-

Associated Antigen 1 (LFA-1).[222] The ligand-receptor interaction leads to CD4 & CD8 positive T cell mediated tumour killing[223,224] along with non-MHC restricted cytotoxicity mediated by NK cells[224-227] and monocytes[228]. In several experimental animal models, the presence of CD54 has been shown to dramatically reduce tumour growth.[223,228,229] Exposure to CD54-transduced tumours has also been shown to be protective against subsequent exposure to the parental tumour.[229] In an *in vitro* human pancreatic cancer cell model, the presence of either ICAM-1 or ICAM-2 (CD102), is required for tumour-specific-antigen independent γδ-T cell mediated tumour killing.[230] In an orthotopic gastric cancer mouse model, expression of ICAM-1 also results in a reduced risk of lymph node metastases.[231] This finding is supported by several clinical patient cohorts where expression of ICAM-1 on lymphomas, gastric, breast and colorectal cancers, was shown to correlate with increased number of tumour infiltrating lymphocytes, reduced risk of lymph node and liver metastases and improved clinical outcomes compared to patients with non-ICAM-1 expressing tumours.[221,232-234] It should be noted however, that whilst the majority of published clinical cohorts show a favourable association between ICAM-1 expression on tumours and clinical outcomes, this was not a universal finding, with some cohorts demonstrating either non-statistical differences or even a reverse correlation between ICAM-1 expression and prognosis.[235,236] CD54/ICAM-1 has been found to be increased in irradiated cell lines and in irradiated normal tissues sampled from patients undergoing radiotherapy treatment.[237-243] CD54 is also found to be elevated in tumour cell lines following exposure to radiation.[238,244] It has also been implicated with both post-irradiation inflammatory reaction and anti-tumour effect.[244,245] The post-irradiation inflammatory effect is abolished in CD54 knockout mice.[246]

3.1.3 CD70

In both humans and mice, CD70 is the ligand for TNFR family member CD27.[247] In both species, CD27 is found on T cells, B cells, and NK cells.[248-250] CD70 is constitutively expressed on epithelial cells of the thymus in both mice and humans.[250-252] Cells of the immune system including DCs[250,253-256], B cells[257], T cells including regulatory T cells[250,257-259] and NK cells[252] are able to transiently express CD70 on activation, and thereby regulate the CD70-CD27 interaction, which is involved in survival, proliferation, and lymphocyte differentiation.[250-261] Transduction onto B16-F10 tumours has been shown to result in some growth delay compared to parental B16-F10.[262,263] These findings have been confirmed in TS/A murine mammary adenocarcinoma tumours, which express high levels of MHC Class I relative to the low levels seen in B16-F10 tumours, using both surface CD70[262] and secretory CD70, the latter of which also resulted in some protection against rechallenge with the parental TS/A tumour.[264] In another murine model, subcutaneous or intracranially implanted tumours were rejected following addition of CD70, which also lead to subsequent protection against the parental glioma cell line.[265] A related experiment demonstrated this effect was abrogated by depletion of $CD8^+$ T cells.[266] As is the case with CD86, tumour infiltrating DCs have been shown to have higher levels of CD70 following irradiation which is postulated to contribute to the beneficial effects of radiotherapy.[218,267]

3.1.4 Experimental Model

The B16-F10 murine melanoma cell line on C57/Bl6 background was transduced with the above three costimulatory molecules individually and in combination. This was performed using lentiviral constructs designed and made by Dr David Sleep and Professor Simon Davis. In addition, a lentiviral construct expressing the non-immunologically active fluorescent protein Discosoma red fluorescent protein (dsRed)[268] was also transduced into the same tumour cell

line for use as a negative control. Expression was validated by FACS analysis. The constructs are illustrated diagrammatically in Figure 3.1 below:

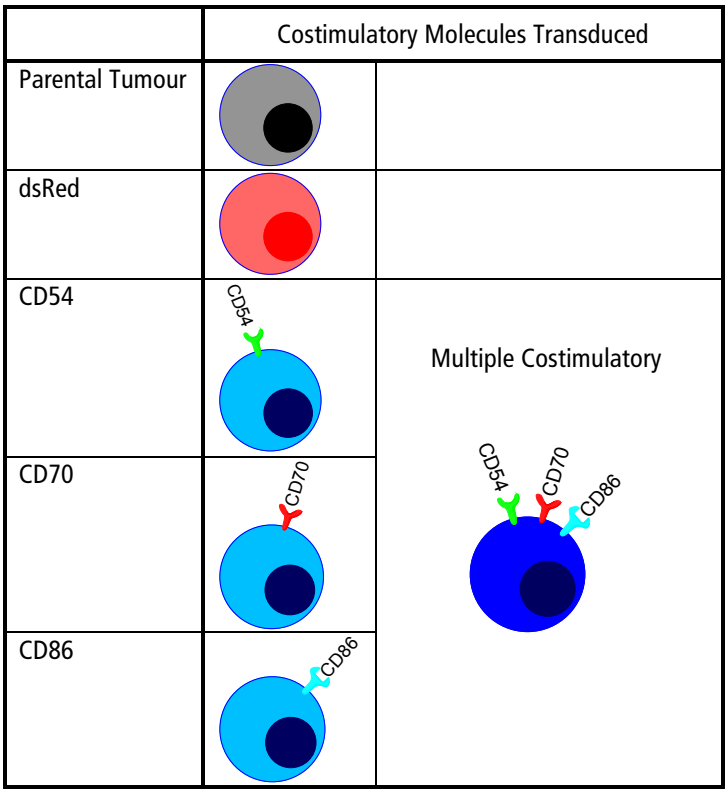


Figure 3.1 Diagrammatic representation of the molecules transduced on to B16-F10 tumours

3.1.5 Pre-clinical Evidence for activity of multiple costimulatory molecules

The literature available linking transduction of the single costimulatory molecules discussed above with enhanced anti-tumour activity *in vitro* and in animal tumour models, as reviewed above, is now quite strong. However, whilst there is evidence that transducing more than one costimulary molecule is possible, the evidence that this confers greater anti-tumour activity is less robust than for the individual costimulatory molecules. Of particular note, the combination of costimulatory molecules assessed here has not previously been assessed. A review of the related literature found two papers supporting the use of multiple costimulatory molecules highlighted only two relevant publications. Grosenbach et al[269] explored the *in vitro* effect up to four transduced costimulatory molecules CD54, CD58 (LFA-3), CD80 and CD252 (OX40L) on

T cells. They demonstrated enhanced initial and sustained activation of naïve and effector T cells, but did not investigate anti-tumour effect *in vivo*. Guinn et al.[215] transduced CD80 and CD86 along with CD137, a member of the tumour necrosis factor receptor family, into the A20 B cell lymphoma cell line and were able to abrogate tumour growth with subsequent protection against the parental tumour.

3.1.6 Clinical Evidence for activity of multiple costimulatory molecules

The literature regarding use of costimulatory molecules in human clinical trials is very sparse. Those few trials that exist, were undertaken as part of cancer antigen vaccination strategies, and were therefore not designed to assess the role of transduced costimulatory molecules. An 18 patient phase I clinical trial by Hörig *et al*,[41] attempted to improve immunisation to the CEA cancer antigen by presenting it in combination with transduced CD80. Patients received serial intramuscular injections with 3 increasing doses of recombinant non-replicating canarypoxvirus expressing both Carcinoembryonic Antigen (CEA) and CD80. The results were somewhat disappointing, with no tumour shrinkage documented. The majority of patients developed progressive disease. Three patients, who received the lowest of the three doses did demonstrate stable disease at 4 months.

PROSTVAC-VF, a phase II randomized controlled trial in patients with metastatic prostate cancer investigated a related approach, combining costimulatory molecules with a cancer antigen rather than whole tumour cells. Patients were injected intradermally with poxviruses (either vaccinia or fowlpox viruses). The poxviruses expressed the cancer antigen, Prostate-Specific Antigen (PSA) along with CD48, CD54 and CD80 injected together with GM-CSF as an adjuvant. In addition to the 82 patients who received the active treatment arm above, an

additional 40 patients received control therapy (2:1 randomisation in favour of active treatment arm). The control arm consisted of empty vector vaccinia and fowlpox virus administered together with normal saline "adjuvant". All patients received the vaccinia based vector as priming followed by 6 fowlpox based vector boosts. Although the trial failed to achieve the primary endpoint of improved disease free survival, there was an improvement in median overall survival (25.1 versus 16.6 months) but no improvement in overall survival, which was similar in both groups.[270] The phase 3 PROSPECT trial, which is further evaluating this approach, is currently underway.[271]

3.2 Results

3.2.1 Genetically Modified B16-F10 tumours *in-vitro* results

To assess whether genetically modified tumours are able to more efficiently prime T Cells, an *in-vitro* CFSE proliferation assay was performed. B16-F10 tumours transduced with the costimulatory molecules CD54, CD70 and CD86, either individually or in combination, were compared to the parental B16-F10 tumour. Irradiated tumour cells pulsed with two concentrations of the influenza nucleoprotein, FluNP, or unpulsed, were co-cultured with CFSE labelled F5 splenocytes, which specifically recognise the FluNP epitope. As illustrated in figure 3.2 A and B below, no increased T Cell proliferation above baseline is observed in the presence of single costimulatory molecules but the presence of multiple costimulatory molecules does result in increased proliferation.

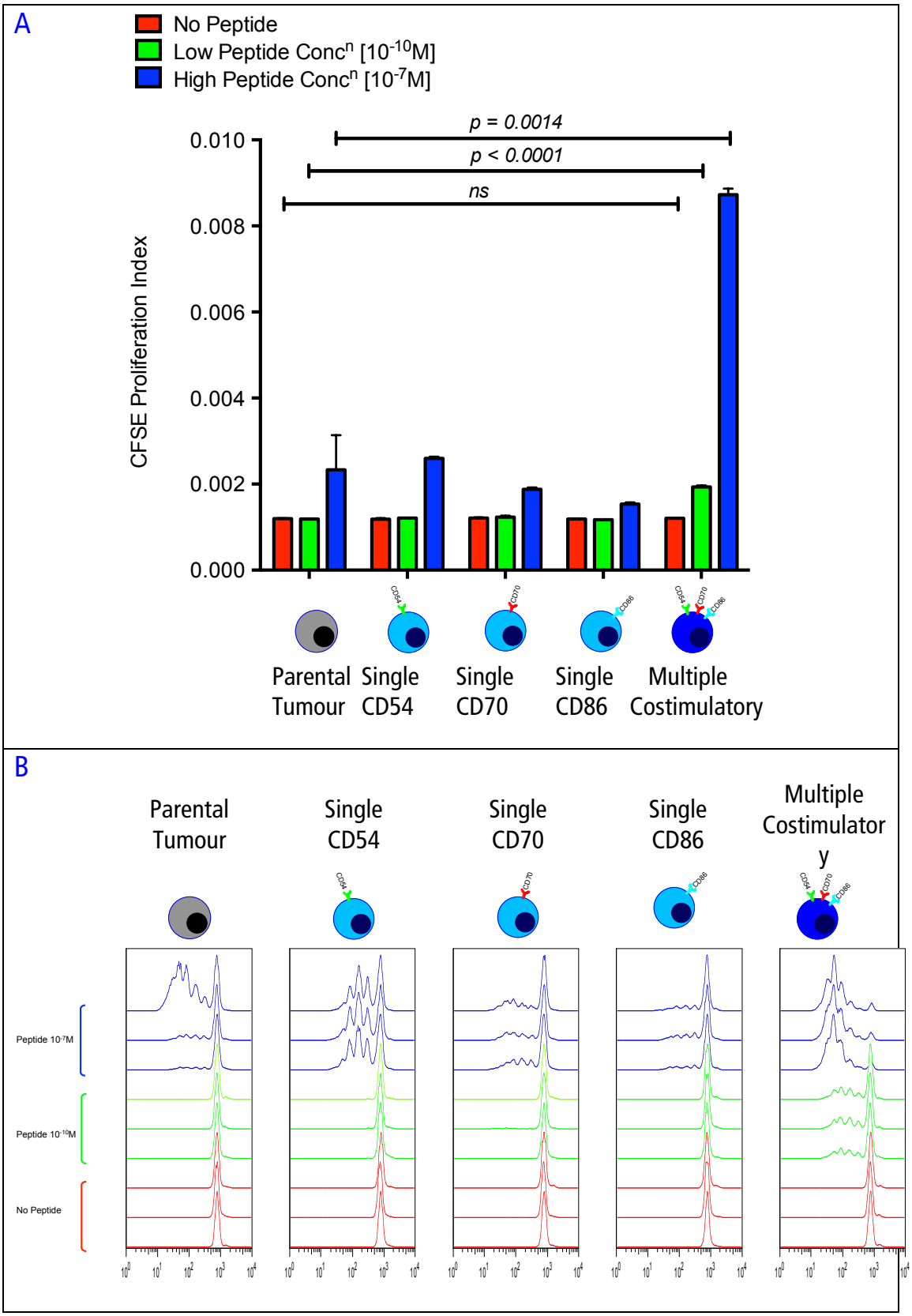


Figure 3.2: A: CFSE Proliferation Index B: CFSE Proliferation Histogram. Improved T Cell priming observed in the presence of multiple costimulatory molecules. CFSE labelled F5 splenocytes proliferation in response to irradiated parental or transduced B16-F10 tumours pulsed with FluNP peptide at concentrations indicated, or unpulsed.

3.2.2 *In-vivo* tumour growth

On the basis of the *in-vitro* results, B16-F10 tumours were injected subcutaneously *in-vivo*. Individual tumour growth is plotted in figures 3.3A-E below and summarised in figure 3.3F. There is a statistically significant difference between the parental tumour size (figure 3.3A) and the tumour transduced with multiple costimulatory molecules (figure 3.3E). By day 14, when the first mice reached the humane endpoint, statistically significant differences (figure 3.3G) were seen between tumour sizes of those tumours transduced with the individual costimulatory molecules CD86 (figure 3.3D) and multiple costimulatory molecules (figure 3.3E) as compared to the parental tumour. Tumours transduced with CD54 (figure 3.3B) and CD70 (figure 3.3C) show a subtle growth delay but this failed to reach statistical significance. As illustrated in the Kaplan Meier plot below (figure 3.3H), all the parental tumour injected mice had reached humane endpoint by day 22, with a mean survival of 16 days and the the single costimulatory molecule tumours, by day 25 (mean survival 20 days). The mean survival in the mice with multiple costimulatory molecules was significantly longer at 35 days with one mouse still tumour free when the experiment was terminated at day 50.

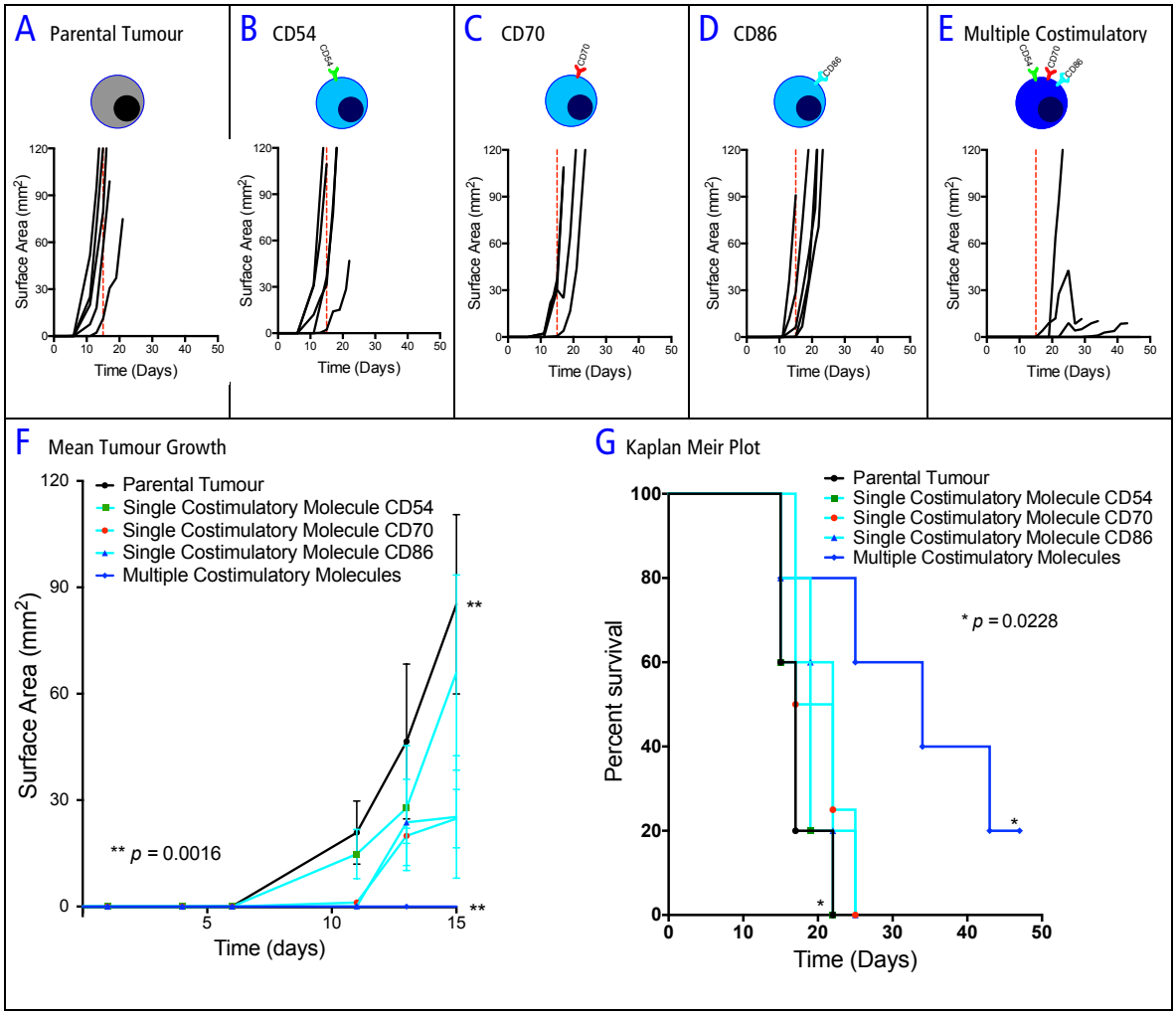


Figure 3.3: Parental B16 tumours versus B16 tumours transduced with costimulatory molecules: The presence of costimulatory molecules impairs tumour growth in an additive manner with three costimulatory molecules resulting in greater effect than any of individual costimulatory molecule. Mice injected with parental B16 tumours were compared to those injected with individual costimulatory molecules (CD54, CD70 or CD86 as indicated) and mice injected with tumours expressing multiple costimulatory molecules (CD54, CD70 and CD86). Experiments performed in C57Bl/6 x HHD F1 mice with 5 mice per group except single B16-CD70 group, which contained 4 mice. **[A-E]** Individual tumour growth plots, illustrating exponential growth in parental **[A]** B16 tumours, with broadly similar growth seen in tumours transduced with **[B]** CD54, subtle growth delay in **[C]** CD70 and **[D]** CD86 expressing tumours, as seen by tumour size at day 15 (red dotted line), and **[E]** significantly impaired growth delay when all three costimulatory molecules present. **[F]** Mean tumour growth with SEM. The difference between B16-parental and B16-Multiple Costimulatory Molecules is statistically significant (p = 0.0014, two-tailed t-test). There was an apparent hierarchy for tumours expressing single costimulatory molecules, with **[B]** CD54 demonstrating least effect, **[C]** CD70 intermediate effect and **[D]** CD86 the most effect, however, with the exception of **[D]** CD86 these differences did not reach statistical significance when compared to **[A]** parental tumour growth. **[F]** Day 15 statistics: Two sided t-test comparing individual tumours to B16-Parental tumours. **[G]** Survival plot – All mice injected with parental B16 tumours or B16 with single costimulatory molecules had reached the humane endpoint by day 25. By contrast, 3 of 5 mice injected with B16-multiple costimulatory molecule tumours are still alive at this time with one mouse tumour still alive and tumour-free at the end of the experiment.

3.2.3 Characterising the role of T & B Cells in tumour growth and rejection

The reduced tumour growth rate seen in-vivo (figure 3.3 above and 3.4A&B below) is dependent on the presence of B or T-Cells. In C57Bl/6 RAG Knock Out mice, which lack these cell types, the difference in growth rates between parental tumours (figures 3.4C) and tumours transduced with multiple costimulatory molecules (CD54, CD70 & CD86) is abolished (figure 3.4D). This is confirmed in the Kaplan Meier plot (figure 3.4E) which shows a statistically significant difference between tumours expressing multiple costimulatory molecules between wild type and RAG mice but not in parental tumours.

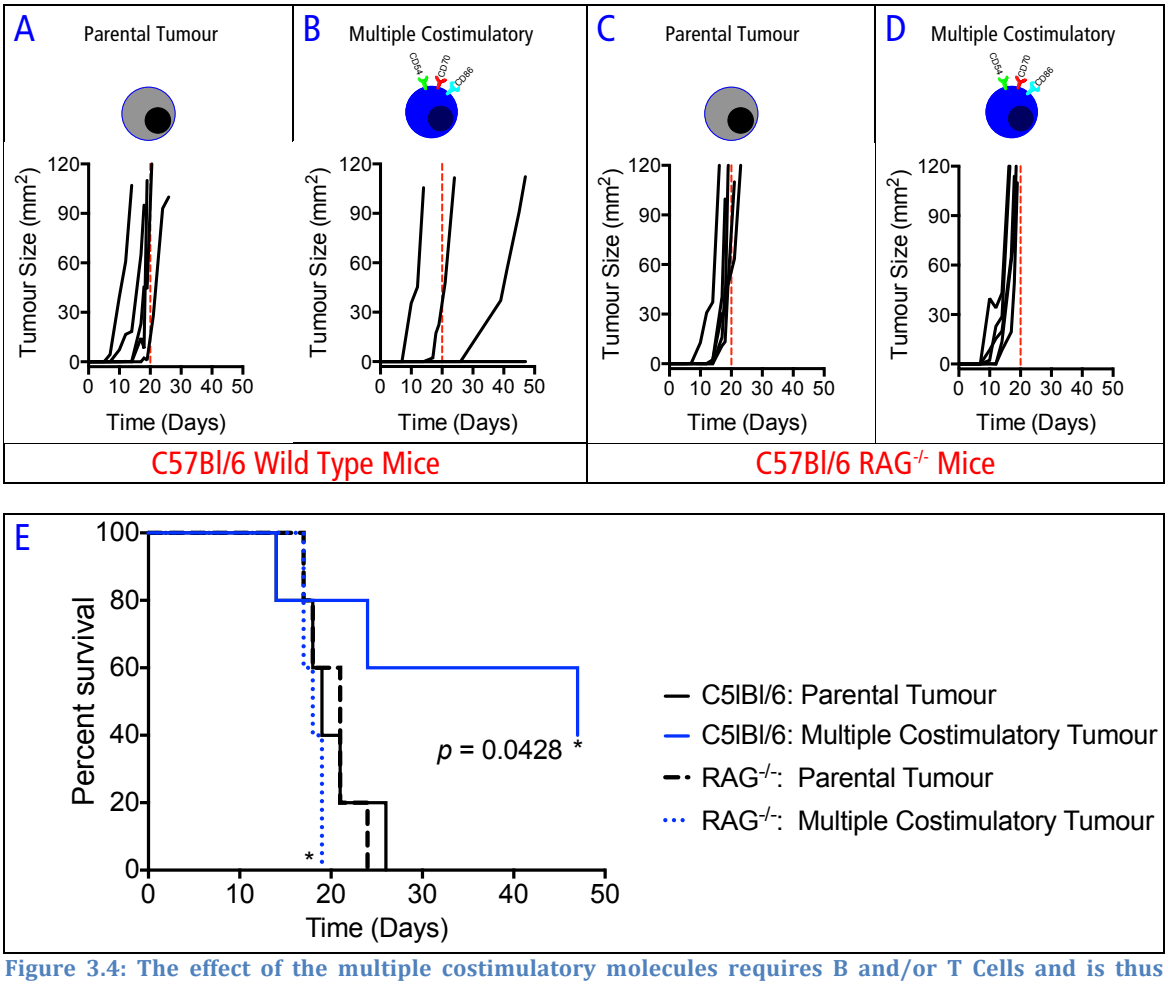


Figure 3.4: The effect of the multiple costimulatory molecules requires B and/or T Cells and is thus abolished in C57Bl/6 RAG$^{-/-}$ mice. B16 parental tumours and B16 tumours expressing multiple costimulatory molecules were injected subcutaneously into C57Bl/6 WT mice and C57Bl/6 RAG$^{-/-}$. n=5/group. Individual tumour growth plots show that in C57Bl/6 wild type mice **[A]** B16 tumours grow normally in all 5 mice, whereas **[B]** B16-Multiple Costimulatory tumours demonstrate delayed or absent growth in 3 of 5 mice, confirming the results in figure 3.3. In contrast, there is no difference between **[C]** B16 tumours and **[D]** B16-Multiple costimulatory molecules in C57Bl/6 RAG$^{-/-}$ mice, with normal tumour growth in all 5 mice in both groups. **[E]** Kaplan Meier Curve demonstrates statistically significant difference (p = 0.0428 by log rank test) when tumours with multiple costimulatory molecules are injected into C57Bl/6 RAG$^{-/-}$ as compared to C57Bl/6 wild type mice.

3.2.4 Characterisation of possible Trp2 antigen specific response to B16-F10

To determine whether the delayed growth in figures 3.3 and 3.4 above is peptide specific, the role of two candidate melanoma associated antigens was assessed. The melanoma associated antigen tyrosinase and tyrosine-related protein-2, Trp2 (SVYDFFVWL)[202,272,273] and Gp100 (EGSRNQDWL)[202,273] along with an irrelevant peptide, influenza nucleoprotein, FluNP (KLGEFYNQMM)[274], were assessed by VITAL in-vivo cell killing assay[201]. On day 22 of the above experiment, described in figure 3.3, the surviving C57Bl/6 mice were injected with peptide-loaded splenocytes, labelled with various concentrations of CFSE, and tail bled 24 hours later. For control purposes, two additional groups of mice were included in the VITAL in-vivo cell killing assay, naïve age and sex matched control mice and F5 strain[194] naïve mice, which specifically recognise the irrelevant FluNP peptide. When comparing mice previously injected with B16 tumours expressing one or more costimulatory molecules to naïve mice of the same strain, no statistically significant decrease in Gp100 peptide loaded splenocytes compared to splenocytes loaded with the irrelevant FluNP peptide is seen (figure 3.5B), however a small but statistically significant decrease in splenocytes labelled with the melanoma antigen Trp2 is seen compared to splenocytes labelled with FluNP (figure 3.5A). As predicted, the Naïve F5 mice specifically reject the FluNP peptide loaded splenocytes (figures 3.5A & B). It is important to note that only two known B16-F10 candidate peptides were assessed and it is therefore possible that other known or unknown B16-F10 epitopes play a greater role in the delayed tumour growth observed.

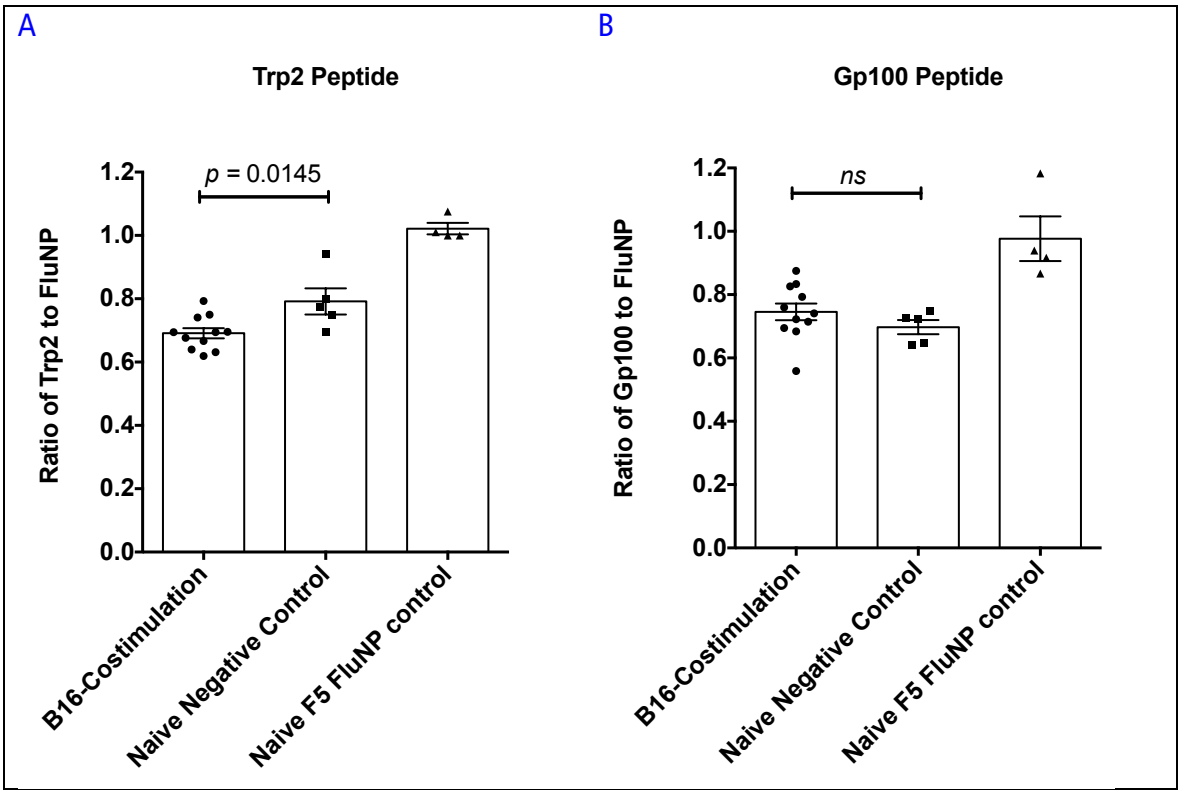


Figure 3.5: Melanoma associated antigen Trp2, but not Gp100, may be implicated in the growth delay observed in B16-F10 tumours expressing costimulaotry molecules. VITAL in-vivo cell killing assay performed to investigate the role of candidate antigens **[A]** Trp2 and **[B]** Gp100 as compared in each case to an irrelevant FluNP peptide.

3.2.5 Effect of multiple costimulatory molecules in a lung metastasis model

As discussed in chapter 1 (Introduction), one potential clinical use of transducing multiple costimulatory molecules onto tumours would be in the metastatic setting. One technique for studying metastatic disease in an experimental model is the intravenous B16 lung metastases model. This technique utilises the black colour of B16-F10 tumours, which derives from their melanocytic origin, to identify metastatic deposits of intravenously injected B16 tumours within the lungs. Parental tumours or tumours expressing control dsRed, CD54, CD70, CD86 or multiple costimulatory molecules (CD54, CD70 and CD86) were injected intravenously into C57Bl/6 mice. After 14 days the mice were culled and lungs removed for FACS analysis and quantification of lung metastases number. In the subcutaneous B16-F10 tumour model used in

the experiments above, the results demonstrate that B or T cells are the most important cell type involved in the differences in tumour growth rates seen. It is known that in intravenous B16-F10 tumour lung metastases models, NK cells play an important role in tumour development.[275]

A single lobe from each mouse was removed to formalin for quantification of the lung metastases (figure 3.6) with the remaining lung tissue collected into R10 media for FACS Analysis. The lungs collected in formalin were photographed, and quantification was performed in Adobe Photoshop® based on a colour threshold, calibrated according to the lungs from naïve mice. The percentage of lung surface area occupied by tumour deposits was used as a marker of tumour burden. Compared to dsRed expressing control tumours there is a statistically significant decrease in the burden of lung metastases that develop in mice injected with tumours expressing CD86 and an even greater decrease in tumours expressing multiple costimulatory molecules (figure 3.6A&B). Lungs of some mice injected with CD54 expressing tumours had a lower tumour burden (figure 3.6A) compared to the control dsRed injected mice but this did not reach statistical significance (figure 3.6B). CD70 expression appeared to offer no protection against the development of lung metastases. (figure 3.6A & B)

The remaining lung lobes taken from mice in figure 3.6 above were processed and stained for FACS analysis. Cells were gated on live cells with additional staining for T Cells (TcR), B Cells (B220) NK cells (NK1.1) and PD-L1. As illustrated in figure 3.7 below there is an increase in T Cells, B Cells, NK Cells and granulocytes within the lungs of the mice injected with tumours expressing multiple costimulatory molecules. In the case of NK Cells, there is a statistically significant increase seen also in the lungs of mice injected with CD86 expressing tumours.

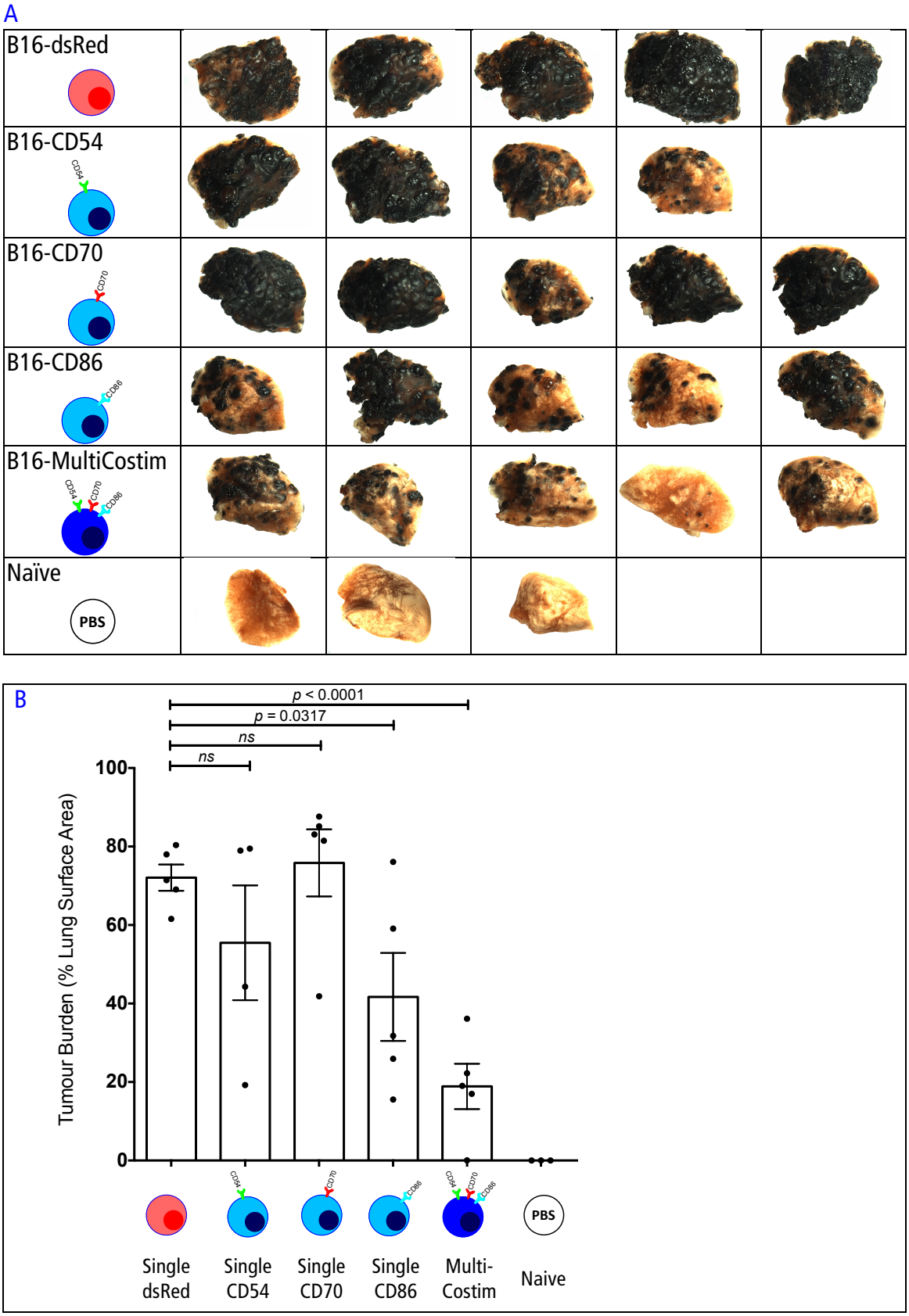


Figure 3.6: Presence of transduced CD86 or multiple costimulatory molecule is partially protective against development of lung metastases [A] Lungs harvested from either naïve mice, or mice injected with tumours as indicated on far left column. Same lobe acquired wherever possible unless damage at time of removal prohibited this. 5 mice per group except B16-CD54 group (n=4) and naïve mice group (n=3). **[B]** graphical representation of percent of lung surface area covered by melanoma metastases, automatically calculated based on pixel colour in Adobe Photoshop®.

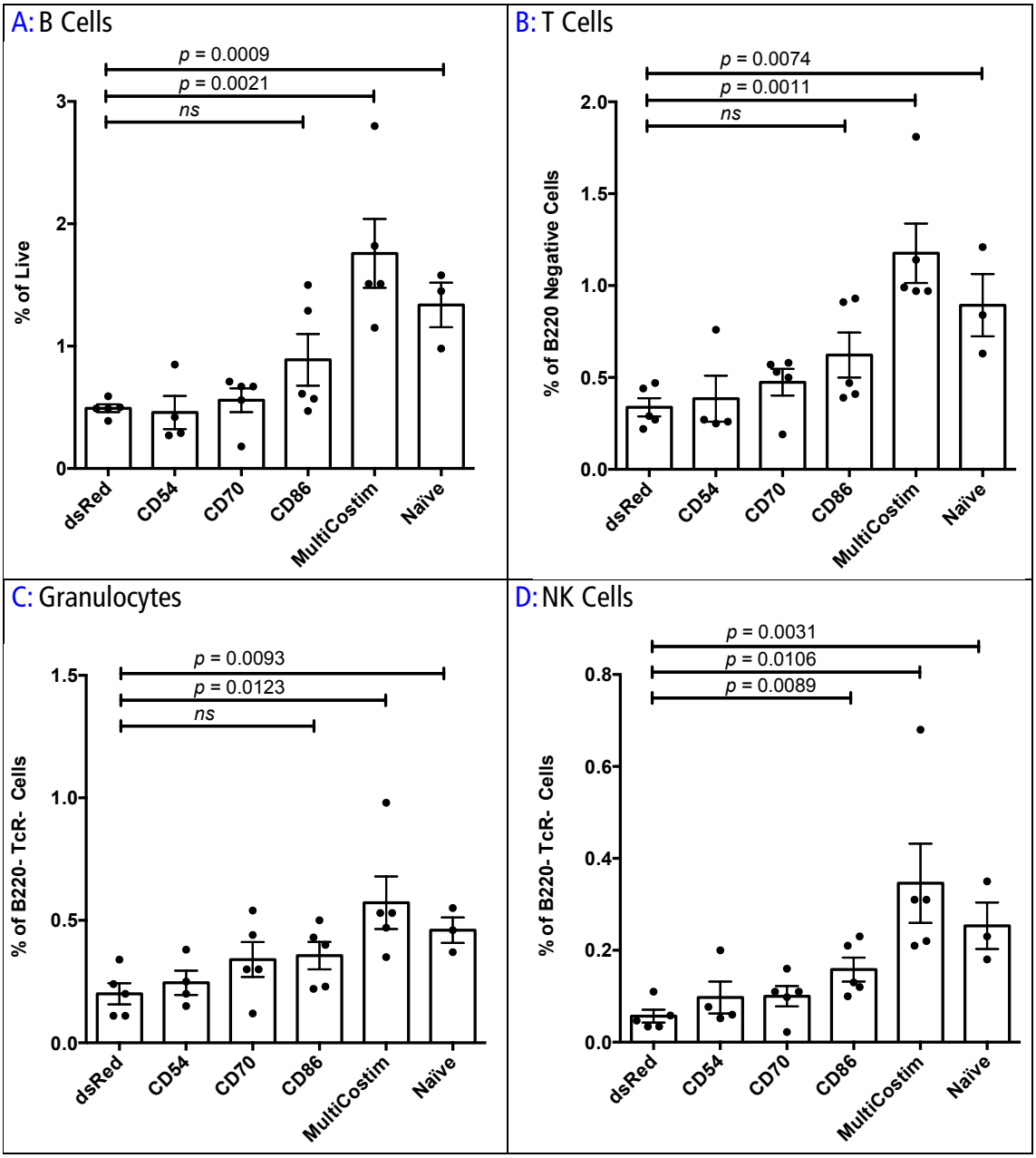


Figure 3.7: FACS analysis of lung tissue corresponding to experiment in figure 3.6 above. One lobe was placed in formalin for assessment of tumour burden. The remaining lung tissue was collected into mR10 for staining and FACS analysis. **[A]** Assessment of number of B Cells, gated on live cells and B220 positive cells. **[B]** Assessment of number of T Cells, gated on live cells and B220 negative, TcR positive cells. **[C]** Assessment of percentage granulocytes as defined by CD11b positivity, gated on live, B220 negative, TcR negative cells. **[D]** Assessment of number of NK cells as defined by NK positivity on live, B220 negative, TcR negative cells

3.2.6 Costimulatory molecules potentiate the effects of PD-1 blockade

As discussed in the introduction, chapter 1, anti-PD-1 blockade has now been shown to be of clinical benefit as an anti-cancer agent in the treatment of melanoma and several other human cancers. To explore the effect of costimulatory molecules in the setting of anti-PD-1 blockade, experiments were conducted using FELIX mice. This homozygous transgenic knock-in mouse strain, on the C57Bl/6 background, expresses human/mouse chimeric PD-1. The exon encoding the ligand binding V-set IgSF domain of the endogenous murine pdcd1 gene has been swapped for the equivalent exon from the human PDCD1 gene.[196] Mice were treated with either intravenous anti-PD-1 antibody or isotype control antibody 5 days prior to intravenous tumour challenge and intraperitoneally with similar antibody, three days post-tumour challenge. Lungs were removed for analysis 13 days post tumour challenge, as opposed to 14 days after challenge in the C57Bl/6 wild type mice above (Figure 3.6) due to concerns for animal welfare from high tumour burden within the lungs. The results of this experiment are illustrated by a representative lung from each treatment group in Figure 3.8A and graphical representation of tumour burden in Figure 3.8B below. In common with C57Bl/6 wild type mice (figure 3.6) there is a trend towards reduced lung metastases in isotype antibody treated mice challenged with CD86 expressing tumours and a further reduction when challenged with tumours expressing multiple costimulatory molecules. In the wild type mice however this trend does not reach statistical significance, possibly as a result of harvesting the lungs a day earlier. In FELIX mice treated with anti-PD-1 antibody however, the reduction in lung metastases tumour burden is far greater in the CD86 expressing tumour group and even more so in the multiple costimulatory tumour group which does reach statistical significance (p=0.0031 for CD86 expressing tumours and p=0.0004 for multiple costimulatory molecules expressing tumours, as calculated using unpaired two-sided t-test). In addition, whereas there is no difference between anti-PD-1 treated mice and isotype control treated mice challenged with B16 parental

tumours, there is a small but non-statistically significant reduction in tumour burden in mice challenged with B16-CD86 tumours and more pronounced statistically significant difference in mice challenged with multiple costimulatory tumours (p=0.0416 by unpaired two-sided t-test). An additional control group incorporating BI6-dsRed were included as part of this experiment but were excluded from the analysis as 2 mice died at the time of initial tumour injection and the remaining mice had fought and received injuries which could potentially have affected the results.

Analysis of both harvested spleens and lungs performed at the completion of the experiment demonstrated an apparent global reduction in PD-1 levels in the anti-PD-1 antibody treated but not isotype antibody treated mice (figures 3.9B & 3.9D) compared with total T-cells in the same organs (figures 3.9A & 3.9C). The in-vivo injected antibody is known to block binding of the commercially available PD-1 antibody (clone J105) used in the FACS analysis. This therefore indicates that the injected anti-PD-1 antibody is still bound 10 days after the final in-vivo injection. Levels of the PD-1 ligands PD-L1 and PD-L2 were measured in the lungs of all mice. Compared to naïve mice, a reduction of PD-L1 is observed in mice injected with B16 and B16 tumours expressing CD86 but not in mice injected with tumours expressing multiple costimulatory molecules. Of note however, whist the tumour injected has an effect on PD-L1 levels, no differences were seen as a result of anti-PD-1 antibody treatment compared to isotype control (figure 3.9E). PD-L2 levels did not appear to be affected by either tumour injection or antibody treatment (figure 3.9F).

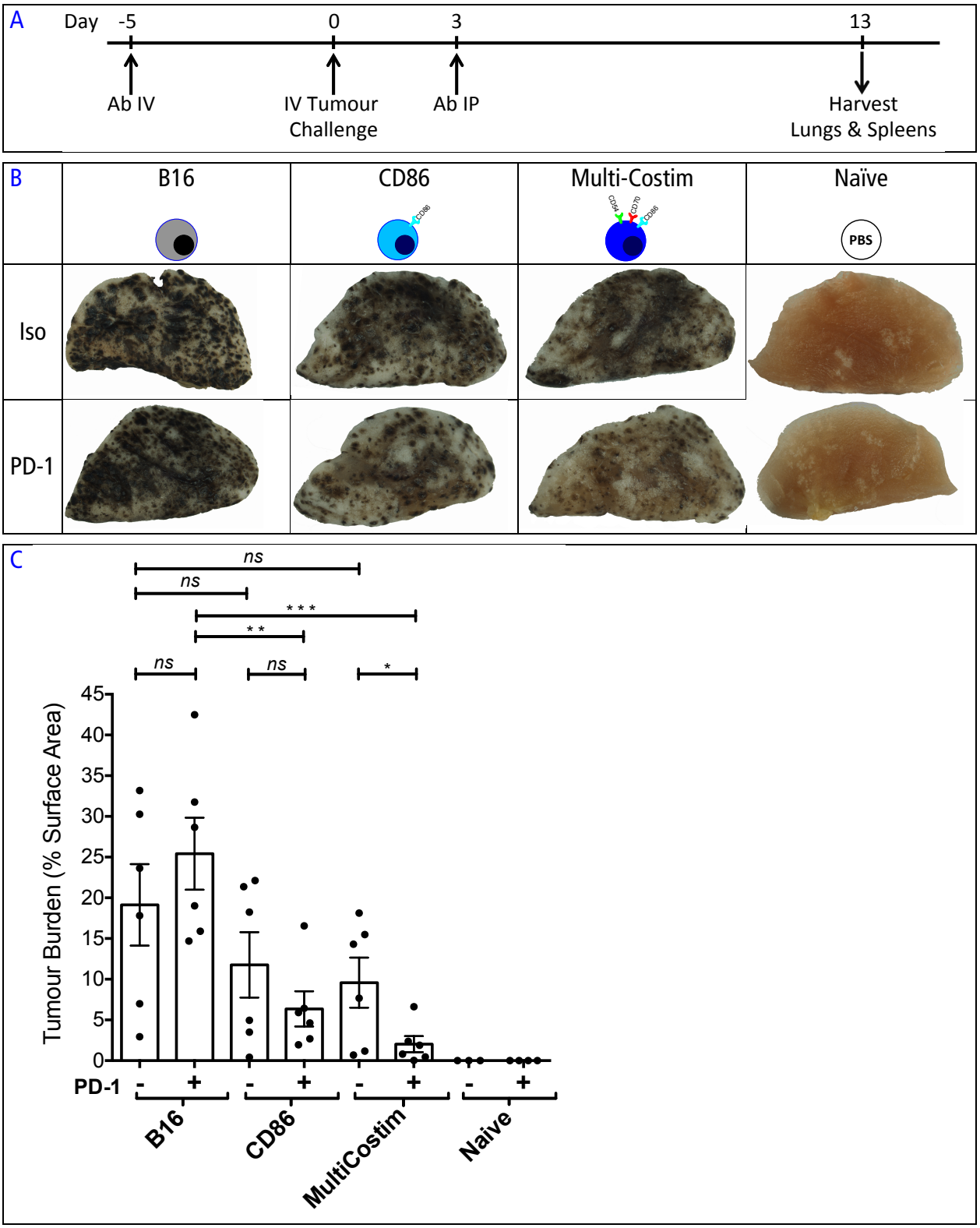


Figure 3.8: Intravenous tumour injection into FELIX mice in the presence of anti-PD-1 antibody or isotype control. [A] Experiment Timeline **[B]** Parental tumour versus CD86 expressing tumours versus tumours expressing multiple costimulatory molecules. One representative lung image shown from each treatment group. Complete set reproduced in ficure 3.8 D overleaf. **[C]** Graphical representation of percentage of lung surface area occupied by tumour metastases, as calculated based on pixel colour in Adobe Photoshop®. When B16 tumours are injected in the presence or absence of anti-PD-1 antibody (PD-1) or isotype control there is no statistically significant difference in the tumour burden. This is also the case where the tumours express CD86, however there is a statistically significant difference following the injection of tumours expressing multiple costimulatory molecules ($p = 0.0416$) expressing tumours. In the presence of isotype control antibody, there is a trend towards reduced tumour burden in the presence of CD86 and multiple costimulatory molecules compared to parental B16, however unlike figure 3.6 above, this does not reach statistical significance. Following treatment with anti-PD-1 antibody there is a more pronounced decrease in tumour burden in the presence of CD86 ($p = 0.0031$) and multiple costimulatory molecules ($p = 0.0004$) compared to B16 parental tumour. **[D]** Complete set of images from figure B above (overleaf).2

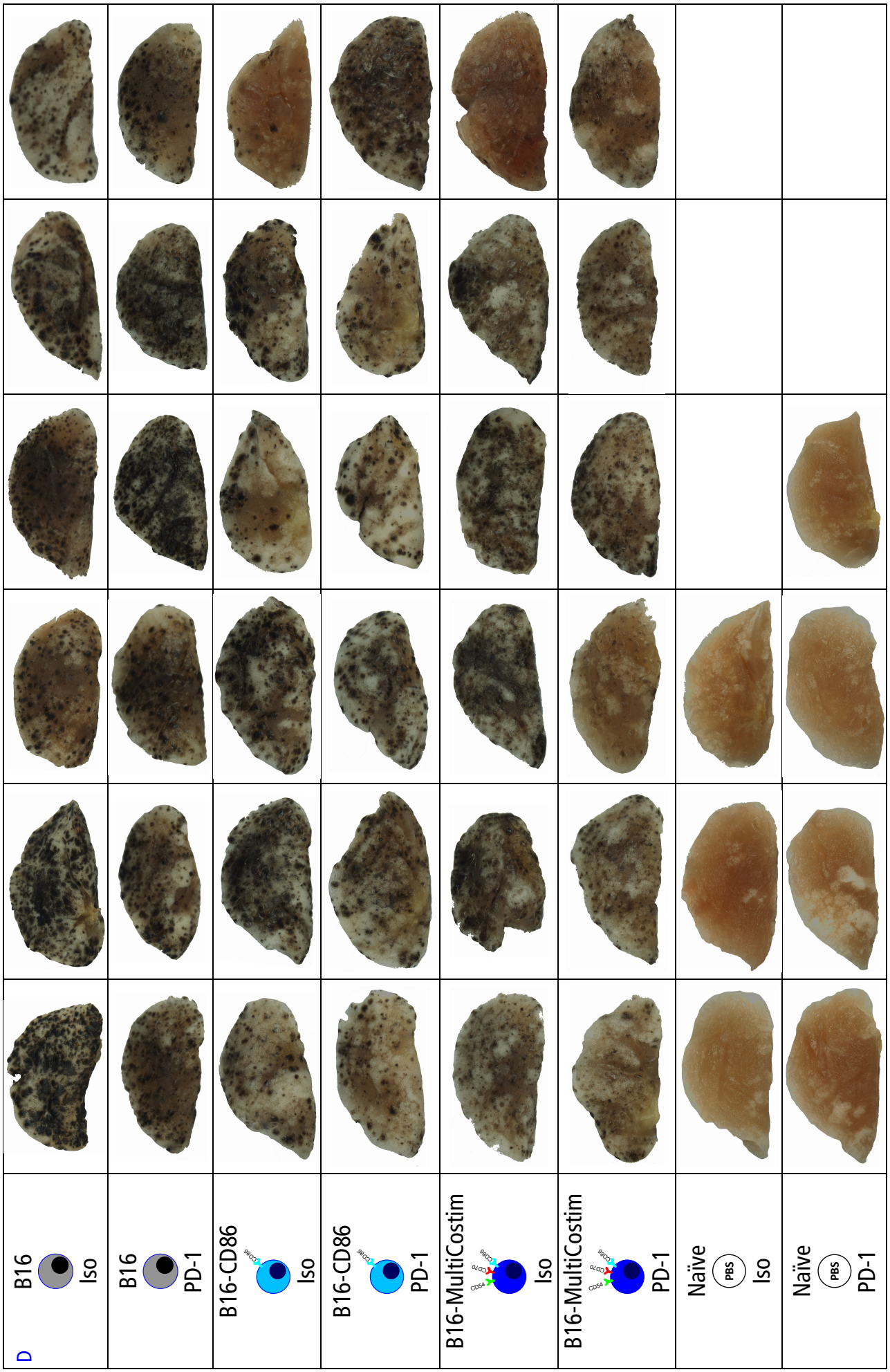


Figure 3.8: [D] Please refer to description on previous page

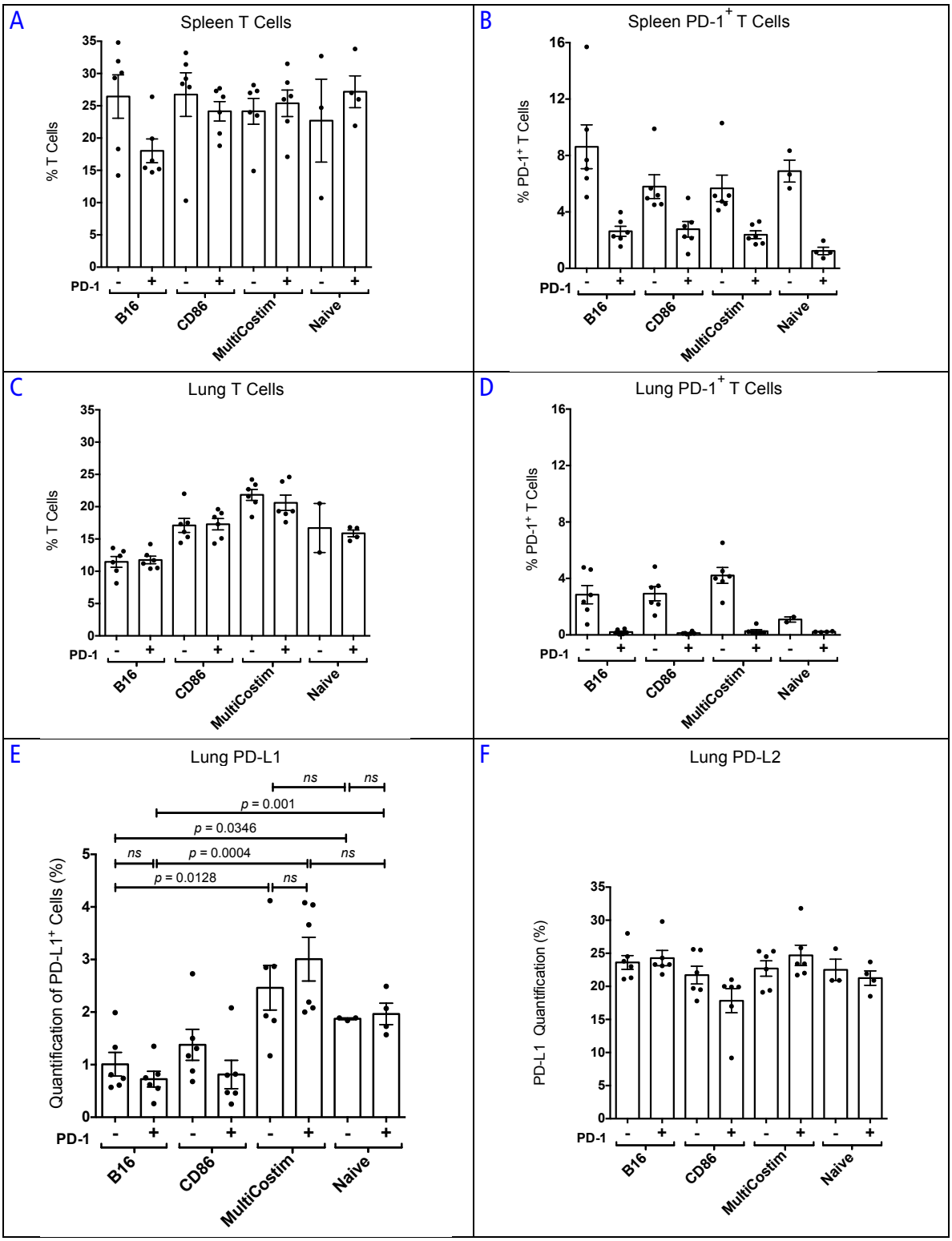


Figure 3.9: FACS analysis of lungs and spleens from mice in figure 3.8 above. [A] Total T cell population in spleens. Gated on Live cells, CD19 negative & CD3 positive cells. No statistically significant differences seen between the treatment groups. **[B]** Drop in PD-1 positive T cells within the spleen in mice treated with anti-PD-1 antibody but not isotype control. Cells gated on live cells and PD-1 positive cells. **[C]** Total T Cells within the lungs demonstrating apparent stepwise increase in T cell population associated with parental B16 tumours, CD86 expressing tumours and multiple costimulatory molecules expressing tumours. **[D]** Complete suppression of PD-1+ T Cells within the lungs in PD-1 antibody treated mice compared to isotope antibody treated controls. The suppression within the lungs is noted to be greater than the systemic reduction as seen in the spleen. Gating strategy as in figure 3.9B. **[E]** Total PD-L1+ cells within the lung. Cells gated on Live PD-L1+ cells demonstrate a non-significant trend towards increased PD-1. As with total T cells, it is possible that the increase in PD-1 seen in the multiple costimulatory treated lungs is apparent rather than actual due to less lung metastases and therefore less total cells. The differences observed are not statistically significant. **[F]** Total PD-L2. Cells gated on Live PD-L2 positive cells. No statistically significant differences between the groups were seen.

3.3 Discussion

The experiments in this chapter have shown enhanced priming in-vitro along with tumour rejection and delayed tumour growth following transduction of costimulatory molecules onto B16 tumours. There was a consistent hierarchy seen throughout all the experiments conducted using this experimental model, with single costimulatory molecules offering greater protection than parental tumours and the presence of multiple costimulatory molecules demonstrating the greatest effect. There is evidence from the RAG knock out mice experiment that the mechanism is B or T cell dependent at least in the subcutaneous tumour model. Attempts to identify antigen specificity concluded that Gp100 is not involved and suggested Trp2 may play a minor role. In the intravenous lung metastasis tumour model (figure 3.6B) the same hierarchy seen previously was again demonstrated, with tumours expressing multiple costimulatory molecules resulting in significantly less lung metastases. As in the earlier experiments, CD86 transduced tumours were found to be the most active of the single costimulatory molecules. The FACS data suggests general influx of several immune cell types including T Cells, B Cells, NK Cells and Granulocytes.

A similar lung metastasis experiment performed in FELIX mice which expresses human/mouse chimeric PD-1 (figure 3.8) confirmed the above result and also showed anti-PD-1 therapy appears to work synergistically with tumours expressing multiple costimulatory molecules.

It is known that PD-1 can be expressed on B Cells, CD4 and CD8 T cells, monocytes, NK cells along with dendritic cells.[152,276-278] CD4 T Cells do not express PD-1 in their resting state but do within 24 hours of activation.[279] It is also known that B16-F10 tumours do not constitutively express PD-L1[159] though can be induced to do so.[160] This has been demonstrated in-vivo through addition of IFNγ.[160] From the results presented here, it is possible that the expression of CD54, CD70 and CD86 on tumour cells are able to cause expression of PD-L1 in tumours

that do not do so in the absence of these molecules. This alone is unable to explain the differences seen in B16 tumours that express CD86 compared to parental B16 tumours. It is therefore likely that an additional mechanism is involved, possibly enhanced recruitment and activation of T cells by the transduced costimulatory molecules. This is supported by the increased number of T cells seen in the lungs (figure 3.9C) but not spleens (figure 3.9A) of mice injected with B16 tumours expressing CD86 and those tumours expressing multiple costimulatory molecules.

Naïve B16 tumours were noted to be unresponsive to the effects of PD-1 blockade (figure 3.8). There was no statistically significant difference in the lung tumour burden following treatment with anti-PD-1 antibody as compared to treatment with isotype control. Tumours transduced to express CD86 or multiple costimulatory molecules are however able to overcome this. A hierarchy is present with regard to the bulk of metastatic disease that develops within the lung. Mice injected with tumours transduced with multiple costimulatory molecules develop smaller volume metastatic disease within the lung than those injected with tumours transduced with CD86 alone.

There has been recent interest in combining tumour irradiation with blockade of CTLA-4[142,280,281] and the PD-1 pathways.[282-284] The effects of such therapy appear to be both additive and synergistic. Radiation is known to result in upregulation of the costimulatory molecules CD54, CD70 and CD86 within tumours or their infiltrating dendritic cells.[218,219,238,244,245,267] It is therefore possible that the mechanism responsible for the response to PD-1 seen in tumours expressing multiple costimulatory molecules in figure 3.8 may also explain the as yet poorly understood effects of combined PD-1 and radiation therapy described above.

At present it is not possible to predict those patients who are likely to respond to anti-PD-1 therapy and those who are not. Whilst the expression of PD-L1 makes the likelihood of responding slightly higher it is not useful as a biomarker, as the majority of tumours expressing PD-L1 will not respond.[169,285] In contrast a modest percentage of patients whose tumours do not express PD-L1 do respond to anti-PD-1 blockade.[169,285] Data from patients treated with anti-PD-1 therapy demonstrate that most tumours will not respond to anti-PD-1 therapy from the outset and are thus said to be 'resistant' to this form of treatment. A subset of patients, however, has tumours that appear to respond to PD-1 blockade initially but then subsequently progress. On the basis of a single case report, this acquired 'resistance' to anti-PD-1 therapy may not be permanent, with subsequent reintroduction of anti-PD-1 therapy following progression actually resulting in a better and more prolonged response to the drug than during the first exposure.[286] The ability to better understand and overcome 'resistance' to PD-1 pathway blockade is of clear clinical interest.

To my knowledge, the experiments discussed in this chapter represent the first instance in which it has been possible to reverse an inherent 'resistance' to PD-1 blocking therapy in an in-vivo tumour model. This approach could therefore be of great therapeutic potential in the metastatic cancer setting.

4. Results Chapter: Transduction of Costimulatory Molecules, cytokines and a chemokine on the CT26 Tumour Cell line

4.1 Introduction

Following on from the first results chapter (chapter 3) I wanted to look at the effect of including additional molecules in addition to costimulatory molecules with a view to concomitantly activating multiple arms of the immune system. Along with the molecules CD54, CD70 and CD86 which were successfully transduced onto B16-F10 tumours individually and in combination, several attempts were made to add an additional costimulatory molecule, CD48 along with a lentiviral cassette containing the cytokines IFNγ, GM-CSF, IL-12 and the chemokine CX3CL1. It was not technically possible to transduce these molecules onto B16-F10. Attempts to transduce up to a total of 8 genes onto the CT26 murine colorectal tumour cell line were however successful. CD54, CD70 & CD86 have been discussed in the previous chapter. Costimulatory molecule CD48, the cytokines IFNγ, GM-CSF, IL-12 and the chemokine CX3CL1 are discussed below. Expression of genes was validated by FACS expression of CD48, CD54, CD70, CD86 and CX3CL1 by FACS analysis.

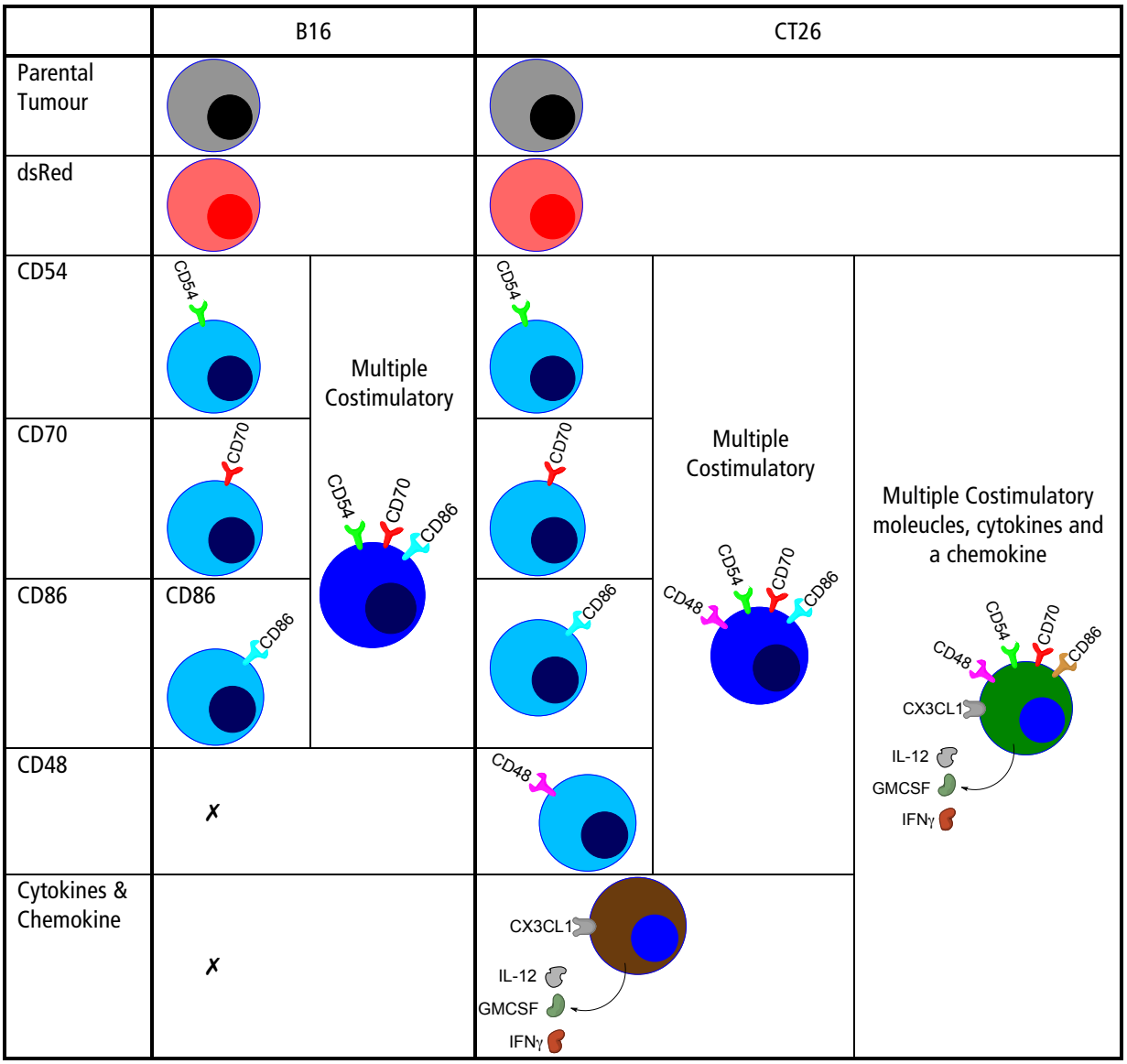


Figure 4.1: Summary of costimulatory molecules, cytokines and chemokine transduced onto B16-F10 and CT26 tumour cell lines

4.1.1 CD48:

CD48 is the murine analogue of human LFA-3.[287] It is a ligand for the immunoreceptors CD2 and CD244, the latter of which is alternatively know as 2B4.[288] It is normally found on the surface of B and T lymphocytes, natural killer cells, dendritic cells, monocytes, neutrophils, mast cells and eosinophils.[289,290] Mice deficient in CD48 develop normally but demonstrate impaired activation of $CD4^+$ T cells.[291] Though allelic polymorphism of CD48 exists in different mice strains it does not appear to affect the binding of CD48 to CD2 and no functional

consequences of this polymorphism have been determined to date.[291,292] The presence of the human analogue LFA-3, on tumour cells may enhance the ability of tumours to metastasize[293] whilst conversely, expression of CD48 has been shown to enhance anti-tumour activity by $CD8^+$ T Cells when expressed either alone or when co-expressed together with CD80 on the surface of poorly immunogenic tumours.[294] The PROSTVAC-VF phase II clinical trial which is discussed in detail in section 3.1.6 included CD48 (LFA-3) as one of the three costimulatory molcuelues that was expressed on poxviruses along with the PSA cancer antigen and injected together with GM-CSF. Whilst the trial failed to achieve its primary endpoint of improved disease free survival, there was an improvement in another secondary endpoint of median survival, which increased by 8.5 months from 16.6 to 25.1. Overall survival however was similar in both groups.

4.1.2 Interferon-gamma (IFNγ)

IFNγ was first identified as a cytokine secreted by lymphocytes with viral inhibitory properties over 40 years ago.[295,296] Over the subsequent decades its importance to both the innate and adaptive immune system and its crucial role, not only against intracellular pathogens but also in tumour immunology has become clearer. It is now known to be secreted by CD4 & CD8 T Cells, NK Cells, B Cells, dendritic cells, macrophages and NKT cells.[297-302] C57Bl/6 mice that lack the receptor for IFNγ develop tumours more rapidly and with greater frequency than wild type mice in response to chemical carcinogens. C57Bl/6 mice that are deficient for both the IFNγ and P53 tumour suppressor gene develop tumours more rapidly than mice deficient in P53 alone.[303] IFNγ deficient BALB/c mice challenged with DA3 mammary cancer cells developed less lung metastases than wild-type controls.[304] In comparing the various murine studies, a degree of mice strain and tumour heterogeneity is apparent. In a two year longitudinal study, 16 out of

32 (50%) C57Bl/6 IFNγ deficient mice were observed to develop spontaneous disseminated lymphoma with 3 additional mice developing other tumours. Over a similar period, none of the 19 BALB/c IFNγ deficient mice developed disseminated lymphoma, though 4 mice did develop other tumours.[305]

IFNγ upregulates MHC Class I and enhances $CD8^+$ T Cell mediated cell killing[306-309] while conversely suppressing recognition by non-MHC-restricted cytotoxic cells.[309] In a phase II clinical trial undertaken in patients with metastatic melanoma, 23 patients received weekly IFNγ subcutaneously. Pre and post HLA class I levels were monitored in 19 patients, and all patients had serum β2-microglobulin measurements as a surrogate marker for HLA class I upregulation. Three patients had a complete response to treatment. Two of 19 patients had downregulation of HLA class I pretreatment both of which were upregulated by treatment however there was no correlation between HLA I upregulation or β2-microglobulin levels and response to therapy.[310] In C57Bl/6 mice, MCA106 fibrosarcoma tumours transduced with IFNγ resulted in tumour growth delay compared to wild type MCA106 tumours. Mice immunized with the irradiated MCA106-IFNγ tumours also demonstrated a degree of protection against subsequent parental tumour challenge.[311] Exogenously added IFNγ has demonstrated non-immunologically mediated cytotoxic effects on ovarian cancer cell lines in vitro. Combined with its immunological properties, this has resulted in a number of early phase and one phase III clinical trial in which patients with ovarian cancer received standard surgery and chemotherapy with randomisation to receive additional subcutaneous IFNγ or not. Patients on the trial arm had an improved progression free survival at 3 years of 51% compared to 38% in the control arm of the trial. Unfortunately no immunological assays have been reported as part of this trial.[312] In a phase II clinical trial, patients with cutaneous lymphomas were treated with

intralesional injections of IFNγ-adenovirus. Half of treated patients had local tumour regression in the injected lesion, whilst one third had regression in distal lesions.[313]

4.1.3 Interleukin-12 (IL-12)

IL-12 is 70kD (p70) heterotrimeric cytokine comprising two disulfind-linked monomers p35 and p40.[314] It is produced by dendritic cells[315] and macrophages[316] and exerts its effects on T and NK cells, in which it induces production of IFNγ.[314,317] Beyond a few early phase trials, systemic treatment with IL-12 has not entered clinical usage owing to its unacceptable side effect profile and high mortality rate in treated patients.[318,319] Transfer of IL-12 genes directly into tumours is a less toxic approach and has been attempted in several animal models along with human clinical trials. Established murine CT26 tumour cells injected with intratumoural adenovirus expressing IL-12 results in delayed tumour growth up to 200 days post tumour challenge, which was found to be largely CD8 dependent.[320,321] A related experiment with murine MC38 adenocarcinoma and murine MCA205 fibrosarcoma confirmed growth delay following intratumoural adenoviral IL-12 injections in these tumour types.[322] A similar effect has also been demonstrated in a metastatic colorectal cancer model in which MCA-26 tumour cells were implanted in the liver. Intratumour IL-12 adenoviral injection was observed to double median survival from 20 to 40 days.[323] Introducing IL-12 into established subcutaneous tumours by intratumoural electroporation has also been investigated and found to be successful in eradicating up to 70% of established B16 tumours via a CD8 dependent mechanism with one third of responding mice protected against subsequent parental tumour rechallenge.[324,325] Using a similar approach, Chen *et al.*[326] injected established subcutaneous murine MB49 bladder tumours with a bi-cistronic adenoviral vector (Ad.mIL-12) carrying cDNAs of both the p35 and p40 subunits of murine IL-12. There were able to show a dose dependent effect, with complete eradication of the tumours following a single injection of 1 x 10^9 pfu.

Mice were protected against subsequent parental tumour rechallenge. Where the rechallenge was subcutaneous, mice were fully protected against development of the parental tumour. Where the parental tumour rechallenge was intravenous, mice showed partial protection, with suppressed development of lung metastases. The original tumour rejection in this instance was found to be both CD4 and CD8 dependent. In humans, a similar approach was used in a phase I clinical trial which recruited 24 patients with melanoma of whom 19 had metastatic disease. All patients received intertumoural injection of plasmid IL-12, followed by electroporation on 3 occasions. Three of 19 patients (15%) had complete regression of all distant metastases, 7 had stable disease (duration 4 to 20+ months) and 9 patients had progressive disease.[327]

4.1.4 Granulocyte-macrophage colony stimulating factor (GM-CSF)

As inferred by its name, GM-CSF is a cytokine originally identified on the basis of its ability to generate granulocytes and macrophage colonies from precursor bone marrow cells.[328]. Its antitumour effects, which are discussed below, derive from its ability to improve the function of antigen presenting cells through the maturation, activation and recruitment of dendritic cells and/or macrophages.[273] GM-CSF deficient mice develop a systemic lupus erythematosis (SLE)-like disorder but have no predisposition to cancer unless simultaneously deficient in IFNγ.[329] GM-CSF transduced subcutaneous 3LL murine lung tumours are rejected, with protection against subsequent parental tumour challenge.[330] GM-CSF transduced B16-F10 tumours were effective at partially protecting C57Bl/6 mice against subsequent subcutaneous[331] or intracranial[332] parental tumour challenge. Irradiated GM-CSF transduced B16-F10 tumours have also been demonstrated to stimulate potent, specific and long-lasting anti-tumour immunity.[122,333] Mice injected with B16-F10 tumours were injected 3 days later with either

irradiated B16-F10 tumour cells or B16-F10 tumours transduced to express GM-CSF. Most of the latter were still alive after 100 days as opposed to a mean survival of 30 days in the former. This was shown to be CD4 and CD8 T cell dependent but NK cell independent by antibody depletion studies. Interestingly whilst still showing some activity in a CT26 tumour model, the same authors showed considerably less protection against subsequent live parental tumour challenge in this tumour type.[122] Successful immunisation with irradiated GM-CSF has also been demonstrated using the A20 murine lymphoma cell line[334] and Lewis lung carcinoma cells[335]. Irradiated B16-F10 tumours transduced with GM-CSF offer greater immunity against subsequent tumour challenge than B16-F10 tumours co-administered with recombinant GM-CSF at the injection site.[273] GM-CSF has been shown to be safe and effective as an adjuvant for protein and peptide-based vaccines. [42,336,337] The larger PROSTVAC-VF phase II trial that was discussed in the introduction to this thesis also utilises GM-CSF in this context, with recombinant GM-CSF adjuvant at the vaccination site together with a poxvirus expressing the cancer antigen PSA, along with costimulatory molecules CD48, CD54 and CD80.[270] In a phase I clinical trial conducted in patients with metastatic melanoma, resected metastases were processed to single cells and transduced in-vitro with human GM-CSF by replication-defective adenoviral vector. Cells were irradiated to 100Gy prior to reinjection weekly for 3 weeks then on alternate weeks until vaccine supply was exhausted or patient removed from the study. Of 34 patients, there was one complete response, one partial response one mixed response and 5 patients with stable disease. The remaining 18 assessable patients had progressive disease. When comparing resected metastases pre and post therapy, 10 of 16 assayed patients showed evidence of having developed a significant $CD4^+$ and $CD8^+$ T Cell and B cell infiltrates associated with tumour destruction following therapy.[182] A phase III clinical trial in which an oncolytic virus engineered to express GM-CSF injected directly into tumours was compared to treatment with subcutaneous GM-CSF has recently closed and shows promising results with a

near doubling in complete responses from 6% in patients treated with subcutaneous GM-CSF to 11% in the oncolytic virus arm. Durable response rates were also significantly improved, with 16% of patients randomised to the oncolytic virus arm as compared to 2% on the subcutaneous GM-CSF arm demonstrating a durable response.[338,339]

4.1.5 Chemokine, C-X3-C motif, ligand 1 (CX3CL1)

Known as fractalkine in humans and neurotactin in mice, CX3CL1 is a cytokine expressed on Natural Killer Cells, monocytes, T Cells and Dendritic Cells characterised by its CX_3C chemokine motif, and is the only known chemokine of this CX_3C class.[340,341] CX3CL1 exists in two forms that have differing biological activities. When anchored to the cell membrane via its long mucin rich stalk, it has properties of an adhesion molecule. In contrast, when its stalk is cleaved by proteolysis, it exists in a soluble form and behaves as a chemokine with effects on NK Cells, T Cells and Dendritic Cells.[340-344] Murine lung 3LL tumours transduced with CX3CL1 were found to both express the membrane-bound, and secrete the soluble form of the molecule. Transduced tumours had reduced growth rate compared to parental tumours, which was shown to be dependent on CD8 T Cells and NK Cells. Increased populations of CD8 and CD4 T cells along with dendritic cells and NK cells within the transduced tumours suggest that the mechanism is via chemoattraction.[345,346] Thymoma EL4 and EG7 tumours transduced with CX3CL1 also have reduced growth rate which was abolished in NK deficient but not B and T cell deficient mice.[347] Established CT26 and B16 tumours transduced with CX3CL1 also have reduced growth rate which was found to be both T and NK cell dependent.[348]. In a murine hepatocellular carcinoma model, CX3CL1 transduction inhibits tumour growth. CD4 and CD8 T Cells were found to be present in 2-3 times the frequency within transduced tumours. These same cell populations were also present at marginally higher frequency in blood which may account for the partial protection against parental tumour rechallenge.[349]

4.2 Results

4.2.1 Tumour Challenge with Multiple Costimulatory Molecules, Cytokines and Chemokine

Tumours were injected subcutaneously on the right flank of BALB/c mice and measured on alternate days until the end of the experiment, or until such time that one of the predefined humane endpoints was reached, as discussed in Methods Section 2.2. In mice injected with untransduced (parental) tumour (figure 4.2A) or tumour transduced with the non-immunologlically active lentiviral control dsRed (figure 4.2B), tumours grew in an exponential manner as would be predicted. In mice injected with tumours transduced with the single costimulatory molecule CD86 (figure 4.2C), most tumours initially developed normally with 5 of the 6 injected tumours becoming detectable between days 10 and day 12. These tumours demonstrated exponential tumour growth until day 18 after which time they were noted to be decreasing in size and were rejected over the following 14 days. The final mouse became tumour-free after an additional 10 days. In mice injected with tumours transduced with multiple costimulatory molecules CD48, CD54, CD70 and CD86 (figure 4.2D) 2 of 6 mice did not develop any palpable tumours, a further 2 mice developed small tumour nodules which were rejected almost immediately, with the final 2 mice developing moderately sized tumours (10-20mm^2) prior to complete rejection by day 28. In the group of mice injected with tumours expressing the secreted molecules IFNγ, GM-CSF and IL-12 along with the chemokine CX3CL1 (figure 4.2E), only 1 of the 6 mice developed a palpable tumour, which was rejected within 5 days. The remaining 5 mice remained tumour-free throughout. In the final group of 6 mice, injected with tumours expressing all 8 transduced molecules, CD48, CD54, CD70, CD86, IFNγ, GM-CSF and IL-12 (figure 4.2F) no tumours were detectable at any time.

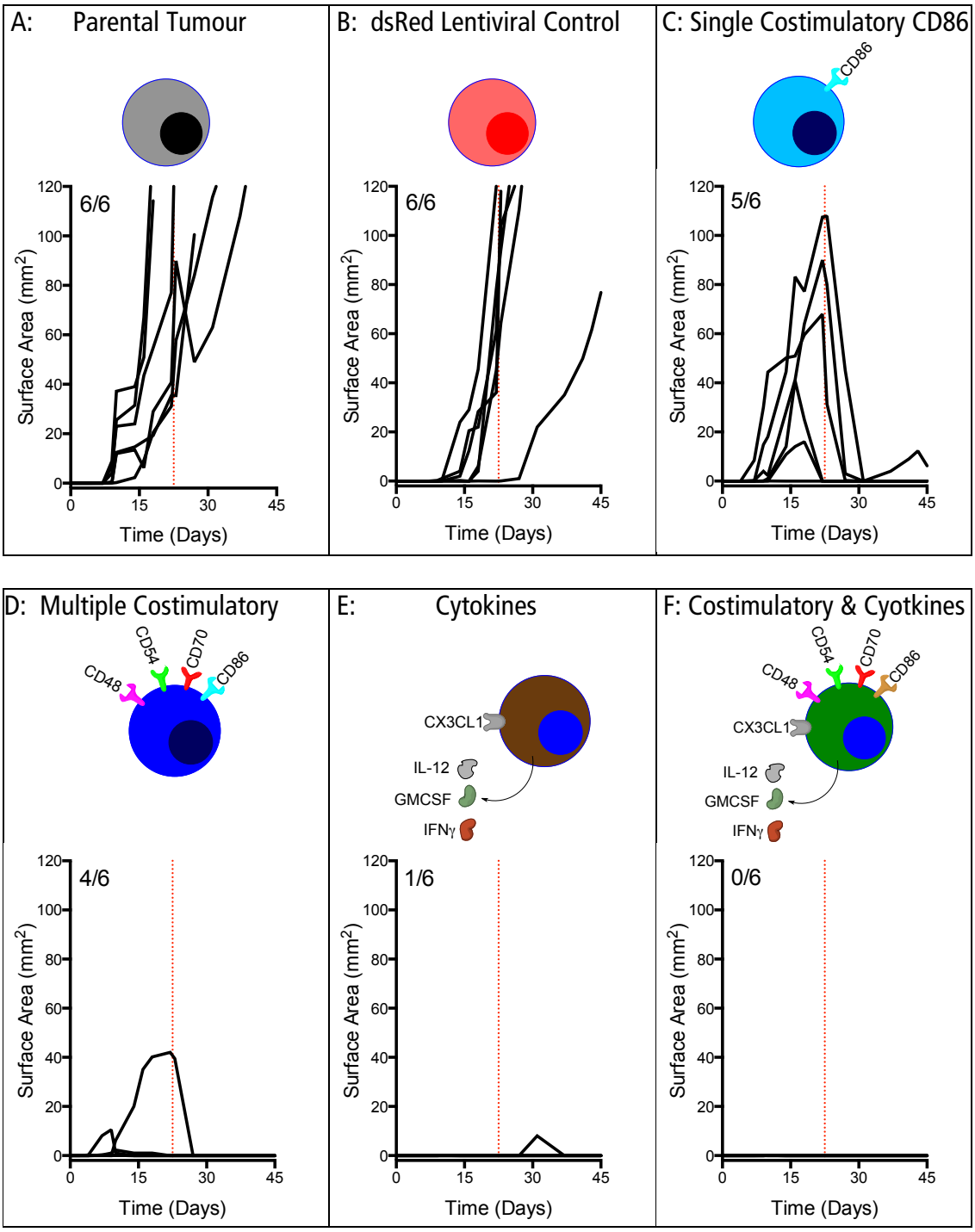


Figure 4.2: Impaired tumour growth and rejection observed in mice injected with tumours expressing costimulatory molecules, cytokines and a chemokine. Growth of parental CT26 tumours and CT26 tumours transduced with various molecules individually or in combination as labelled. The number in the top left of each graph indicates the number of mice in which tumour development was observed. Statistical analysis of tumour size, calculated on day 18 when the first mouse reached the humane endpoint, demonstrated statistically significant differences between the size of the parental tumour and tumours expressing multiple costimulatory molecules (p = 0.0298), cytokines and chemokines (p = 0.0138) and costimulatory molecules, cytokines and chemokines (p = 0.0138)

4.2.2 Serum from mice that rejected tumours fails to react with parental CT26

To explore the mechanism of rejection observed above, the involvement of antibodies was examined. Surviving mice from Figure 2 above were tail bled and serum isolated and incubated with fresh CT26 tumours. Secondary anti-mouse IgG antibody was added and percentage IgG positive cells quantified by FACS analysis (Technique based on reference [202]) As can be seen in figure 4.3 below, no statistically significant differences were identified between the mice from figure 4.2 as compared to naïve mice (figure 4.3A below) as contrasted with serum obtained from C57Bl/6 mice previously immunised with intraperitoneal CT26 tumours (figure 4.3B).

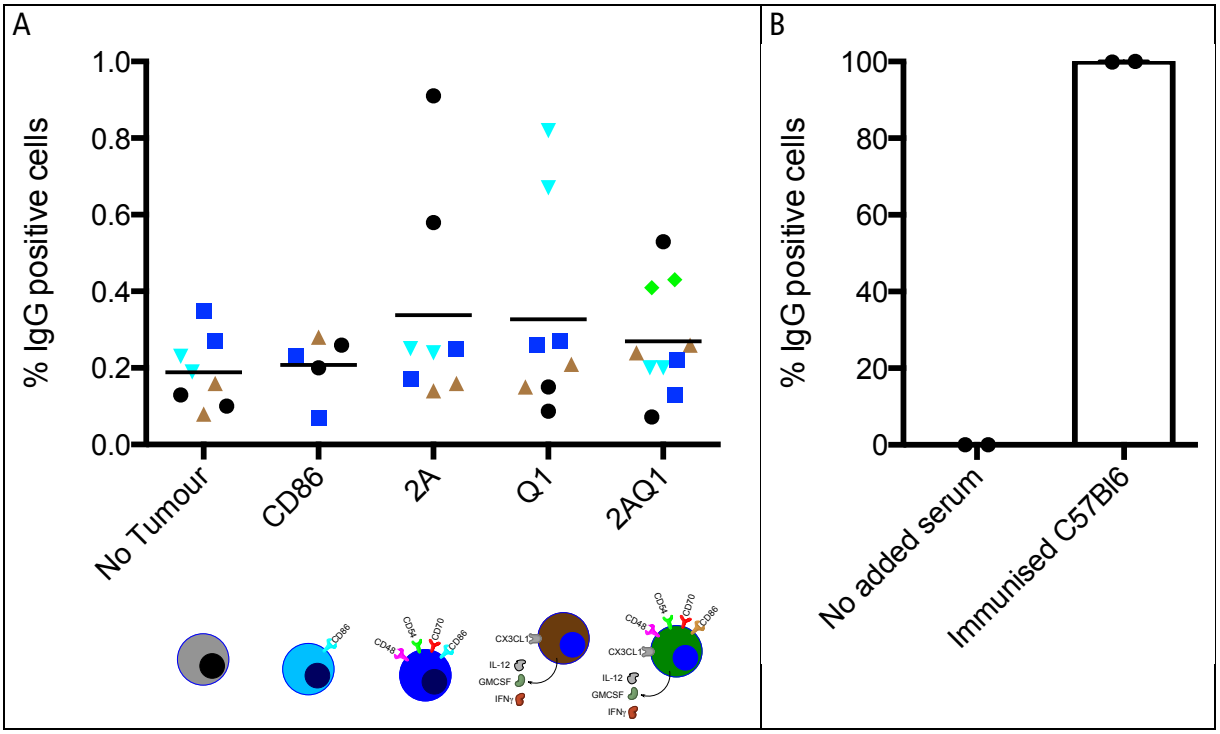
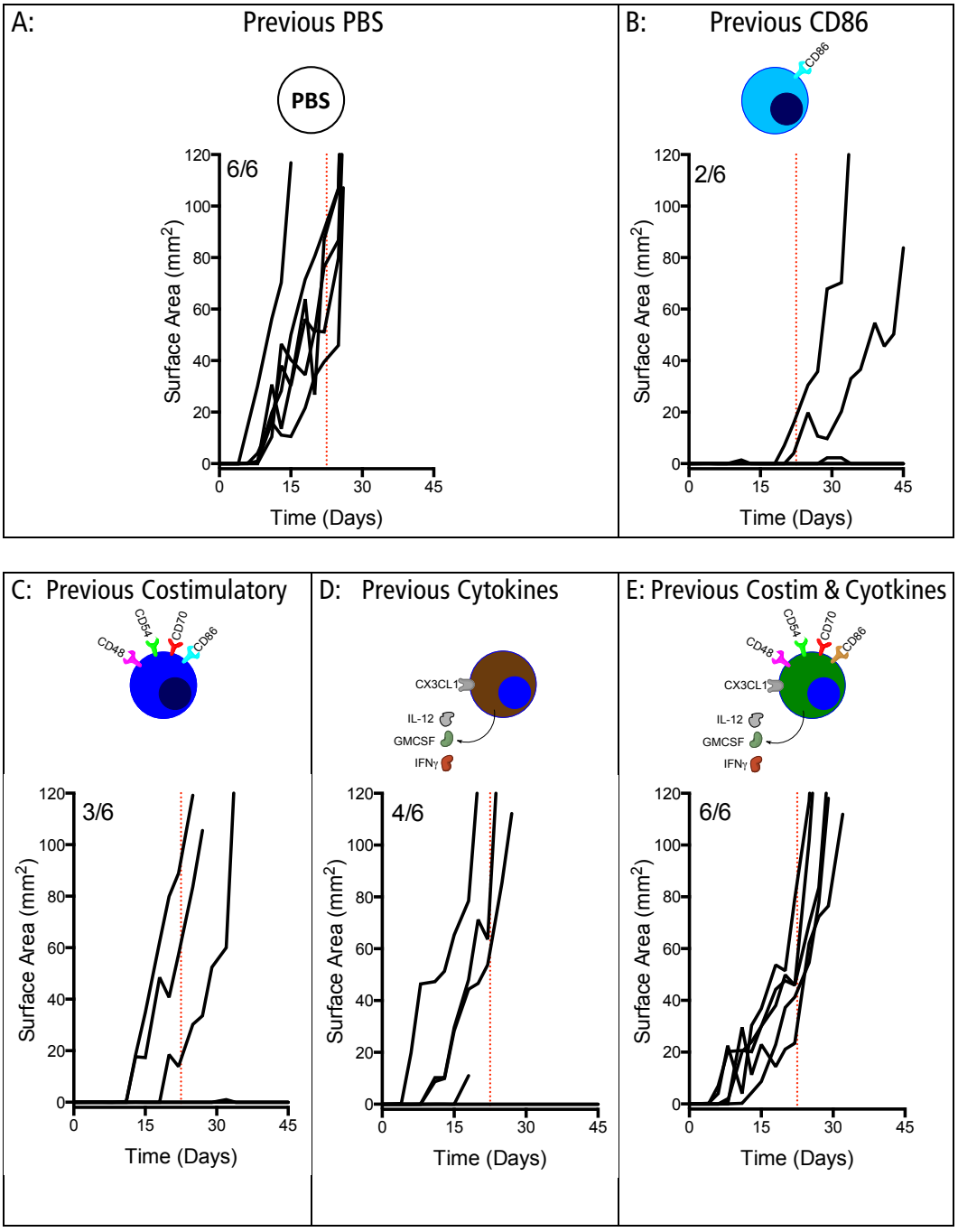


Figure 4.3: Antibodies specific for cell surface proteins were not enhanced in any treatment group. Serum was obtained from surviving mice in figure 2 and incubated in duplicates with $0.5x10^6$ fresh CT26 tumours at a ratio of 1:25. Cells were then washed and Goat anti-mouse IgG was added and FACS analysis performed with dead cells exluded by live staining and percentage IgG positive cells calculated. Within each tumour treatment group, the duplicate samples are identified by identical coloured shapes. [A] No differences in IgG antibodies to CT26 tumours identified in the serum of mice, with all mice having less than 1% positive cells [B] Positive and negative controls plotted with a different Y-axis scale. Positive control serum obtained from C57Bl/6 mouse immunised by intraperitoneal injection of live CT26 tumours 2 weeks earlier.

4.2.3 Rechallenge of Surviving mice with parental CT26

To further assess the mechanism of rejection observed above, the mice that successfully rejected the tumour were rechallenged with parental, untransduced CT26 tumours. Control mice that had previously been injected with PBS alone were also challenged with parental CT26 tumours. As illustrated in figure 4.4 A-G below, mice previously injected with tumours expressing the costimulatory molecule CD86 demonstrate the greatest protection against parental tumour rechallenge. Mice previously challenged with multiple costimulatory molecules also retain some protection. In contrast, mice previously exposed to tumours expressing cytokines and chemokines either in the presence or absence of multiple costimulatory molecules are not protected against subsequent tumour rechallenge. Figure 4.4F is a Kaplan Meier graph, plotting survival against time and confirms that there is a statistically significant improvement in survival in mice following parental CT26 tumour challenge in mice previously injected with tumours expressing single costimulatory molecule CD86 or multiple costimulatory molecules CD48, CD54, CD70 and CD86. When comparing all the survival curves to control mice, previously treated with PBS only, in addition to the two mice groups discussed above, the Log Rank (Mantel-Cox) test also confirms a statistically significant difference in mice previously challenged with tumours expressing all 8 molecules. The magnitude of this difference is however small with only a two-day improvement in median survival from 26-28 days following parental tumour rechallenge. This illustrates the difference between a meaningful and statistical difference. Whereas in the cytokines and chemokine exposed mice two mice remain tumour free at the end of the experiment, there is a wide variability in the response seen, with both groups having a median survival of 26 days, and there is no statistical significant difference in survival. In contrast, in mice that had previously been exposed to tumours expressing multiple costimulatory molecules, cytokines and chemokines, their median survival is 2 days longer at 28, a statistically significant difference, but one which is unlikely to

be therapeutically meaningful if extended to a clinical setting.



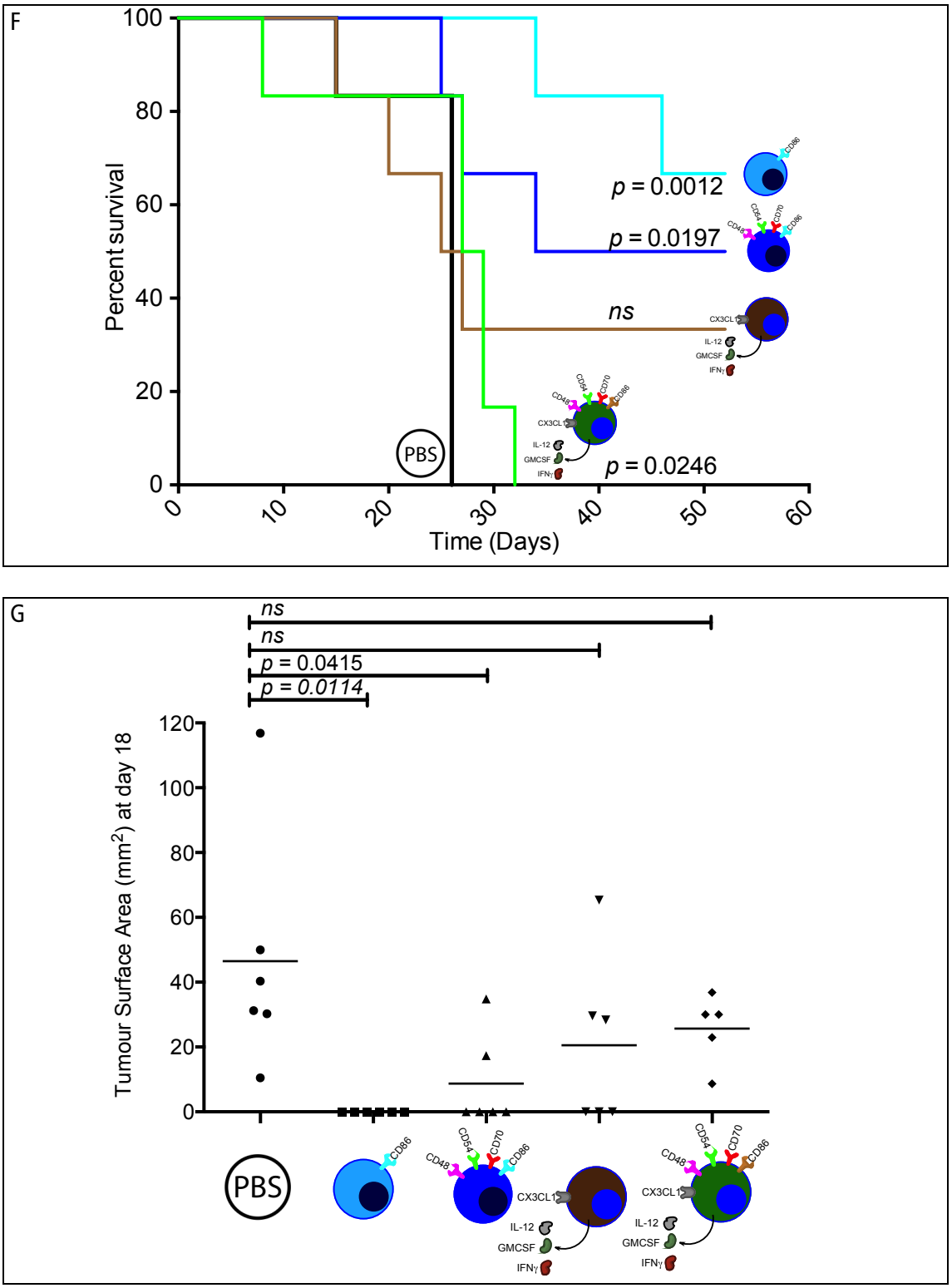


Figure 4.4: Rechallenge with parental CT26 tumours. Compared to **[A]** Naïve mice challenged with Parental CT26 tumours, **[B]** Previous exposure to CT26 transduced with single costimulatory molecule CD86 offers the greatest protection against subsequent parental tumour rechallenge. **[C]** Previous exposure to tumours expressing multiple costimulatory molecules confers some degree of protection but less than tumours expressing CD86 alone. [D] Previous exposure to tumours expressing cytokines and a chemokine offers no statistically significant protection against subsequent parental tumour rechallenge. **[E]** Previous exposure to tumours expressing all 8 molecules confers a very small but statistically significant delay in tumour growth **[F]** Kaplan Meir curve plotting time to humane endpoint, including p values for results A-E above. **[G]** Statistical analysis of tumour size, calculated on day 15 when the first mouse reached the humane endpoint, demonstrated statistically significant differences between the size of the tumours in mice previously exposed to CD86 expressing tumours (p = 0114) and mice previously exposed to tumours expressing multiple costimulatory molecules (p = 0.0415).

4.2.4 Investigating the importance of B Cells, T Cells and NK cells in the tumour delay and rejection seen

To further investigate the immune cells implicated in the rejection described in figure 4.2 above, the effect of the modified CT26 tumour growth was compared between wild type BALB/c mice and BALB/c γ-chain$^{-/-}$ RAG$^{-/-}$ mice which lack B Cells, T Cells along with the common γ-chain, and hence NK cells.

The results seen in the wild-type BALB/c mice (figures 4.5A-E below) confirm the results described in figure 4.2 above. In contrast however, when injected mice that lack B, T and NK cells, with similar tumours, all the mice develop tumours (figures 4.5F-J below). There is however a delay in tumour growth in mice that were injected with tumours expressing cytokines and chemokine either in the presence or absence of multiple costimulatory molecules as illustrated by the red dotted line in figures 4.5F, 4.5I and 4.5J. This translates into a statistically significant improvement in survival (figure 5L) in these two mouse populations as compared to mice injected with the parental CT26 tumours.

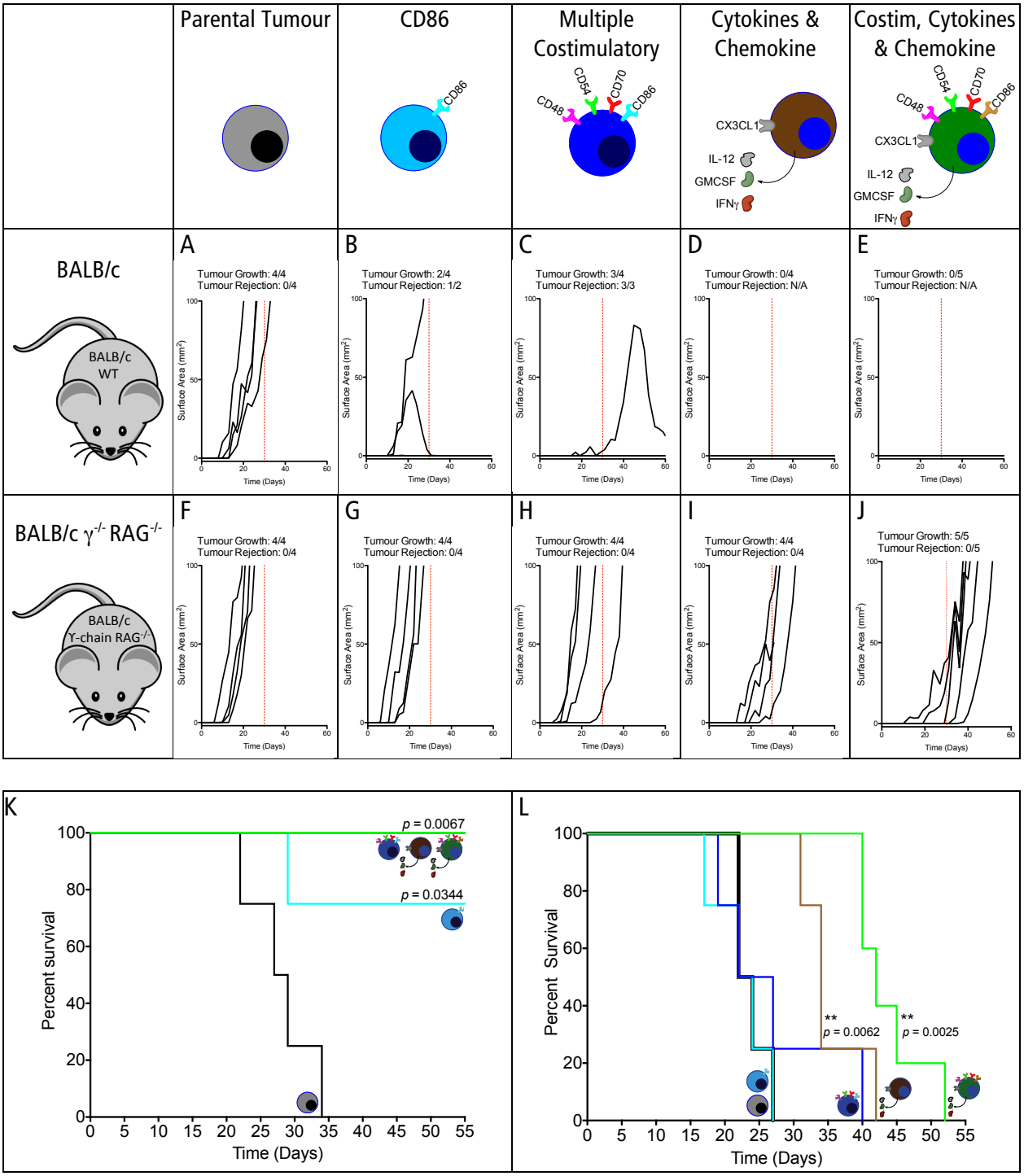


Figure 4.5: Tumour delay and rejection in the presence of Cosimulatory molecules is dependent on the presence of B Cells, T Cells and NK Cells. [A-E] Tumour growth in wild type BALB/c mice **[F-J]** Tumour growth in BALB/c γ-/- RAG-/- mice . When comparing figures B with G and C with F it is apparent that the growth delay and rejection observed in mice treated with costimulatory molecules is no longer seen when tumours are injected into mice that lack B Cells, T Cells and NK Cells. In contrast when comparing figures D to I and E to J, the growth delay and rejection observed in wild type BALB/c mice is only partially dependent on B Cells, T Cells and NK cells in the presence of cytokines and chemokine and whilst in their absence the tumours grow, there is evidence of growth delay which persists even in the absence of these cell types. **[K]** and **[L]** which are Kaplan Meier plots for wild type BALB/c mice and BALB/c γ-/- RAG-/- mice respectively. The p values represent log rank test comparison to the parental tumour growth. Comparing these two plots demonstrate that whilst all the transduced tumours grow in the absence of B, T and NK cells, a growth delay persists even in the absence of these cell types leading to differences in survival when cytokines and a chemokine are present.

4.2.5 Transduced molecules exert a systemic effect

As discussed in the previous chapters, one of the potential clinical applications of this technique is in the metastatic setting where an accessible tumour or metastasis at one site could be transduced with selected molecules. The hope is that the tumour cells at the transduced site could then effectively behave as antigen presenting cells by presenting tumour specific antigen to T cells leading to a systemic anti-tumour response.

To test this approach a pseudo-metastatic mouse model was used in which modified tumours were injected on one flank of the mouse and the parental tumour on the opposite (contralateral) flank. There is a statistically significant decrease in the parental mean tumour size at day 26, in mice that were injected with tumours expressing all 8 molecules on the opposite flank compared to mice that received no contralateral tumour. This translates into a statistically significant survival advantage in this mouse population despite the fact that the tumours expressing 8 genes failed to grow. Mice that were injected with CD86 expressing tumours or tumours expressing multiple costimulatory molecules on the opposite flank also appear to develop smaller parental tumours however this difference does not reach statistical significance. There does however appear to be a hierarchy with regard to the parental tumour growth. In mice with no contralateral tumour, the ipsilateral tumour grows fastest, those with a contralateral tumour expressing single costimulatory molecule CD86 have a subtle growth delay, those injected with contralateral tumours expressing multiple costimulatory molecules a more apparent delay and those with tumours expressing all 8 genes the greatest parental tumour growth delay.

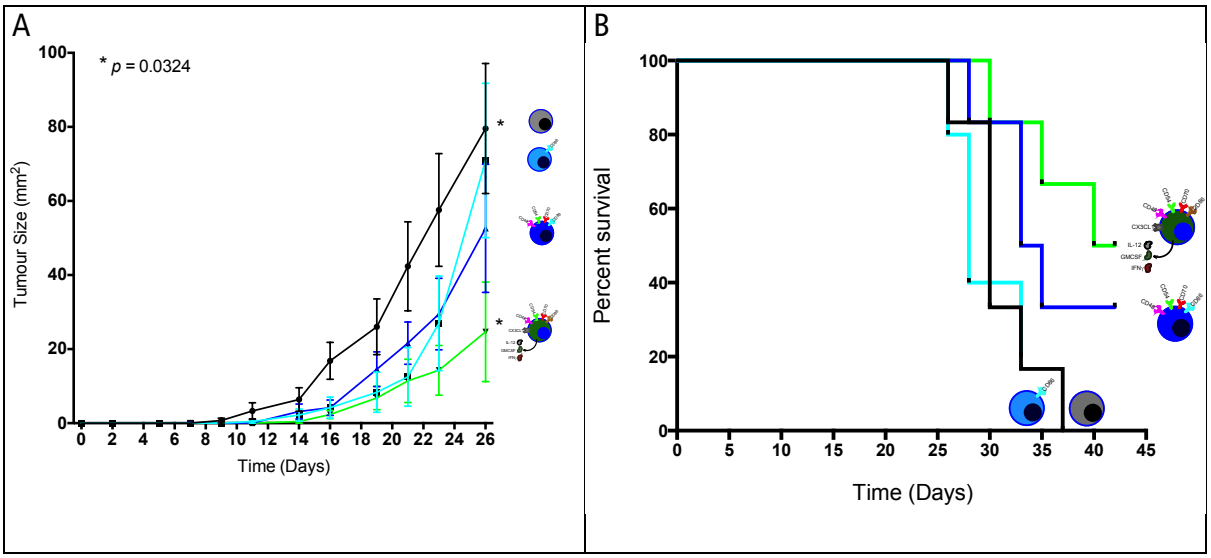


Figure 4.6: Injection of tumours expressing multiple costimulatory molecules, cytokines and a chemokine exerts a statistically significant systemic effect resulting in reduced growth of the contralateral parental tumour even in the absence of any measurable transduced tumour. It is possible the injection of tumours expressing only multiple costimulatory molecules is having a similar effect though this does not reach statistical significance either by tumour size at day 26 or by log rank (Mantel Cox) test.

4.2.6 Attempting to quantify the degree of systemic versus local effect of transduced molecules

In an attempt to quantify the extent to which the effect seen in Figure 4.6 above is due to local versus systemic effect, modified and control tumours were injected either on the same flank or opposite flanks.

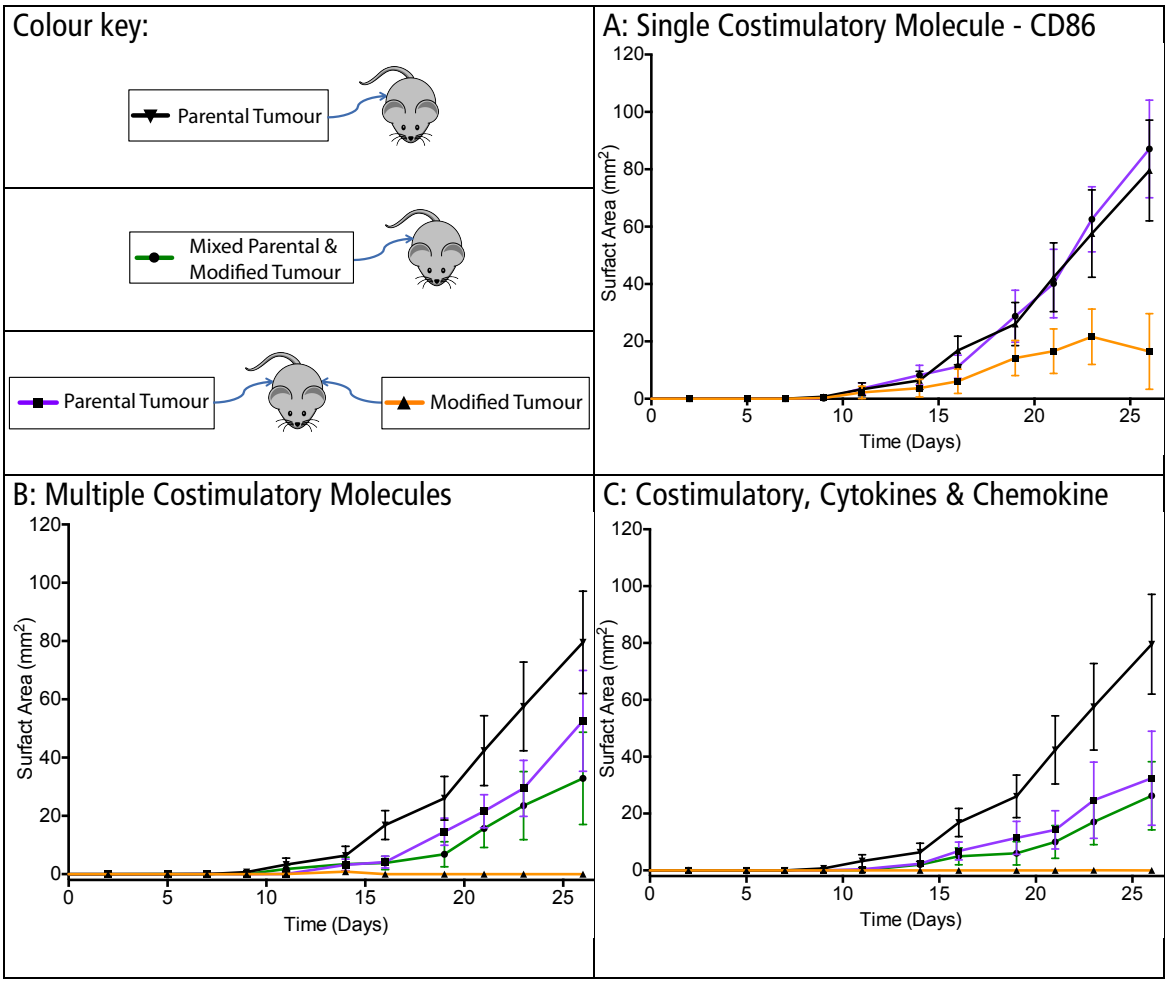


Figure 4.7: Local and systemic effects appear equally important in the case of tumour transduced with multiple costimulatory molecules with or without cytokines and a chemokine. In contrast CD86 appears to exert only local effects. 50,000 tumour cells injected at each site as indicated in colour key above. If the transduced molecules exerted purely local effects, the purple line and the black line should be superimposed. The closer the purple line is to the yellow line, the greater the systemic effect exerted by the transduced tumour on the contralateral parental tumour. In addition, the closer the green and purple lines appear together, the greater the systemic effect. On the basis of this **[A]** CD86 exerts its effects locally whereas both **[B]** multiple costimulatory molecules and **[C]** multiple costimulatory molecules together with cytokines and a chemokine exert both local and systemic effects.

4.3 Discussion

The initial experiments in this chapter confirmed that the results from the previous chapter, with regard to tumour growth of tumours transduced with costimulatory molecules, holds true in a different tumour model and in mice of a different background. The B16-F10 tumour used in the previous chapter is known to be particularly non-immunogenic.[262] It is therefore not surprising that the effects of the transduced molecules in the CT26 tumour model are more pronounced than those seen with B16-F10. Tumours transduced with CD86 (figure 4.2C & 4.5B) were noted to regress following initially normal tumour growth. Tumours transduced with multiple costimulatory molecules (CD48, CD54, CD70 and CD86) demonstrating reduced growth from the outset followed by tumour regression (figure 4.2D & 4.5C). Additional molecules transduced in the CT26 molecule were added in the hope of engaging multiple arms of the immune system simultaneously and the results in figure 4.2, confirmed in figure 4.5, demonstrate that this is indeed the case, with the near complete lack of tumour growth in tumours transduced with cytokines and chemokine (figure 4.2E & 4.5D) and total abolishment of any tumour growth in mice challenged with tumours expressing all 8 costimulatory molecules, cytokines and chemokine (figure 4.2F & 4.5E). The rapidity of the rejection in the final two groups of tumours (those transduced with cytokines and chemokine and costimulatory molecules, cytokines and chemokine) possibly attests to engagement of the innate rather than adaptive immune system. The lack of memory observed when these tumours were rechallenged with parental tumour further strengthens this hypothesis (figure 4.4D-G). The same experiment demonstrates that the total tumour exposure is the single most important predictor of protection against subsequent parental tumour challenge. Total tumour exposure is measured by the area under the curve in a tumour size versus time graph and therefore incorporates tumour size along with the duration over which the tumour was present. Mice previously injected with CD86 expressing tumours therefore have the greatest immunity

to subsequent parental tumour challenge followed by mice previously exposed to tumours expressing multiple costimulatory molecules. Previous exposure to tumours expressing cytokines and chemokine either in the presence or absence of multiple costimulatory molecules did not offer any protection against subsequent parental tumour challenge. Mechanistically, there are two alternative explanations for the complete lack of any protection to parental tumour rechallenge following exposure to tumours expressing cytokines and chemokine. As discussed above it is possible these tumours are rejected on the basis of the innate immune system and these mice therefore have no immunological memory. It is also possible that the lack of memory is a result of IFNγ expression by these tumours. There is some evidence that whilst beneficial in primary anti-tumour response, IFNγ mediates loss of CD4+ T cells which can impair secondary anti-tumour responses.[350] In the literature review pertaining to IFNγ in the introduction to this chapter, whilst there are many examples of the anti-tumour properties of transduced IFNγ only one paper was identified that reports evidence of immunological memory with evidence of exposure to IFNγ transduced tumours being able to confer protection against parental tumours in subsequent rechallenge.[311]

Comparing the results in wild type BALB/c mice to BALB/c $\gamma^{-/-}$ $RAG^{-/-}$ illustrates that some or all of the following cell types; B Cells, T Cells and NK Cells, are responsible for the rejection seen in the presence of costimulatory molecules in wild type BALB/c mice (4.5B versus 4.5G and 4.5C versus 4.5H). The results from figure 4.3 indicate that the rejection is not antibody mediated. Regarding the rejection observed in tumours expressing cytokines and chemokines, the same cells types are clearly involved. There is however still evidence of growth delay when these cells are absent (Figure 4.5D versus 4.5I and 4.5E versus 4.5J) indicating that other cells or molecules of the immune system must also play a role, though the experiments described here have not identified these cells.

To assess to what degree the transduced molecules were exerting a local effect and to what extend the effect was systemic, mice were injected with a parental tumour on one flank and a modified tumour on the contralateral flank (figure 4.6). This experiment confirms that the transduced tumours expressing a combination of multiple costimulatory molecules, cytokines and a chemokine together is able to induce a systemic effect that leads to tumour growth retardation of the parental tumour on the contralateral flank. There is the suggestion that expression of single costimulatory molecule CD86, and multiple costimulatory molecules may also have an effect (figure 4.6A & 4.6B). Whilst both failed to reach statistical significance, the fact that tumours modified with these molecules follow the same hierarchy present consistently in both the previous and current chapters lends credence to these molecules exerting a genuine effect but suggests this effect is likely weak compared to tumours expressing multiple costimulatory molecules, cytokines and a chemokine. The results of figure 4.7, again suggest that in the case of tumours expressing multiple costimulatory molecules with or without transduced cytokines and chemokine, that the effects that they exert are systemic in addition to local.

5. Results Chapter: Transduction of IL-2 & IL15 fused to their respective receptors

5.1 Introduction

In the previous chapters it was possible to achieve excellent anti-tumour responses following transduction of costimulatory molecules, cytokines and a chemokine. However mice that completely rejected the transduced tumours did not show uniform protection against subsequent parental tumour challenge (Results chapter 4 Figure 4.4). As will be discussed further below, both IL-2 and IL-15 are involved in the generation of memory responses. The experiments in this chapter attempt to improve anti-tumour immunity using novel constructs which fuse IL-15 to the α-subunit of the IL-15 receptor and IL-2 either fused to, or present alongside, the α-subunit of the IL-2 receptor.

Both IL-2 and IL-15 have anti-cancer properties. IL-2 is licensed by the FDA for the treatment of metastatic melanoma and renal cell carcinoma.[351] In metastatic renal cell cancer complete response rates of 7-10% have been reported.[352] Marginally lower rates have been reported in patients with metastatic melanoma.[353,354] Most data for IL-15 comes from preclinical studies[355,356] IL-15 is also being assessed in a couple of phase I clinical trials, one of which has recently closed but not yet reported.[357,358]

The receptors for IL-2 and IL-15 are structurally similar heterotrimeric receptors. The γ-chain, CD132, is shared with several other interleukins; IL-4, IL-7, IL-9 and IL-21.[359-361] The β-subunit, CD122, is specific to the IL-2 and IL-15 receptors.[362,363] Finally they each have a unique α-subunit IL-2Rα (alternatively known as CD25) and IL-15Rα respectively.[364,365]

Transpresentation of IL-15 by its high affinity receptor IL-15Rα by dendritic cells is important in controlling both the production and the presentation of IL-15 to NK and $CD8^+$ memory T cells.[366-369] Murine tumour models utilising this approach have been able to show improved in-vitro activity to treatment with IL-15/IL-15Rα in combination as compared to IL-15 alone.[370-372]

Daclizumab is a humanised monocolonal antibody against IL-2Rα-chain, which is licensed for clinical use in the prevention of organ transplant rejection and which is currently undergoing clinical trials in multiple sclerosis. Neither IL-2 nor daclizumab exert a direct effect of on dendritic cell maturation. It is has therefore been suggested that CD25 on dendritic cells transpresents IL-2 to T cells in a similar manner to IL-15.[373]

IL-2 and L-15 share many important functional similarities but also some significant differences. Both IL-2 and IL-15 are involved in the proliferation and differentiation of T Cells.[374-377] They also play a role in the proliferation, maintenance and activation of natural killer cells[378,379] and in the proliferation of B cells where they are involved in regulating synthesis of immunoglobulins.[380,381] In addition to the above, IL-2 is involved in activation-induced cell death (AICD), maintenance of regulatory T Cells and elimination of self-reactive T Cells.[382-385] In contrast, IL-15 plays a crucial role in the proliferation of memory-phenotype CD8+ T cells. [386-390] IL-15 is also able to inhibit the role of IL-2 in activation-induced cell death.[391]

5.1.1 Introducing the IL-2/IL-2Rα and IL-15/IL-15α Constructs

In the experiments that follow in this chapter IL-2 and IL-15 have been transduced into tumours in association with their respective receptors using the lentiviral constructs below:

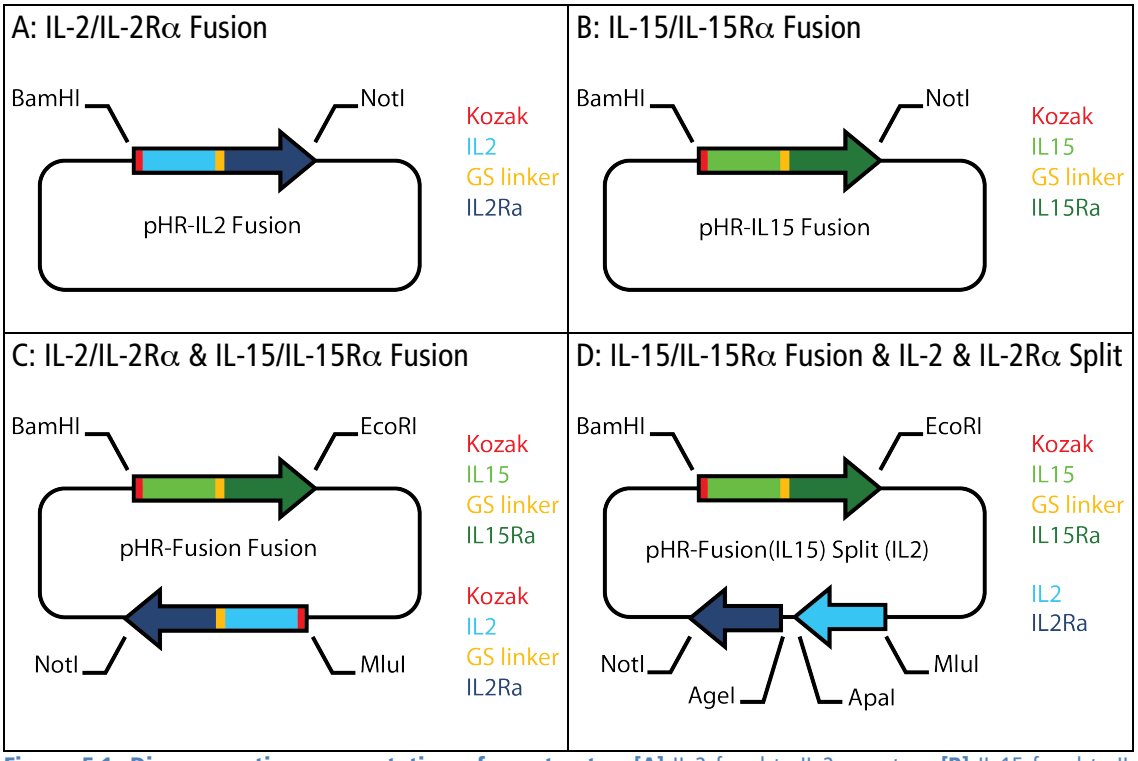


Figure 5.1: Diagrammatic representation of constructs. **[A]** IL-2 fused to IL-2 receptor. **[B]** IL-15 fused to IL-15 receptor. **[C]** IL-2 and IL-15 fused to their respective receptors. **[D]** IL-2 and IL-2 receptor present but not fused along with IL-15 fused to the IL-15 receptor.

The above constructs were transduced onto the EG7 tumour cell line which had previously been transduced with Cyan Fluorescent Protein (CFP). EG7 is an ovalbumin transduced EL4 thymoma cell line. CFP was selected on the basis of the peak excitation and emission wavelengths of 434 and 477 nm respectively. It can therefore be distinguished, by flow cytometry, from Green Fluorescent Protein (GFP) which has peak excitation and emission wavelengths of 488 and 507 nm respectively.[392,393] This difference is exploited using the GFP-OT1 animal model discussed later in this chapter.

For purposes of simplification, the following nomenclature will be used in the remainder of this chapter:

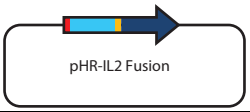
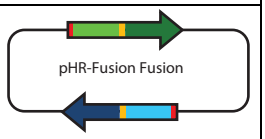
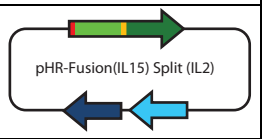
Abbreviation	Definition	Lentiviral Construct
EG7-CFP	EG7 tumours transduced with Cyan Fluorescent Protein but not with any IL-2 or IL-15 constructs	
EG7-CFP-IL-2	EG7-CFP cells transduced with IL-2 fused to the α-subunit of the IL-2 Receptor	
EG7-CFP-IL15	EG7-CFP cells transduced with IL15 fused to the α-subunit of the IL-15 Receptor	
EG7-CFP-FF	EG7-CFP cells transduced with both IL-2 and IL-15 fused to the α-subunits of their respective receptors	
EG7-CFP-FS	EG7-CFP cells transduced with IL-15 fused to the α-subunit of its receptor, along with IL-2 and the α-subunit of its receptor which are not fused (split)	

Figure 5.2: Nomenclature and definitions used in this chapter

5.2 Results

5.2.1 Confirming Biological activity of the IL-2 and IL-15 constructs

To confirm that the generated cell lines had biologically active IL-2 and IL-15 cells were cultured with CTLL-2, an IL-2 and IL-15 dependent cell line.[203,204] Confirmation that CTLL-2 is unable to survive in the absence of IL-2 or IL-15 is demonstrated in the second column from the left in Figure 5.3A & 5.3B below. CTLL survival was titrated using exogenous human IL-2. This shows that greater than 50 U/ml of human IL-2 is required for normal CTLL-2 survival. The EG7-CFP tumour lines expressing IL-2 and or IL-15 along with their respective receptors were also cultured with CTLL-2. The tumour cells were excluded from analysis by gating out of all CFP positive cells. The viability of the remaining CTLL-2 cells was assessed using a live/dead stain to calculate percentage survival. At 48 hours after incubation approximately 10% of CTLL cells cultured with EG7-CFP have survived. Of those CTLL-2 cells cultured with IL-2 and IL-15 expressing constructs, this figure increases to approximately 60% CTLL-2 cell survival. Interestingly the EG7-CFP-FF construct which expresses both IL-2 and IL-15 fused to their respective receptors appears to result in slightly less CTLL-2 survival than the other constructs. This experiment has been repeated in triplicate including at different time points and is a consistent finding. Finally, tumours transduced with the various constructs were labelled with CFSE labelled and cultured in-vitro. This confirmed that the constructs did not affect in-vitro tumour growth rate.

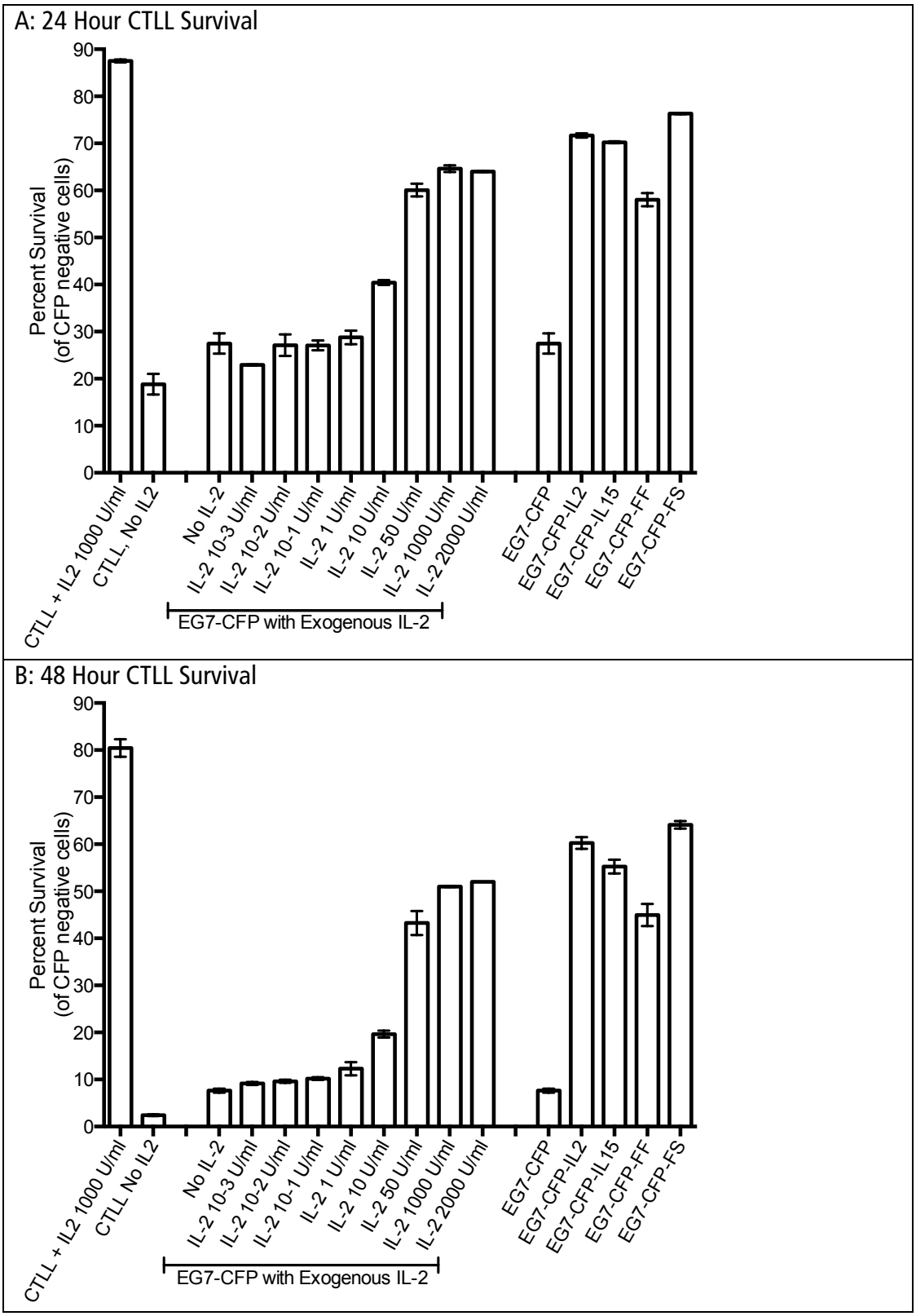


Figure 5.3: Confirming biological activity of the IL-2 and IL-15 constructs. The CTLL cell line requires the presence of IL-2 or IL-15 to survive. CTLL survival was titrated with exogenous human IL-2 in the presence of EG7-CFP. The various IL-2/IL-15 expressing construct were then compared. The results above demonstrate CTLL survival after **[A]** 24 hours and **[B]** 48 hours of culture with irradiated tumour cells. Results shown are representative of 3 repeat experiments. Of note, CTLL survival was noted to be slightly lower in the presence of EG7-CFP-FF in two of the three repeats. Compared to EG7-CFP, the differences for EG7-CFP-IL2, EG7-CFP-1L15, EG7-CFP-FF and EG7-CFP-FS all are statistically significant using an unpaired two-sided t-test. At 24 hours p = 0.0025, p = 0.0025, p = 0.0070 and p = 0.0019 respectively, and following 48 hours co-culture, p = 0.0006, p = 0.0010, p = 0.0041 and p = 0.0003 respectively.

5.2.2 Effect of transduced molecules on OT-1 T Cell proliferation

OT-I transgenic mice are characterised by having MHC class I-restricted, ovalbumin-specific, CD8+ T cells[199,200] and are thus capable of recognising EG7 tumours. As discussed in the introduction to this chapter, both IL-2 and IL-15 are involved in T Cell activation and proliferation. To assess the effect of OT-1 specific T cell proliferation, the transduced tumours along with positive and negative controls were co-cultured with CFSE labelled OT-1 T cells. As expected, OT-1 T cells fail to proliferate in the presence of non-OVA expressing EL4 tumours but do proliferate when the same cells are loaded with OVA peptide. Similarly CFSE dilution, which corresponds to proliferation, is seen in the presence of OVA expressing EG7 tumours. The presence of transduced IL-15/IL-15Rα but not IL-2/IL-2Rα results in enhanced OT-1 proliferation beyond that seen in EG7-CFP tumours. The EG7-CFP-FF and EG7-CFP-FS constructs also show slight increase in proliferation in some of the repeats, but this was not a consistent finding.

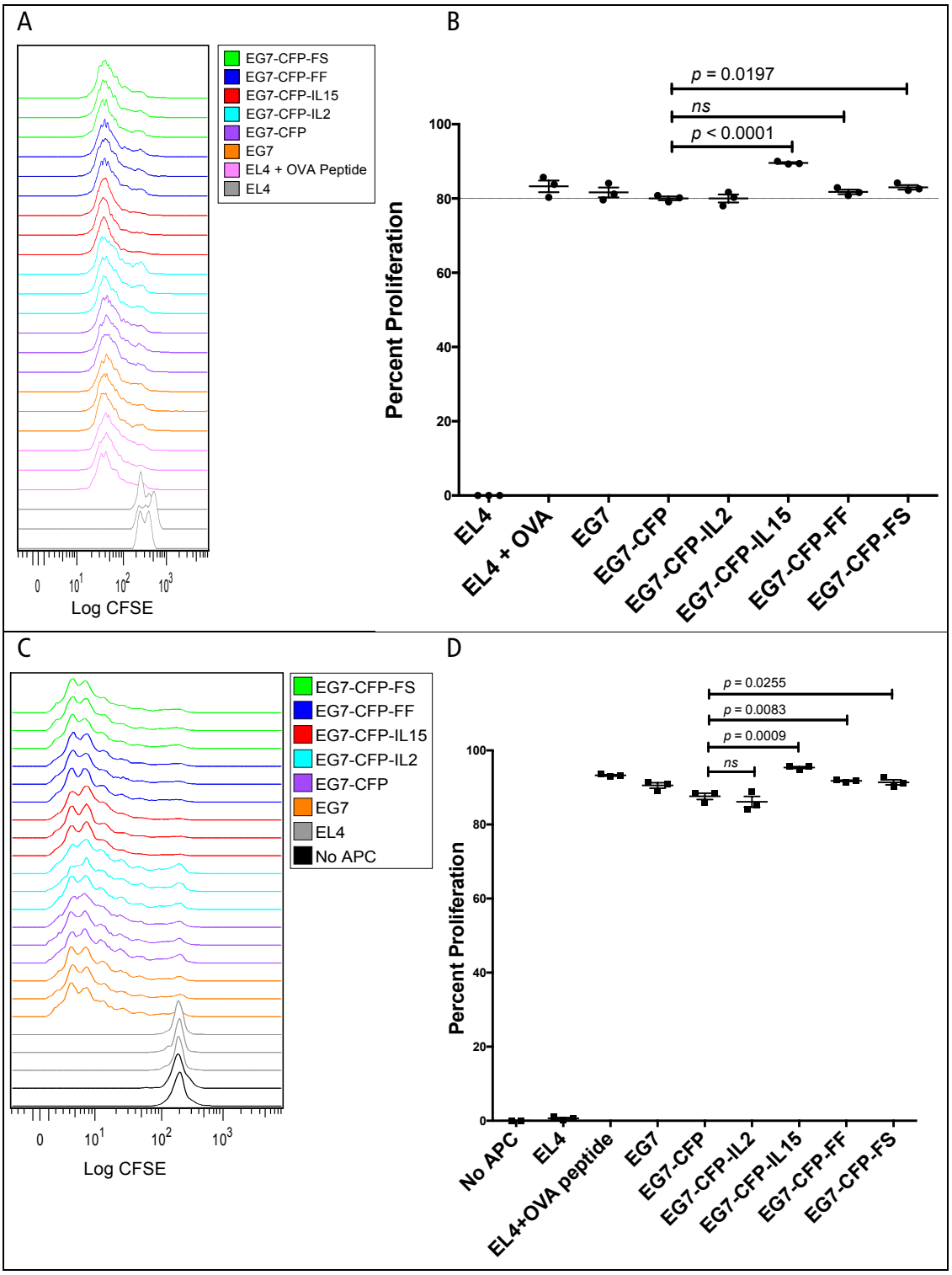


Figure 5.4: Improved *in-vitro* CFSE proliferation of OT-1 in presence of IL-15/IL-15Rα but not IL-2/IL-2Rα expressing tumours. **[A]** Log CFSE plot **[B]** Percentage proliferated cells. Performed following 40 hours co-incubation of CFSE labelled OT-1 splenocytes with irradiated tumours. EL4 (OVA-negative control) or EG7 tumours with transduced constructs as indicated. Cells depleted for B220+ CD11c+ cells. Gated on Live cells. Representative of 3 experiments. In each case, presence of transduced IL-2/IL-2Rα cells did not result in improved proliferation whereas presence of IL-15/IL-15Rα tumours did. The two cell lines which express both IL-2 and IL-15 along with their respective receptors appeared to subtly improve proliferation though did not reach statistical significance in every instance. **[C]** Log CFSE plot **[D]** Percentage proliferated cells. Performed as above but without B220 and CD11c depletion following 66 hours incubation.

5.2.3 GFP-OT1 Mouse model

As discussed above, OT-I transgenic mice are characterised by having MHC class I-restricted, ovalbumin-specific, CD8+ T cells capable of recognising EG7 tumours.[199,200] To generate Ovalbumin-specific T cells that can be easily monitored after being adoptively transferred into C57Bl/6 mice, this transgenic strain was crossed with C57BL/6-GFP mice that express a transgene coding for green fluorescent protein (GFP) under control of the human ubiquitin C promoter. These Ub-GFP transgenic mice have previously been characterised and whilst found to express GFP in all examined tissues, have particularly high levels in cells of haematopoietic origin. There is differential GFP levels within haematopoietic cells such that it is possible to differentiate T cells from B cells on the basis of distinct GFP levels.[197] As demonstrated in figure 5.5 below, the F1 offspring of the OT1 transgenic and Ub-GFP transgenic mice were characterised and found to have approximately 80% CD8+ T cells of which 94% are double GFP and OT-1 positive.

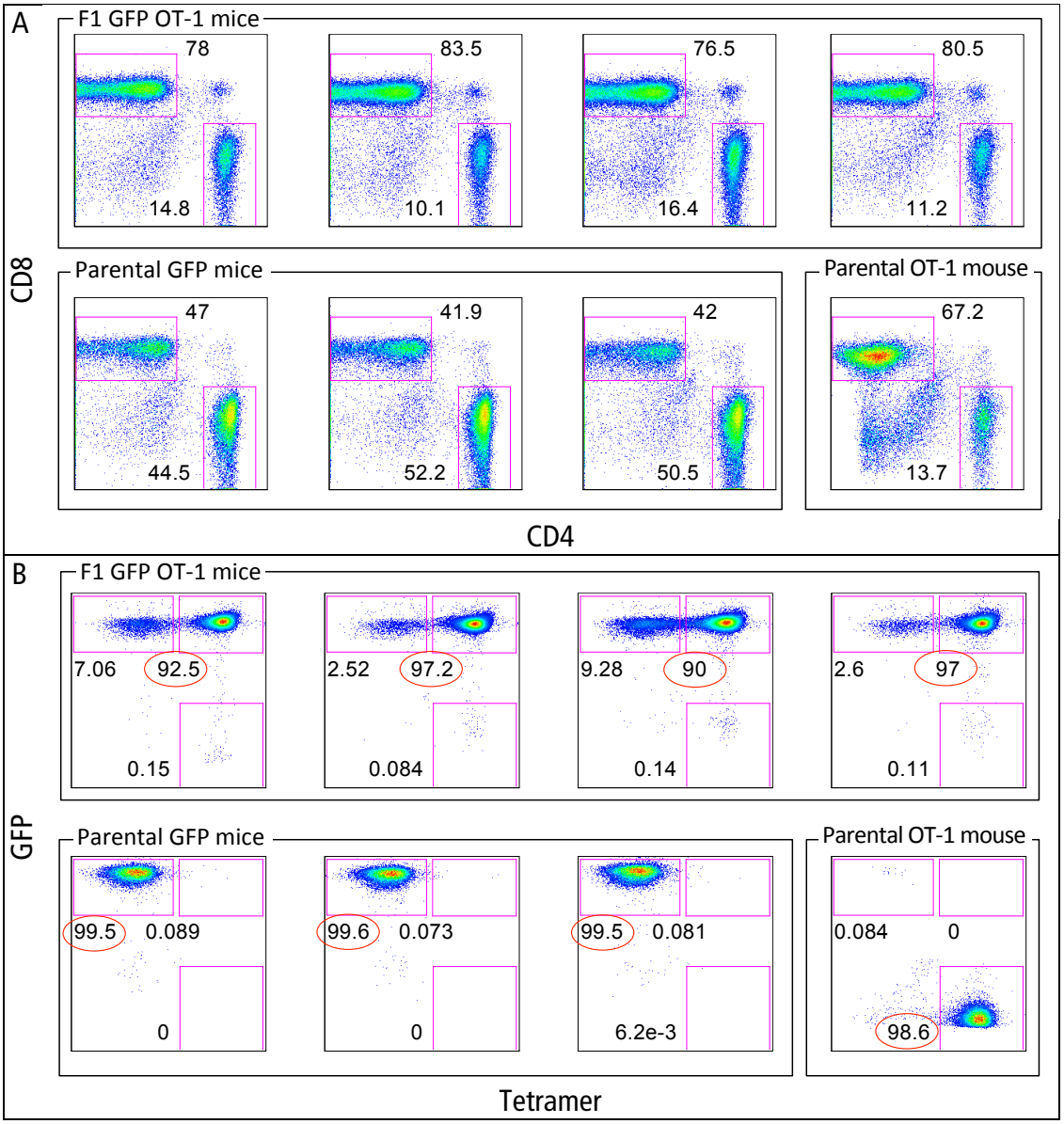


Figure 5.5: Characterisation of GFP-OT1 F1 mice T Cells. **[A]** T Cells are predominantly CD8⁺. Cells were first gated on Singlet cells, then live cells, B220⁻ and TcR⁺ cells. **[B]** CD8⁺ T cells predominantly GFP⁺ and OT-1 tetramer positive. Gating strategy as above but additionally gated on CD8⁺ cells.

5.2.4 GFP-OT1 T-Cell Adoptive transfer model

The GFP-OT-1 T Cell adoptive transfer model which utilises the generated tumours and transgenic mice described above is a useful in-vivo model for understanding the effect of the IL-2/IL-2Rα and IL-15/IL-15Rα constructs. CFP-transduced ovalbumin expressing tumours are injected subcutaneously into C57Bl/6 mice. The mice can then be injected with donor GFP-OT1 ovalbumin specific T cells. It is then possible to perform FACS analysis on removed spleens, tumours and lymph nodes to look for differences in number and characteristics of the adoptively transferred T Cells.

A pilot experiment confirmed that it was possible to identify adoptively transferred T cells within blood, spleen, tumour, draining and non-draining lymph-nodes of adoptively transferred mice, and suggested that adoptively transferred cells may selectively localise to ovalbumin expressing tumours and possibly its draining lymph node:

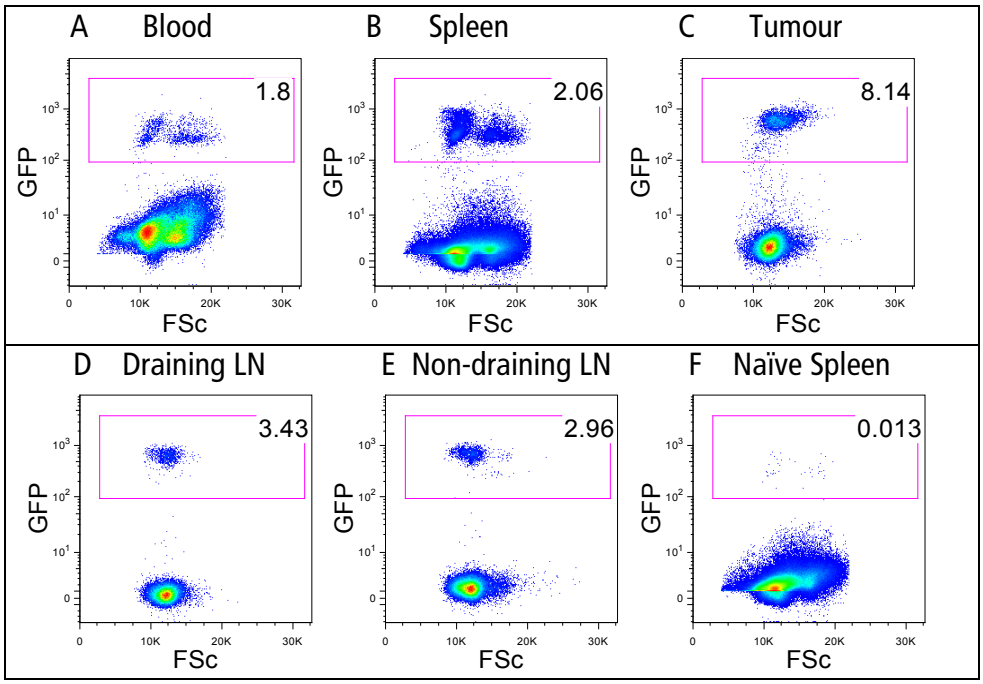


Figure 5.6: Identifying adoptively transferred T cells within tissues indicated 48 hours after intravenous injection of 20x10⁶ splenocytes. **[A]** Blood, **[B]** Spleen, **[C]** Tumour, **[D]** Draining lymph node, **[E]** Non-draining lymph node. **[F]** This GFP positive population is absent in spleen from a naïve mouse, not adoptively transferred with GFP-OT1 splenocytes. This pilot experiment suggests above baseline levels of adoptively transferred GFP-OT1 splenocytes accumulate within the OVA expressing tumour along with draining and possibly non-draining lymph nodes.

5.2.5 Differences in in-vivo growth

Having shown no differences in *in-vitro* tumour growth rate for tumours expressing the various constructs, tumours were injected subcutaneously into C57Bl/6 mice. As can be seen from the tumour growth plot in figure 5.7 below, obvious differences in tumour growth based on the transduced molecules are seen *in-vivo*.

All three mice challenged with EG7-CFP tumours developed tumours, but in 2 of the 3 mice, the tumours were noted to have started regressing beyond day 14. Two of the 3 mice challenged with EG7-CFP-IL2 developed tumours whilst one did not. In both cases, the tumours were noted to have started regressing, beyond day 9 and 12 respectively. None of the 3 mice challenged with EG7-CFP-IL15 developed tumours and only one of the 3 mice challenged with EG7-CFP-FF developed a tumour, which began regressing after day 9.

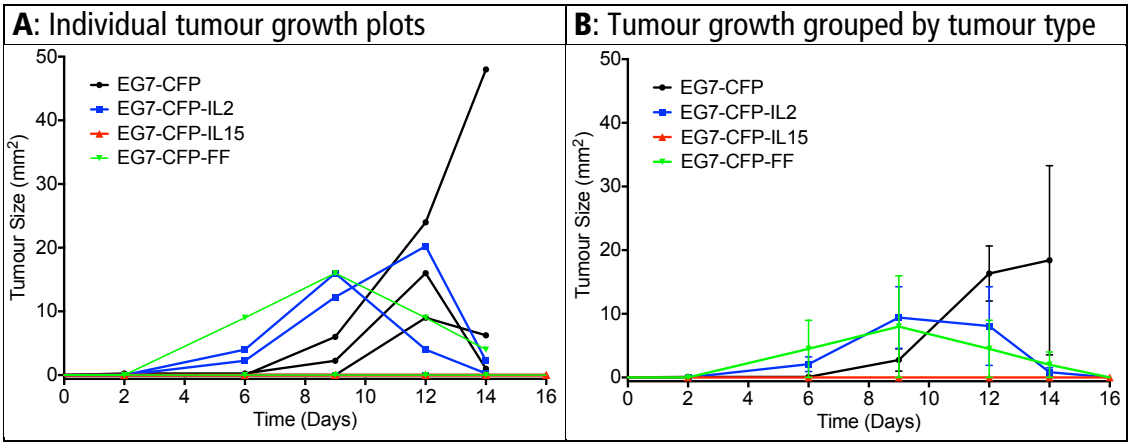


Figure 5.7: Tumour Growth. Mice were injected with $1x10^6$ tumours into right flank and tumour growth measured over time. Three mice per group were injected with EG7-CFP, EG7-CFP-IL2, EG7-CFP-IL15 or EG7-CFP-FF. **[A]** Tumour growth for individual mice plotted **[B]** Tumour growth grouped by tumour type. At day 12 there is a statistically significant difference in tumour size between those mice injected with EG7-CFP-IL-15 tumours of which none had developed tumours and EG7-CFP tumours, which had all developed tumours. $p = 0.0196$ by unpaired Student's t-test. Other differences observed do not reach statistical significance.

Although the original plan had to be to adoptively transfer mice with palpable tumours on day 14, it was noted on day 12 that tumours in some mice were beginning to regress. In view of this, mice with palpable tumours were injected with $20x10^6$ B220 depleted splenocytes from GFP-OT1 F1 mice and culled 48 hours later. Unfortunately by day 14 most of the tumours were very small or had completely regressed and it was therefore not possible to perform a valid comparison between the groups. It was however possible to look for differences between the draining and non-draining lymph-nodes of mice injected with either EG7-CFP or EG7-CFP-IL2 tumours. In comparing the percentage of GFP+ CD8 T cells within draining and non-draining lymph-nodes normalised to the same cell population within the spleen, no statistically significant differences observed either between the tumours or between draining and non-draining lymph-nodes. There were insufficient number of tumour bearing mice in the other tumour groups to allow statistical analysis. It should be emphasized that the tumours were spontaneously regressing prior to adoptive transfer of the GFP-OT1 F1 T cells and it may therefore be unsurprising that no differences between the groups was observed.

5.2.6 Overcoming tumour regression

In an effort to overcome the tumour rejection observed in figure 5.7 above, the number of tumour cells injected was titrated between $0.5x10^6$ and $5x10^6$ tumour cells. This was undertaken for EG7-CFP tumours along with EG7-CFP-IL2 and EG7-CFP-IL15 tumours.

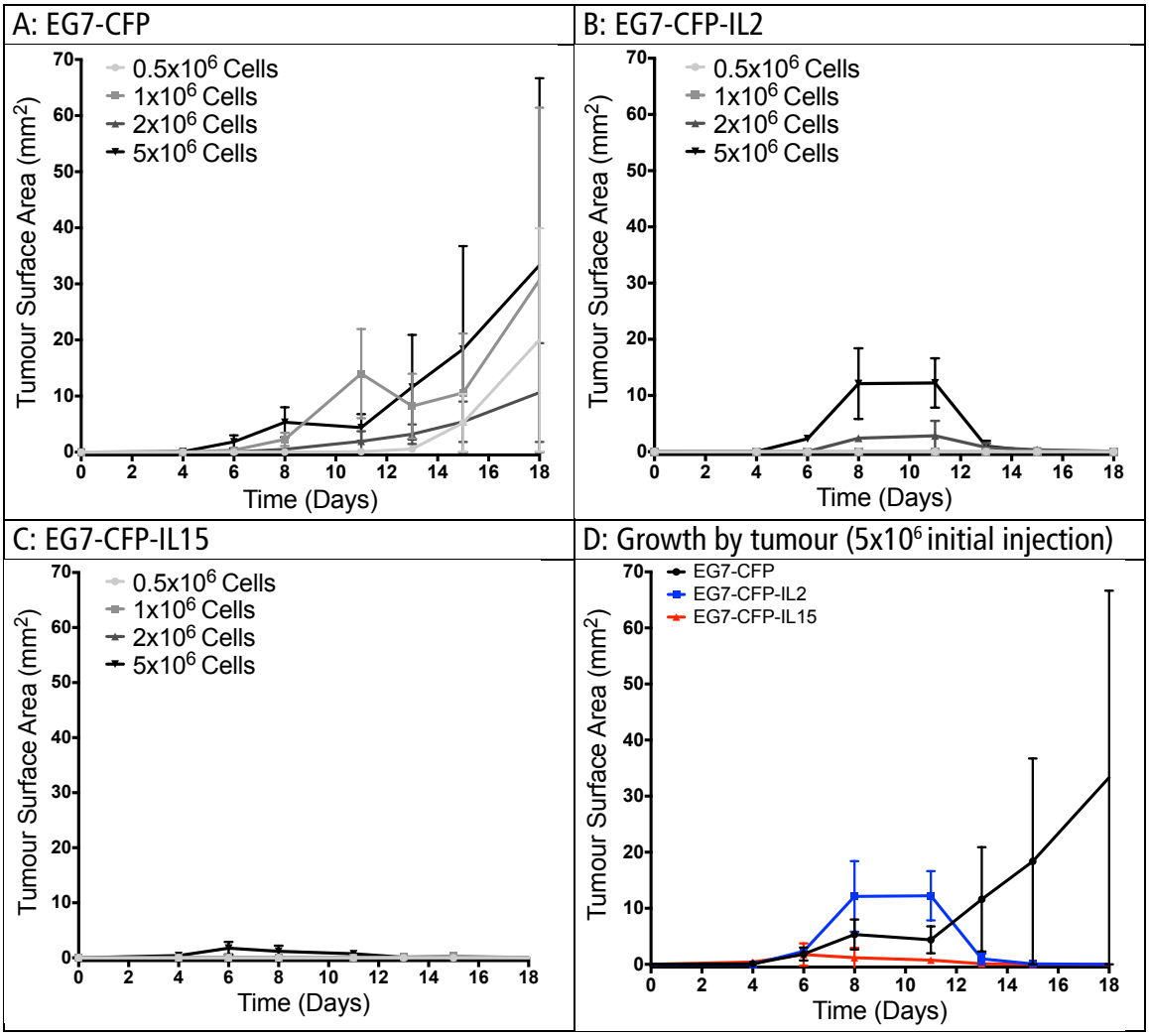


Figure 5.8: Effect of increasing initial tumour cell number on EG7-CFP, EG7-CFP-IL2 and EG7-CFP-IL15 tumours. n=3 mice per group, 12 groups in total **[A]** EG7-CFP tumours grow regardless of initial tumour number, however there is a non-statistically significant trend towards increased tumour size with higher initial tumour number. Tumour regression was observed regardless of initial tumour number and accounts for the large error bars seen at the later time points. **[B]** EG7-CFP-IL2 tumour growth was observed in all mice who received an initial tumour challenge greater or equal to $2x10^6$ cells, but not in any mice that received a lower initial tumour challenge. Tumour regression was observed in 100% of tumour bearing mice. **[C]** EG7-CFP-IL15 tumours developed in a percentage of mice who were injected with or above $1x10^6$ tumour cells, however by enlarge tumours never grew beyond a palpable nodule except in the highest tumour challenge where one mouse developed a tumour measuring $3.24mm^2$. **[D]** Comparison by tumour type in mice injected with $5x10^6$ tumour cells. In the EG7-CFP group, tumours regressed in 2 of the 3 mice, with only one tumour reaching the humane endpoint, thus accounting for the large error bars seen in this group at the later time points.

EG7-CFP tumours grow regardless of initial tumour cell number within the range assessed but most though not all tumours that develop subsequently reject regardless of initial cell number. Tumours that express IL-2 linked to its receptor grew providing the initial tumour challenge contains $2x10^6$ or more cells, however regression was observed in 100% of cases. Mice injected with tumours that express IL-15 linked to its receptor developed only small tumour

nodules that regressed almost immediately with initial tumour challenge of $1x10^6$ cells or above. Even at the highest tumour cell number assessed, $5x10^6$ cells, where all 3 mice developed tumours, the peak sizes were small compared to the other tumour types assessed with peak sizes of 0.25, 1 and 3.24mm^2 respectively. Similarly, 100% of EG7-CFP-IL2 tumours that developed also subsequently regressed.

In summary, it is possible to partially overcome the resistance to tumour development in all tumour types by increasing the initial tumour burden, but it was not possible to prevent tumour regression in EG7-CFP-IL2 or EG7-CFP-IL15 tumours even with initial tumour injection of $5x10^6$ cells.

5.2.7 Expression of IL-2 & IL-15 linked to their respective receptors inhibits tumour growth through a local rather than systemic effect

The presence of transduced tumours results in differences in tumour growth in vivo. To explore whether this was a local or systemic effect, a bilateral tumour model was used. Mice were injected with either EG7-CFP tumours on both flanks, or EG7-CFP on one flank and EG7-CFP-FF on the contralateral flank as illustrated in figure 5.9A below. As illustrated in figures 5.9 B & C, EG7-CFP-FF tumours failed to grow, however this had no impact on the growth of the contralateral EG7-CFP tumours indicating that that the mechanism of tumour rejection is as a result of a local rather than systemic effect.

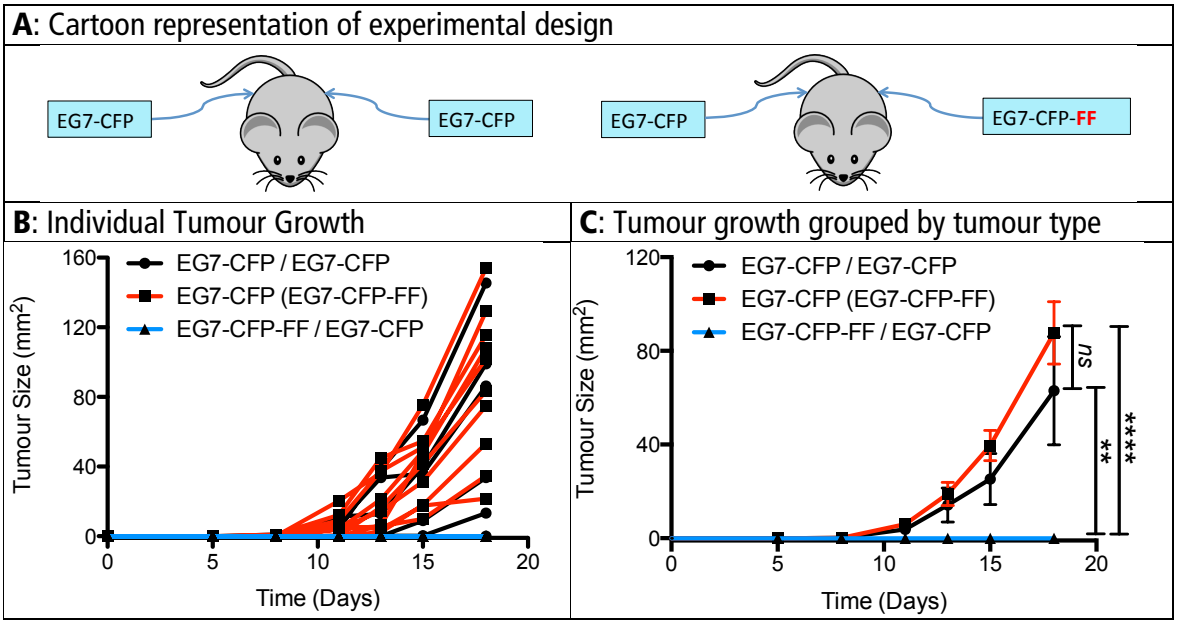


Figure 5.9: Transduced IL-2 & IL-15 linked with their respective receptors prevents tumour growth but has no effect on contralateral EG7-CFP tumours. Three mice were injected with EG7-CFP tumours on both flanks. Tumours developed at five of the six injection sites. Ten mice were injected with EG7-CFP tumours on one flank and EG7-CFP-FF on the contralateral flank. Tumours grew at all EG7-CFP injection sites but no tumours developed at EG7-CFP-FF injection sites. **[A]** Cartoon representation of experimental design. **[B]** Individual tumours growth plotted. **[C]** Tumour growth plotted by tumour group. No statistically significant growth difference was seen between EG7-CFP tumours injected opposite similar tumours as compared to EG7-CFP-FF tumours. In the key for figures B & C that follows the first tumour is the tumour plotted and the second tumour is the contralateral tumour. A bracket around the contralateral tumour indicates that although injected, a palpable tumour never developed.

5.2.8 Priming adoptively transferred T cells prior to tumour challenge

To investigate differences in the ability of transduced tumours to prime T Cells, mice were adoptively transferred with GFP-OT1 F1 splenocytes prior to tumour challenge. Following this, mice were injected with irradiated EG7-CFP or EG7-CFP-IL15 tumours ($5x10^6$ tumour cells irradiated to 84Gy) or PBS. An assessment of proliferation of the adoptively transferred cells was made by tail-bleeding and FACS analysis of the GFP+ CD8+ T cell population 13 days later (Figure 5.10A below). As this showed no differences between the groups, repeat challenge with identical irradiated tumours or PBS was undertaken at day 20 with assessment of proliferation made by tetramer staining in addition to GFP+ CD8 T Cells (Figure 5.10B below). Three of the 5 mice in the group injected with EG7-CFP-IL15 showed evidence of increased proliferation of the adoptively transferred cells. A third challenge using the same irradiated tumours or PBS was undertaken on day 29 with further tail-bleeding 7 days later. This showed no differences in proliferation of the adoptively transferred T Cells in mice who received either irradiated EG7-CFP and EG7-CFP-IL-15 tumours. Both appeared to be slightly higher than the baseline PBS values but this does not reach statistical significance (Figure 5.10C below). Regarding Figures 5.10A-C, it should be noted that no specificity control was used in these experiments and the ability to pick-up and interpret differences between the groups is thus limited. On day 37, all mice were challenged with live EG7-CFP tumours on the opposite flank and tumour growth observed. As illustrated in figure 5.10D below, 4 of the 5 mice primed with PBS develop tumours greater than $0.5mm^2$. In contrast, 3 of the 5 mice primed with irradiated EG7-CFP tumours develop tumours greater than $0.5mm^2$ (figure 5.10E) and only 2 of the 5 mice primed with irradiated EG7-CFP-IL-15 (figure 5.10F) tumours develop tumours greater than $0.5mm^2$. As illustrated in figures 5.10G and H below, in addition to the number of mice that develop tumours, there is also a statistically significant difference in tumour size in mice primed with irradiated EG7-CFP-IL15 as compared to PBS. This is not the case for mice primed with

irradiated EG7-CFP.

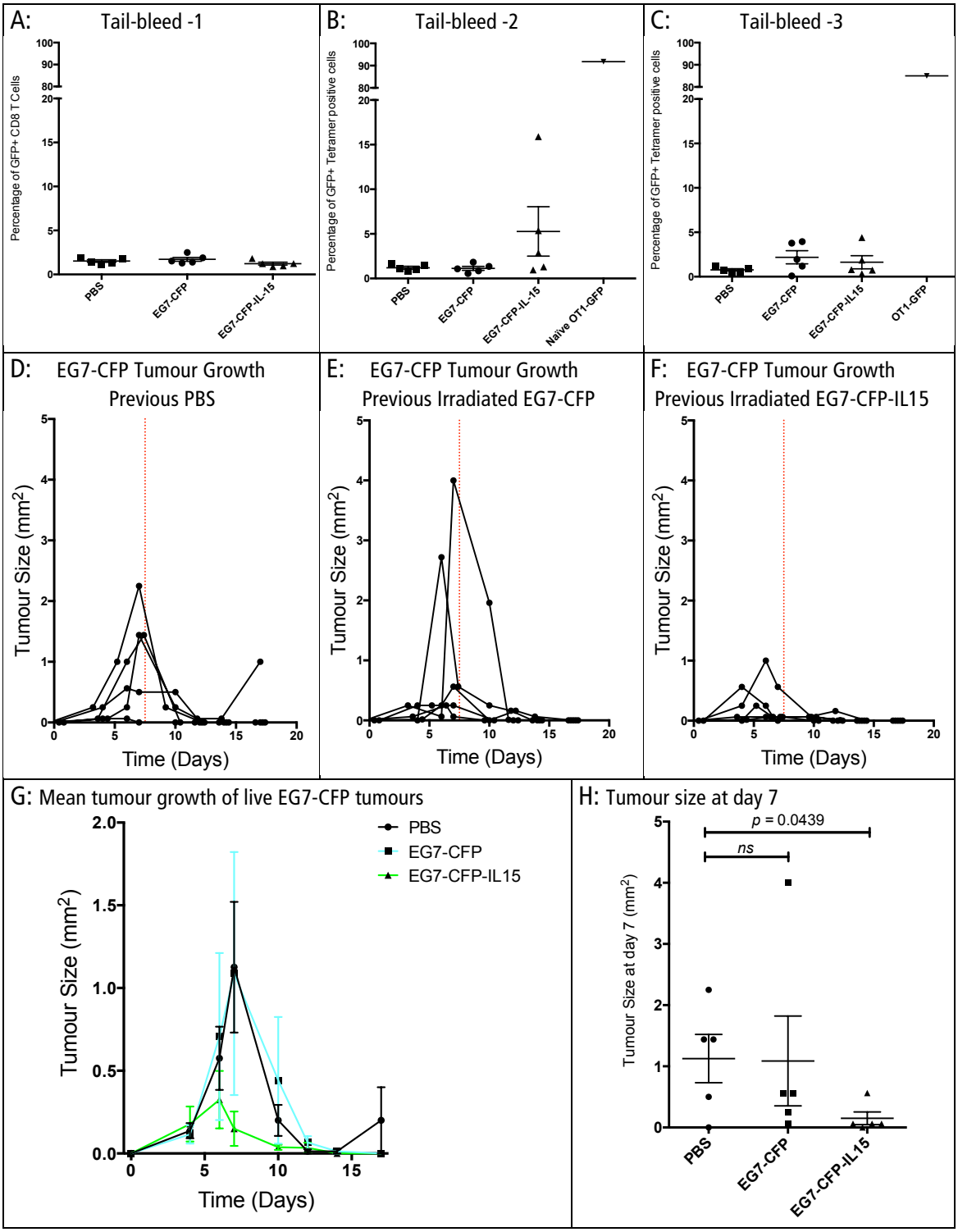


Figure 5.10: Priming with irradiated EG7-CFP-IL-15 results in reduced size of subsequent parental tumour. Mice were adoptively transferred with 20x10^6 splenocytes each and injected with irradiated EG7-CFP, EG7-CFP-IL-15 or PBS (5 per group) 48 hours later. **[A]** At first tail bleed 13 days following initial challenge there is no differences in adoptively transferred GFP+ population between the groups. **[B]** At second tail-bleed, 7 days following rechallenge with identical irradiated tumours or PBS there is a non-significant trend towards increased proliferation in mice injected with irradiated EG7-CFP-IL15. **[C]** At 3rd tail-bleed a non-significant increase above PBS baseline is seen in mice irradiated with either EG7-CFP or EG7-CFP-IL15. **[D-H]** Mice previously challenged with **[D]** PBS, **[E]** Irradiated EG7-CFP and **[F]** Irradiated EG7-CFP-IL15, above were challenged with live EG7-CFP tumours injected on the opposite flank. [G] Mean tumour growth of live EG7-CFP and [H] Tumour size at day 7 illustrate that compared to mice injected with PBS, there is a reduction in tumour size observed in mice primed with irradiated EG7-CFP-IL15 but not EG7-CFP. This result is statistically significant, p = 0.0439 (unpaired two-tailed Student's t-test)

5.2.9 Rejection of IL-2/IL-2Rα and IL-15/IL-15Rα expressing tumours is partially B or T cell mediated

To investigate the role of T and B cells in the tumour rejection and regression observed in the experiments above, the tumours EG7-CFP, EG7-CFP-IL2 and EG7-CFP-IL15 were injected into either C57Bl/6 or C57Bl/6 $RAG^{-/-}$ mice that lack these cell types. EG7-CFP tumours maintain a broadly similar pattern of growth and rejection in both C57Bl/6 and $RAG^{-/-}$ mice. However for tumours that express IL-2 or IL-15 linked to their respective receptors, differences do emerge. C57Bl/6 mice injected with IL-2/IL-2Rα expressing tumours develop small tumours that rapidly regress. In $RAG^{-/-}$ mice, tumours develop in a broadly similar manner to control EG7-CFP tumours. The same is true for mice injected with IL-15/IL-15Rα expressing tumours. No tumours develop in C57Bl/6 mice whereas in $RAG^{-/-}$ mice tumour growth is broadly similar to EG7-CFP tumours that do not express IL-2 or IL-15 along with their receptors.

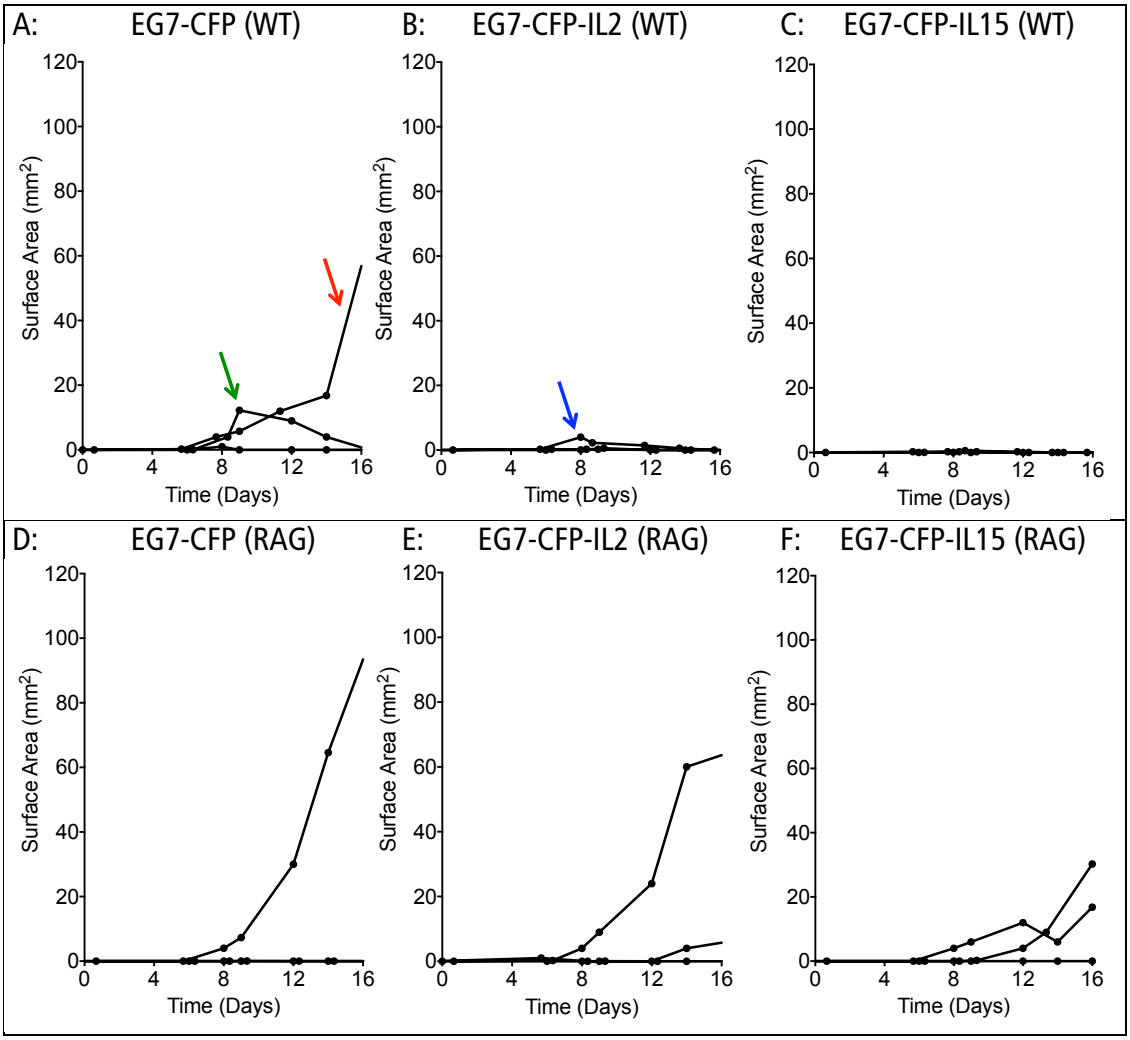


Figure 5.11: Rejection observed is partially B or T Cell mediated. Figures A-C plot EG7-CFP, EG7-CFP-IL2 & EG7-CFP-IL15 tumour growth in C57Bl/6 mice whilst figures D-F plot similar tumours in C57Bl/6 RAG$^{-/-}$ mice. n=3 per group. **[A]** EG7-CFP tumours in C57Bl/6 mice: two tumours grow one to 57mm^2, whilst a second grows to 12mm^2 and then regresses **[B]** EG7-CFP-IL2 tumours in C57Bl/6 mice: Two tumours develop small nodules which regress rapidly. **[C]** EG7-CFP-IL15 tumours in C57Bl/6 mice: No tumour growth observed. **[D]** EG7-CFP tumours in RAG$^{-/-}$ mice. No significant differences compared to WT C57Bl/6 mice. One tumour grows to 93mm^2, whilst a second tumour develops a small nodule which regresses immediately. **[E]** EG7-CFP-IL2 tumours in RAG$^{-/-}$ mice. Two tumours grow, with no regression observed. **[F]** EG7-CFP-IL15 tumours in RAG$^{-/-}$ mice, again two tumours grow and no regression is observed.

A comparison of splenocytes, draining and non-draining lymph-nodes obtained from the above mice and naïve control mice was performed. Compared to the naïve controls, there was noted to be a statistically significant increase in the percentage of activated T cells as assessed by CD69 positivity in the lymph-nodes in only those mice that had developed tumours greater than 4mm^2 at any time during the experiment. There was no statistically significant difference between the draining and non-draining lymph-nodes in this regard. Two of the mice with tumours greater than 4mm^2 had received EG7-CFP tumours whilst one had received EG7-CFP-

IL2. More detailed statistical analysis between these two tumour types was not possible owing to the small number of mice. A similar trend was also noted within the spleens of the same three mice though this did not reach statistical significance.

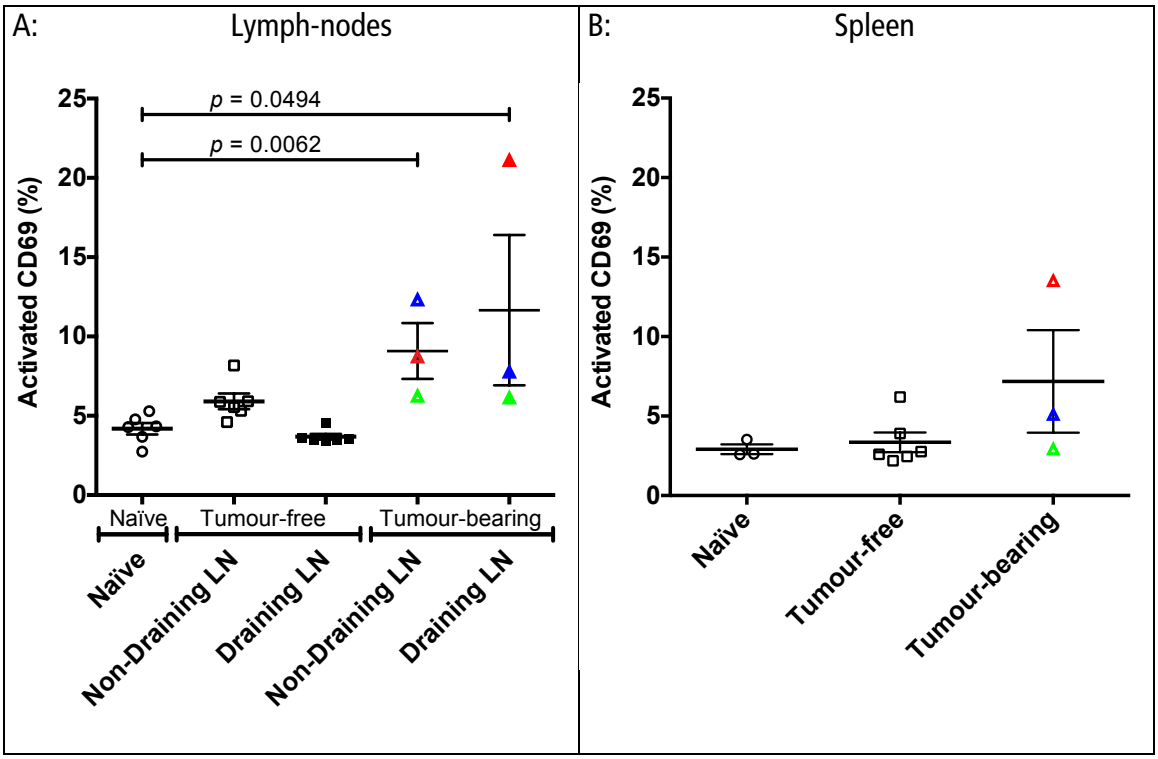


Figure 5.12: Mice that develop tumours have more activated T Cells. Higher proportion of activated, $CD4^+$ TcR^+ $CD69^+$ T cells are observed in mice who develop tumours greater than $4mm^2$ in size. **[A]** This finding is observed within both draining (p=0.0494) and non-draining (p=0.0062) lymph-nodes. Statistical analysis performed using unpaired Student's t-test. **[B]** Splenic T cells show a similar trend in the same animals but does not reach statistical significance. The coloured animals correspond to the same colours used in figure 5.11 above.

5.2.10 Histological analysis of transduced tumours

In an attempt to further understand the mechanisms leading to the observed tumour rejection and regression, C57Bl/6 mice were injected with EG7-CFP tumours, EG7-CFP-IL2 tumours or EG7-CFP-IL15 tumours. On the basis of the results in figure 5.8 above, $5x10^6$ tumour cells were injected on the right flank of each mouse. Once tumours became palpable, mice were culled and tumours collected, and split with one half collected as fresh samples into formalin and the other half preserved as a frozen specimen for subsequent histological analysis.

H&E shows possible differences in the degree of tumour necrosis between the different tumour groups with IL-15 expressing tumours demonstrating the most necrosis ($p = 0.0165$ compared to EG7-CFP tumour). To understand the underlying mechanism for both rejection and necrosis, immnunohistochemical stains including CD4, CD8, DX5 and LY6G were undertaken. No differences were noted between the different tumour groups. CD4 was difficult to interpret as EG7 tumours themselves stain positive for this marker owing to their thymoma origin, and subtle differences in infiltrating lymphocytes may therefore not have been obvious. The DX5, natural killer cell, stain did not show differences between the tumours, however the level of positive staining was very low in general. This may be an accurate reflection of few natural killer cells being present but could also indicate a technical problem with the staining. In addition to the above stains, additional immunohistochemical stains CD3 and B220 were attempted but stained non-specifically and could therefore not be accurately interpreted.

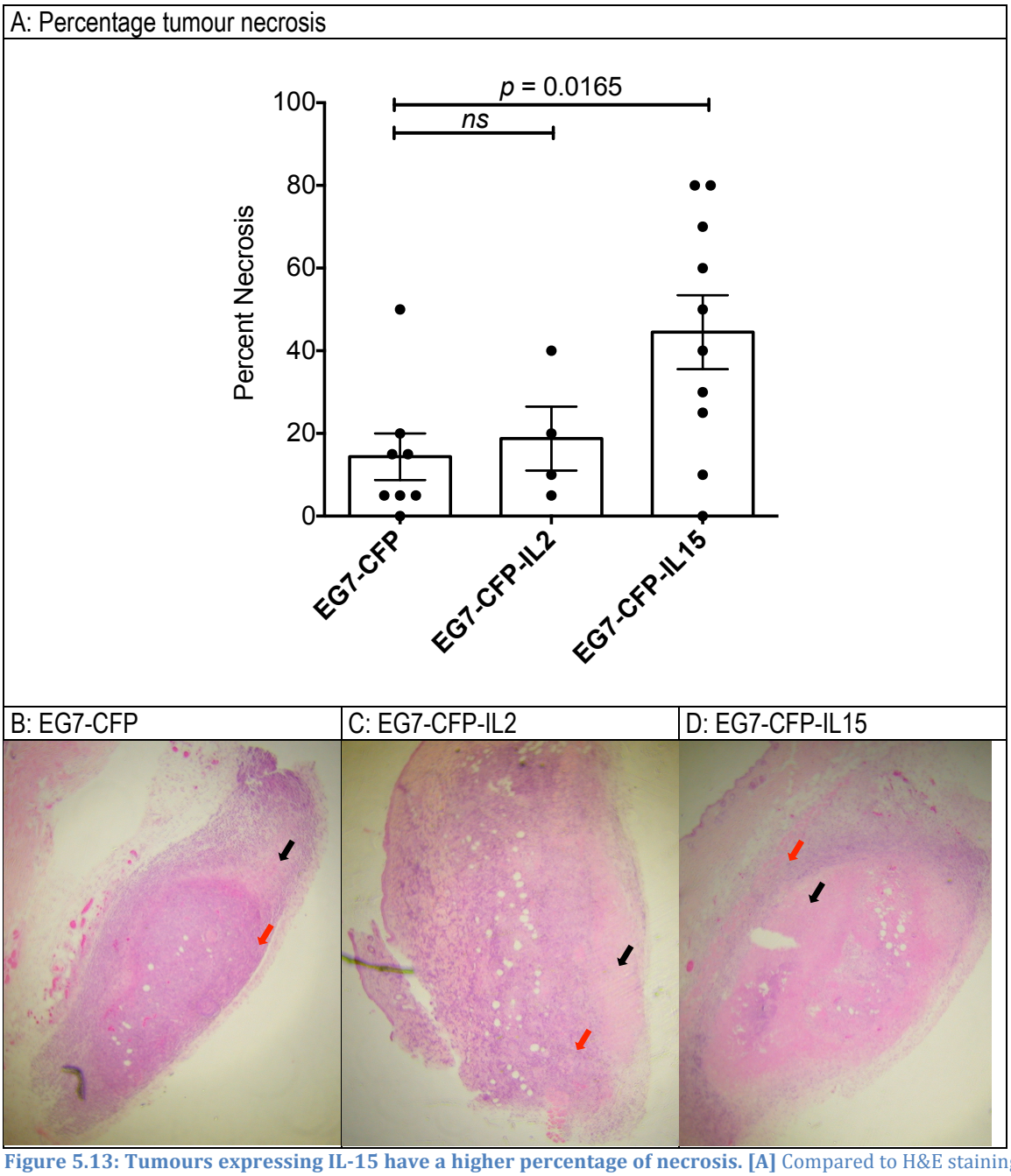


Figure 5.13: Tumours expressing IL-15 have a higher percentage of necrosis. [A] Compared to H&E staining of EG7-CFP tumours, tumours that express IL-15 shows higher percentage of the tumour composed of necrotic tissue. Tumours expressing IL-2 do not demonstrate a statistically significant difference. **[B-D]** Low magnification H&E stains of representative H&E stain. The red arrows point at viable tumour whereas the black arrows illustrate areas of necrosis **[B]** EG7-CFP tumour with small (15%) area of necrosis **[C]** EG7-CFP-IL2 slide demonstrating 40% tumour necrosis **[D]** EG7-CFP-IL15 tumours with a small rim of viable tumour surrounding a necrotic centre accounting for 80% of total tumour. Necrosis scoring was undertaken, in a blinded manner, by Dr Daniel Royston, Histopathology Consultant at Oxford University Hospitals.

5.3 Discussion

In the in-vitro experiments presented in this chapter, it has been demonstrated that the transduced IL-2 and IL-15 are biologically active as assessed by their ability to maintain the IL-2/IL-15 dependent CTLL-2 cell line (figure 5.3). Also in-vitro it has been shown that the five generated tumour lines continue to express ovalbumin as assessed by OT-1 proliferation (figure 5.4). The same series of experiments also indicated that the tumours transduced with IL-15 fused to its receptor enhanced ovalbumin specific proliferation compared to the other four cell lines. In-vivo (figure 5.7), this same cell line EG7-CFP-IL15 failed to grow in mice challenged with $0.5x10^6$ cells which was a sufficient number for control EG7-CFP and EG7-CFP-IL2 tumours to grow. EG7-CFP-FF which expresses both IL-15 and IL-2 linked to their respective receptors also had reduced in-vivo growth. It was shown that even by increasing the number of injected tumour cells ten fold (figure 5.8), there was very restricted tumour growth and those tumours that did develop regressed rapidly. EG7-CFP-IL2 tumours grew to a greater degree but 100% of the tumours were noted to regress which was not the case in the EG7-CFP cell line. With regards to expression of the IL-2 & IL-15 constructs, the effects of the transduced molecules appear to be local rather than systemic. When EG7-CFP-FF and EG7-CFP were injected on opposite flanks of the same mouse and the EG7-CFP-FF tumours failed to grow but the contralateral EG7-CFP grew normally, as compared to control mice injected with EG7-CFP tumours bilaterally (figure 5.9. As predicted by the in-vitro OT-1 proliferation experiment (figure 4), irradiated EG7-CFP-IL-15 tumours appeared able to improve priming of adoptively transferred OT1-GFP T cells in-vivo and partially protect against subsequent challenge with live EG7-CFP tumours as compared to mice who were "primed" with only PBS (figure 5.10). Mice primed with irradiated EG7-CFP not expressing IL-15/IL-15Rα had no protection against subsequent live EG7-CFP tumour challenge.

Attempts to characterise which cell types are involved in the rejection of the IL-2/IL-2Rα and IL-15/IL-15Rα expressing tumours were only partially successful. Injecting these tumours into RAG mice (figure 5.11) suggested that B or T cells plays a key role in the rejection of IL-/IL-2Rα expressing tumours (Figure 5.11E). These cell types are also involved in the rejection of IL-15/IL-15Rα expressing tumours, however in the absence of T and B cells (Figure 5.11F) IL-15/IL-15Rα expressing tumours grow where they do not in WT mice (Figure 5.11C), however their growth was possibly attenuated and it is therefore possible that other cell types are also involved. The results from figure 5.12 suggest inconclusively that more activated T Cells are present in mice that develop tumours compared to those that do not. Figure 5.13, histology, was unable to determine the cell types involved in the observed tumour rejection but did confirm statistically significant differences between EG7-CFP-IL-15 tumours and EG7-CFP tumours with regard to the degree of necrosis present within the tumours. Unfortunately immunohistochemical staining was unhelpful in further determining the cell type underlying these changes.

As discussed in the introduction to this chapter, it is now well established that IL-15 requires transpresentation by IL-15Rα to exert its effect. There is also a single paper suggesting that IL-2 may similarly require IL-2Rα for transpresentation.[373] The results presented in this chapter are consistent with this finding but do not offer further evidence in support of this. It would be interesting to repeat the experiments involving EG7-CFP-IL2, EG7-CFP-FF and EG7-CFP-FS in a transgenic IL-2Rα knock out mouse model[394-396] with and without daclizumab, the monoclonal antibody to the IL-2Rα discussed in the introduction to this chapter with the aim of providing further evidence in support of IL-2 transpresentation.

6. Conclusion

The experiments presented in this thesis all utilise the transduction of multiple genes into tumours to improve anti-tumour immunity. This approach, if successful, has the potential to improve tumour control, not only at the site of the transduced tumour but also at sites of distant metastatic disease, which have not been transduced.

Transduction of the costimulatory molecules CD54, CD70 and CD86 onto B16-F10 murine melanoma tumours enhances priming in-vitro (figure 3.2) and causes tumour rejection and delayed tumour growth in-vivo (figures 3.3 & 3.4). This finding was confirmed using CT26 tumours transduced with CD48, CD54, CD79 and CD86 in BALB/c mice (figures 4.2 & 4.5). In both tumour models, the effect was demonstrated with single costimulatory molecules but was more pronounced when multiple costimulatory molecules were transduced and is most likely to be either B or T cell dependent (figures 3.4 & 4.5). Attempts to identify antigen specificity concluded that Gp100 is not involved, but suggested Trp2 may play a minor role (figure 3.5). Results similar to the subcutaneous tumour model were obtained in an intravenous B16-F10 lung metastasis model (figures 3.6 & 3.8) where T Cells, B Cells, NK Cells and Granulocytes all appear to be involved (figure 3.7). Work in a human/mouse chimeric PD-1 model shows that transduction of multiple costimulatory molecules is able to overcome intrinsic unresponsiveness to anti-PD-1 therapy (figure 3.8). Accumulating data from several human trials demonstrates that between 65-80% of cancer patients treated with anti-PD-1 therapies will not respond to this therapy from the outset and are thus said to be 'resistant' to this form of treatment.[166,397-399] The ability to better understand and overcome 'resistance' to PD-1 pathway blockade is of clear clinical interest. To my knowledge, the experiments discussed in this thesis represent the first and only instance in which it has been possible to reverse an inherent 'resistance' to PD-1

blocking therapy in an in-vivo tumour model. There is a suggestion, currently unproven, from a number of trials in patients with melanoma that it is possible to improve response rates from anti-PD-1 therapy by combining these agents with other immunotherapeutic agents. Whilst great caution is required in drawing concrete conclusions when comparing arms across different clinical trials, objective response rates with single agent anti-PD-1 therapy in melanoma are generally reported to be in the order of 24-31%[166,397] whilst in combination with CTLA-4 blockade, response rates in two separate trials of between 40-61% have been reported.[400,401] Similar results have not to date been seen in lung cancer or any other tumour type.[398,399] The approach described in this thesis that was able to overcome 'resistance' to anti-PD-1 therapy through expression of costimulatory molecules is therefore of great potential interest in the metastatic cancer setting.

The transduction of eight molecules, the costimulatory molecules CD48, CD54, CD70 and CD86 together with the cytokines IFNγ, GM-CSF, IL-12 and the chemokine CX3CL1 resulted in total abolishment of any tumour growth (figures 4.2 & 4.5). Those tumours transduced with cytokines and chemokine were rejected almost immediately suggesting involvement of the innate rather than adaptive immune system. The lack of memory observed when these tumours were rechallenged with parental tumour (figure 4.4) supports this hypothesis. This is in contrast to mice challenged with tumours expressing single and multiple costimulatory molecules. IFNγ, despite its recognised anti-tumour properties[303-310,312,313] could also be implicated in this lack of memory to the parental tumour, as it has been demonstrated to cause loss of $CD4^+$ T cells which can impair secondary anti-tumour responses.[350] The rejection seen in the presence of costimulatory molecules is abolished in the absence of B cells, T cells and NK cells whereas the rejection seen in the presence of cytokines and chemokine is only partially abolished indicating other immune cells are involved (figure 4.5). Tumours transduced with a

combination of multiple costimulatory molecules, cytokines and a chemokine together are able to induce a systemic effect (figure 4.7) that leads to tumour growth retardation of a parental tumour on the contralateral flank.

In an attempt to enhance anti-tumour memory responses, IL-2 and IL-15 linked to their respective receptors were transduced onto EG7 tumour cells which express the tumour antigen Ovalbumin.[191] Irradiated EG7-CFP-IL-15 tumours improved the priming of adoptively transferred OT1-GFP T cells in-vivo and partially protected against subsequent challenge with live EG7-CFP tumours as compared to mice who were "primed" with only PBS or irradiated EG7-CFP not expressing IL-15/IL-15Rα (figure 5.10). B or T Cells were shown to be involved in the effects seen (figure 5.11). It is however possible that other cell types are also involved. Histologically (figure 5.13), EG7-CFP-IL-15 tumours have significantly more necrosis than non-IL-15 expressing tumours.

One possible clinical application of the work presented in this thesis is in the metastatic disease setting. If these findings are transferable to humans, it is possible that transduction of multiple genes, for example by electroporation, into an accessible site of tumour in the context of PD-1 blockade could result in a systemic anti-tumour effect.

In vivo electroporation utilises high-intensity electric fields that transiently increase plasma membrane permeability and thus allow delivery of DNA plasmids or other agents into tumour or other cells.[402] This approach has been attempted with some success in several animal models where GM-CSF and CD80 plasmids were inserted into JPS murine fibrosarcoma tumours, and CT26 murine colorectal tumours by intratumoural electroporation with eradication of both local and distal tumours.[403] Similar experiments were also undertaken in a JPS murine fibrosarcoma

model[404] and a B16-F10 murine melanoma tumour model[405], in which GM-CSF and CD80 intratumoural plasmid electroporation was combined with systemic depletion of regulatory T Cells using a CD25 depleting antibody, also demonstrated good activity.[404,405]

In humans, most of the work with electroporation has utilised the increased membrane permeability to improve chemotherapy delivery into tumour cells. A number of single patient case reports and small patient series show the technique to be well tolerated with some promising results.[406-410] There have also been attempts to use the technique for inserting immunomodulatory plasmids directly into tumour cells. Richards et al.[411] have inserted IL-2 plasmids into cutaneous melanoma lesions, but have not yet reported any results. Daud et al.[327,402] have undertaken a phase 1 clinical trial, inserting plasmid IL-12 into cutaneous melanoma lesions of 24 patients with advanced melanoma. Overall 76% of electroporated lesions demonstrated some necrosis. Of the 19 patients with measurable distant disease, over half had a clinical benefit from this treatment, with 3 patients (16%) having a durable complete response lasting between 18 - 20 months and an additional 7 patients (37%) having stable disease over a 4 – 20 month interval. The procedure itself was well tolerated with only transient pain at the time of electroporation.[327,402] Although to date, this technique has only been used with 1 or 2 genes, there is no known technical barrier to using a larger number. Whilst it is possible that not all genes will be expressed on all tumour cells, it may be that having all genes expressed within the tumour microenvironment is sufficient. To take this project further in this direction, would require the establishment of a metastatic animal model with known tumour antigen and collaboration with one of the centres with experience in tumour electroporation.

Oncolytic viruses preferentially infect and kill cancer cells and can be used as a vehicle for delivery of immunotherapy genes into tumour cells.[412] A recent Phase III study inserting GM-CSF into tumours using an HSV-1 based oncolytic virus, reported in abstract only, shows the treatment to be well tolerated and although primary endpoint of overall survival was not met, the delivery technique did result in improved durable response rates.[413] Several other phase I-III trials are currently in progress or recently completed.[412,414,415] To date, only single genes have been introduced in humans using this method. It has however been possible to insert up to two genes in experimental animal models.[416-418] If in the future it becomes technically possible to insert more genes using this technique, oncolytic viruses could be an alternative to electroporation.

Another potential clinical application for the findings discussed in this thesis is the combination of radiotherapy with anti-PD-1 directed therapy. Radiation is known to result in upregulation of the costimulatory molecules CD54[237-246], CD70[218,267] and CD86[218,219] within tumours or their infiltrating dendritic cells. The experiments presented here would therefore predict that tumours that are intrinsically 'resistant' to anti-PD-1 therapy could become 'sensitive' to its effects, if the treatment is combined with radiation. This would be of most clinical value if the enhanced sensitivity to anti-PD-1 therapy occurred not just within the irradiated tumour, but also at other sites of metastatic disease. The abscopal effect is a recognised phenomenon within the field of radiation oncology, in which radiation to disease at a single site results in tumour regression at distant disease sites. It is an extremely rare and unpredictable event and, to date, no therapeutic manipulation has been able to reproduce it in a controlled manner. Experiments using radiotherapy combined with a CTLA-4 inhibitor demonstrated that fractionated but not single-dose radiotherapy induced an immune-mediated abscopal effect in murine breast and colon cancer models.[280] A case report from a single patient with metastatic

melanoma appeared to confirm this finding[281], though a 799 patient randomised phase 3 study of patients with metastatic prostate cancer demonstrated no statistically significant difference between CTLA-4 inhibitor or placebo combined with radiotherapy.[419] There is less data available on the combination of radiation with anti-PD-1 directed therapy. In a murine breast cancer model[282] and intracranial glioma model[283], the effects of radiotherapy and anti-PD-1 therapy were synergistic in terms of local tumour growth.[282,283] No studies have looked at the effect of combining anti-PD-1 therapy and local tumour radiation on distant sites of disease. The experiments in this thesis suggest that the combination of radiotherapy with anti-PD-1 could enhance tumour sensing and recognition by the adaptive and innate immune systems and achieve such a systemic effect. On-going work within the lab to further investigate this is currently in progress.

Over the one hundred and fifty years since Virchow first postulated a linkage between the immune system and cancers, our understanding of the complex interactions between the two has grown considerably. The last few years in particular have seen new immunotherapy drugs entering clinical practice at an unprecedented rate. The potential of the immune system in fighting cancer is finally beginning to be unleashed. The challenge over the years ahead will be to harness the immense power of the immune system to cure cancers that until now have been considered incurable. Now is an exciting time in the field of tumour immunology.

7. References

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